



รายงานวิจัยฉบับสมบูรณ์

โครงการ: สรีรวิทยาทางไฟฟ้าและพยาธิสรีรวิทยาของหัวใจ
Electrophysiology and Pathophysiology of the Heart

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คำนำ

การเสียชีวิตโดยเฉียบพลันจากหัวใจ เป็นสาเหตุหลักของการเสียชีวิตในผู้ป่วยโรคหัวใจที่เกิดจากหลอดเลือดที่ไปเลี้ยงกล้ามเนื้อหัวใจอุดตัน โดยการเสียชีวิตโดยเฉียบพลันนี้ ส่วนใหญ่มาจากการที่เกิดภาวะหัวใจเต้นผิดจังหวะชนิดร้ายแรง ซึ่งจะทำให้เสียชีวิตภายในเวลาไม่กี่นาที หากไม่ได้รับการรักษาอย่างทันท่วงที นอกจากนั้นแล้ว หากผู้ป่วยสามารถรอดชีวิตจากภาวะนี้มาได้ ปัญหาที่ตามมาภายหลัง จะเกิดจากการที่กล้ามเนื้อหัวใจตายบางส่วน จากการที่ขาดเลือดไปเลี้ยง ดังนั้นการค้นคว้าวิจัยในปัจจุบัน จึงพยายามหาวิธีการเพิ่มประสิทธิภาพ การรักษาภาวะ heart attack นี้โดยการที่จะพยายามจะลดการตายของกล้ามเนื้อหัวใจลงให้ได้มากที่สุดและเพิ่มประสิทธิภาพการรักษาภาวะหัวใจเต้นผิดจังหวะชนิดร้ายแรง ซึ่งต้องใช้ในการปล่อยกระแสไฟฟ้าแรงสูงเข้าไปช็อคหัวใจโดยตรง เพื่อหยุดภาวะหัวใจเต้นผิดจังหวะชนิดร้ายแรงนี้

ในรายงานการวิจัยฉบับนี้ ได้รวบรวมเนื้อหางานวิจัยที่ได้มีการใช้ยาหลายชนิด รวมถึงยาต้านเบาหวานเพื่อพัฒนาวิธีการรักษาภาวะกล้ามเนื้อหัวใจขาดเลือดให้ได้ประสิทธิภาพสูงสุด คณะผู้วิจัยหวังเป็นอย่างยิ่งว่าผลงานค้นคว้าวิจัยในรายงานฉบับนี้จะเป็นประโยชน์อย่างยิ่งต่อนักวิจัยและแพทย์ที่สนใจ เพื่อนำไปต่อยอดเพื่อให้เกิดประโยชน์สูงสุด คือ แก่ผู้ป่วย และประชาชนทั่วไป ในอนาคตอันใกล้

สำหรับงานวิจัยในครั้งนี้ ผู้วิจัยขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) (RTA5280003) และคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่ให้การสนับสนุนงานวิจัยในครั้งนี้เป็นอย่างดี ทำให้งานวิจัยสำเร็จลุล่วงไปได้โดยตลอด

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สิงหาคม 2555

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บทคัดย่อ (ภาษาอังกฤษ)

Heart diseases have been responsible for high morbidity and mortality in most countries worldwide. Acute myocardial infarction is a condition in which coronary artery cannot provide blood supply to myocardium due to the occlusion of this vessel. This can lead to fatal cardiac arrhythmia, i.e. ventricular fibrillation, which can kill the patient in a few minutes if immediate treatment by defibrillation is not provided. Although myocardial reperfusion has been a successful therapy for acute myocardial infarction, reperfusion itself is also known to cause myocardial damage, and is known as reperfusion injury. Since the infarct size has been shown as a good predictor of cardiac function and mortality after acute myocardial infarction, interventions that can reduce the infarct size as well as decrease fatal arrhythmia incidence during ischemia-reperfusion can be useful therapeutic strategies. Currently, several drugs have been shown to provide cardioprotective effects. These drugs include granulocyte-colony stimulating factor (G-CSF), antidiabetic drugs rosiglitazone and vildagliptin, and p38 inhibitor. Nevertheless, the effects of these drugs on the ischemia-reperfused hearts have never been tested.

In the present study, we found that G-CSF, vildagliptin and p38 inhibitor could decrease the infarct size as well as stabilized cardiac electrophysiology by preventing cardiac arrhythmias. However, rosiglitazone provided dual effects, i.e. beneficial and harmful, to the heart. During ischemia-reperfusion, rosiglitazone could decrease the infarct size, but increase the ventricular fibrillation incidence. At the mitochondria level, G-CSF, vildagliptin and p38 inhibitor could improve cardiac mitochondrial dysfunction caused by ischemia-reperfusion injury, and may explain their effects on infarct size reduction. Recent clinical study investigated the role of incretins in acute myocardial infarction patients supported our preclinical studies. In contrast, rosiglitazone did not improved cardiac mitochondrial dysfunction even though the infarct size was decreased. Our finding indicated that the anti-apoptotic effect of rosiglitazone might be via the mitochondrial independent pathway.

Regarding thalassemic heart, we have demonstrated **for the first time that** T-type calcium channel could be mainly responsible for iron uptake into cardiomyocytes of thalassemic mice. Normally, T-type calcium channel is not expressed in normal adult heart except at the electrical conducting pathway inside the heart. However, in some pathological condition such as myocardial infarction and heart failure it can re-expressed. We demonstrated that thalassemic hearts also expressed T-type calcium channel and played an important role in iron entry into cardiac cells. We also demonstrated that heart

rate variability (HRV) obtained from 24-hour holter monitoring might be used to detect cardiac autonomic balance in thalassemic mice and thalassemia patients at the early state when no cardiac dysfunction was found. These finding could lead to the new strategies for prevention and treatment in thalassemia to prevent iron overload cardiomyopathy, which is responsible for many deaths in this group of patients.

In summary, the output of this research project includes 34 articles listed in PubMed, 42 abstract presentation at national and international scientific meeting, PhD graduates, 11 MSc graduates, 7 current PhD students, and 13 international and national research awards. Furthermore, the entire career research support from the Thailand Research Fund in the past ten years also plays a major role in the Outstanding Scientist Award that this principal investigator received this year.

บทคัดย่อ (ภาษาไทย)

โรคหัวใจเป็นสาเหตุสำคัญของการเสียชีวิตในประเทศต่างๆ ทั่วโลก ภาวะกล้ามเนื้อหัวใจตายเฉียบพลันเป็นภาวะที่เกิดจากการที่หลอดเลือดแดงที่ไปเลี้ยงกล้ามเนื้อหัวใจเกิดจากอุดตันทำให้ไม่มีเลือดไปเลี้ยง ภาวะนี้จะสามารถก่อให้เกิดการเต้นผิดจังหวะของหัวใจห้องล่างชนิดร้ายแรงที่เรียกว่า ฟีบริลเลชัน ซึ่งเมื่อเกิดขึ้นแล้วสามารถคร่าชีวิตผู้ป่วยได้ในเวลาไม่กี่นาที หากไม่ได้รับการรักษาอย่างทันท่วงที โดยการทำดีฟีบริลเลชัน ถึงแม้ว่าการรักษาโดยการสวนหัวใจ เพื่อไปเปิดเส้นเลือดที่อุดตันจะช่วยให้กล้ามเนื้อหัวใจได้รับเลือดกลับไปเลี้ยงเหมือนเดิม แต่การรักษาแบบนี้เป็นที่ทราบกันดีว่า สามารถทำให้เกิดการเสียหายต่อกล้ามเนื้อหัวใจได้ และรู้จักกันในชื่อของความเสียหายต่อหัวใจจากการมีเลือดกลับมาเลี้ยงหัวใจได้ ในปัจจุบันขนาดของกล้ามเนื้อหัวใจที่ตาย เชื่อว่าเป็นตัวพยากรณ์ถึงการทำงานของหัวใจในอนาคต รวมถึงการเสียชีวิตในผู้ป่วยกลุ่มนี้ได้ด้วย ดังนั้นการรักษาที่สามารถลดขนาดของกล้ามเนื้อหัวใจที่ตายลง และรวมถึงการรักษาที่สามารถลดอัตราการเกิดภาวะหัวใจเต้นผิดจังหวะชนิดร้ายแรง จึงน่าจะเป็นวิธีการรักษาที่เหมาะสม ปัจจุบันนี้ มียาหลายตัวที่มีรายงานว่าผลในการป้องกันหัวใจ ยาเหล่านี้ ได้แก่ G-CSF, ยารักษาเบาหวาน Rosiglitazone และ Vildagliptin รวมถึงยาใหม่ที่ยับยั้งการทำงานของ p38 อย่างไรก็ดีตาม ผลของยาเหล่านี้ต่อการป้องกันหัวใจในขณะที่เกิดความเสียหายจากภาวะกล้ามเนื้อหัวใจขาดเลือด และมีเลือดกลับไปเลี้ยงยังไม่มีการศึกษาวิจัย

ในโครงการวิจัยนี้ เราพบว่ายา G-CSF, Vildagliptin และยายับยั้ง p38 สามารถลดขนาดของกล้ามเนื้อหัวใจที่ตายและยังช่วยป้องกันการเกิดภาวะหัวใจเต้นผิดจังหวะได้อีกด้วย อย่างไรก็ดีตามยา rosiglitazone ให้ทั้งผลดีและผลเสีย กล่าวคือ สามารถลดขนาดของกล้ามเนื้อหัวใจที่ตายลงได้ แต่กลับเพิ่มโอกาสของการเกิดภาวะหัวใจเต้นผิดจังหวะชนิดร้ายแรง ในการศึกษาวิจัยระดับโมโตคอนเดรียของหัวใจ เราพบว่า G-CSF, Vildagliptin และยายับยั้ง p38 ช่วยป้องกันความเสียหายต่อไมโทคอนเดรียของหัวใจ ที่เกิดความเสียหายจากการที่กล้ามเนื้อหัวใจขาดเลือดและมีเลือดกลับมาเลี้ยงได้ จึงน่าจะเป็นสาเหตุที่ทำให้ขนาดของกล้ามเนื้อหัวใจตายมีขนาดลดลงได้

สำหรับในหัวใจในโรคธาลัสซีเมีย เราค้นพบเป็นครั้งแรกว่า ทีไทพ์แคลเซียม แชนแนล อาจเป็นช่องทางที่นำเหล็กเข้าหัวใจในโรคธาลัสซีเมียได้ ตามปกติแล้ว ช่องทางชนิดนี้จะไม่พบในหัวใจของคนปกติ แต่จะสามารถพบได้ในหัวใจที่มีพยาธิสภาพ ได้แก่ ในโรคกล้ามเนื้อหัวใจตาย และหัวใจล้มเหลว เราพบเป็นครั้งแรกว่าในหัวใจของหนูธาลัสซีเมีย จะมีการแสดงออกของช่องทางนี้ขึ้นในเซลล์กล้ามเนื้อหัวใจ และมีบทบาทสำคัญต่อการนำเข้าสู่เซลล์กล้ามเนื้อหัวใจ นอกจากนี้เรายังได้ค้นพบว่า ค่าความแปรปรวนของหัวใจ (HRV) สามารถใช้ในการบ่งบอกค่าความผิดปกติของการควบคุมระบบประสาทอัตโนมัติในหัวใจของโรคธาลัสซีเมียได้ ตั้งแต่ระยะต้นของโรค ก่อนที่จะตรวจพบการทำงานที่ผิดปกติของหัวใจ การค้นพบนี้อาจนำไปสู่การพัฒนาวิธีป้องกันและการรักษาใหม่ๆ ในโรคธาลัสซีเมียที่เกิดภาวะหัวใจล้มเหลว จากการที่มีเหล็กสะสมในหัวใจมากเกินไป และเป็นสาเหตุการเสียชีวิตหลักในผู้ป่วยกลุ่มนี้

กล่าวโดยสรุป ผลลัพธ์จากโครงการวิจัยนี้ประกอบไปด้วย ผลงานวิจัยที่ดีพิมพ์ในวารสารระดับนานาชาติ ที่อยู่ในฐานข้อมูล Pubmed จำนวน 34 เรื่อง, ได้มีการนำเสนอผลงานประชุมในงานประชุมระดับนานาชาติ และระดับชาติจำนวน 42 เรื่อง, ผลิตบัณฑิตระดับปริญญาเอกได้ 11 คน และปริญญาโท 7 คน, มีนักศึกษาระดับปริญญาเอกที่เกี่ยวข้องกับทุนวิจัยนี้ 2 คน, และได้รับรางวัลงานวิจัยระดับชาติและนานาชาติจำนวน 13 รางวัล นอกจากนี้แล้ว ผู้รับทุนส่งเสริมกลุ่มวิจัยยังได้รับรางวัลนักวิทยาศาสตร์ดีเด่น ประจำปี 2555 จากมูลนิธิส่งเสริมวิทยาศาสตร์และเทคโนโลยีในพระบรมราชูปถัมภ์อีกด้วย

Executive Summary

In this research project, two main aims including the pharmacological intervention to attenuate the severity of cardiac ischemia-reperfusion injury and the mechanism of iron entry in thalassemic hearts for iron-overload cardiomyopathy prevention have been extensively investigated. We found that several drugs including the granulocyte-colony stimulating factor (G-CSF), anti-diabetic drug vildagliptin, and a new compound SB203580 that inhibits the action of p38 MAPK could decrease the infarct size as well as stabilized cardiac electrophysiology by preventing cardiac arrhythmias caused by ischemia-reperfusion injury. However, anti-diabetic drug rosiglitazone provided both beneficial and harmful effects to the heart, by decreasing the infarct size, but increasing the ventricular fibrillation incidence. The beneficial effects of G-CSF, vildagliptin and p38 inhibitor could be due to their protection on cardiac mitochondrial function.

Regarding thalassemic heart, we have demonstrated **for the first time** that T-type calcium channel could be mainly responsible for iron uptake into cardiomyocytes of thalassemic mice. Normally, T-type calcium channel is not expressed in an adult heart except at the electrical conducting pathway inside the heart. However, in some pathological condition such as myocardial infarction and heart failure it can re-expressed. We demonstrated that thalassemic hearts also expressed T-type calcium channel and played an important role in iron entry into cardiac cells. We also demonstrated that heart rate variability (HRV) obtained from 24-hour holter monitoring might be used to detect cardiac autonomic balance in thalassemic mice and thalassemia patients at the early state when no cardiac dysfunction was found. These findings could lead to the new strategies for prevention and treatment in thalassemia to prevent iron overload cardiomyopathy, which is responsible for many deaths in this group of patients.

In summary, the output of this research project includes 34 articles listed in PubMed, 42 abstract presentation at national and international scientific meeting, PhD graduates, 11 MSc graduates, 7 current PhD students, and 13 international and national research awards. Furthermore, the entire career research support from the Thailand Research Fund in the past ten years also plays a major role in the Outstanding Scientist Award that this principal investigator received this year.

เนื้อหางานวิจัย

1. งานวิจัยในโครงการนี้สามารถแบ่งเนื้อหางานวิจัยออกได้เป็น 2 เรื่องใหญ่ ได้แก่ การศึกษาวิจัยในภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน และในหัวใจที่มีปริมาณเหล็กสะสมมากเกินไปในโรคธาลัสซีเมีย ดังนี้

ใน ischemic hearts

- 1.1 ได้ทำการศึกษาถึงผลของ G-CSF ต่อ cardiac electrophysiology ในสภาวะที่เกิดภาวะกล้ามเนื้อหัวใจขาดเลือด สารสำคัญของงานวิจัยมีดังนี้

Effects of granulocyte colony-stimulating factor (G-CSF) on cardiac electrophysiology during ischemic/reperfusion (I/R) period are unclear. We hypothesized that G-CSF stabilizes cardiac electrophysiology during I/R injury by prolonging the effective refractory period (ERP), increasing the ventricular fibrillation threshold (VFT) and decreasing the defibrillation threshold (DFT). In this study, 30 pigs were used. In intact heart protocol, pigs were infused with either G-CSF or vehicle (n=7 each group) without I/R induction. In I/R protocol, pigs were infused with G-CSF (0.33ug/kg/min) or vehicle (n=8 each group) for 30 minutes prior to a 45-minute left anterior descending artery occlusion and at reperfusion. Diastolic pacing threshold (DPT), ERP, VFT and DFT were determined in all pigs before and during I/R period.

We found that neither G-CSF nor vehicle altered any parameter in intact-heart pigs. During ischemic period, G-CSF significantly increased the DPT, ERP and VFT without altering the DFT. In the vehicle group, only the DPT was increased during ischemia. During reperfusion, G-CSF continued to increase the DPT without altering other parameters. Systolic and diastolic pressures were significantly decreased from the baseline in both groups during I/R period. Although the area at risk was not different between the two groups, the infarct size was significantly decreased in the G-CSF group, compared to the vehicle.

All of these findings indicate that G-CSF increases the DPT, ERP and VFT and reduces the infarct size, which may help stabilizing the myocardial electrophysiology, thus preventing fatal arrhythmia in ischemic myocardium. However, G-CSF does not improve defibrillation efficacy during I/R injury.

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- 1.2 ได้ทำการศึกษาถึง ผลของ G-CSF ต่อการป้องกัน mitochondrial damage จาก H_2O_2 -induced oxidative stress ใน isolated cardiac mitochondria ของหนูและกลไกการออกฤทธิ์ของ G-CSF บน cardiac mitochondria สารสำคัญของงานวิจัยมีดังนี้

Ischemic heart disease is one of the major causes of death in most nations. Ischemic heart disease/myocardial infarction causes the increase of reactive oxygen species (ROS), leading to oxidative stress in cardiomyocytes. In the heart, it is known that mitochondria are the principal source of ROS production. Previous studies suggested that ischemic heart disease could cause mitochondrial dysfunction, leading to increased oxidative stress. Recently, granulocyte-colony stimulating factor (G-CSF) has been reported that it has a protective effect on cardiomyocytes following ischemic heart disease. G-CSF was demonstrated to improve doxorubicin-induced mitochondrial damage in cultured cardiomyocytes. However, the effects of G-CSF in isolated cardiac mitochondria have never been investigated.

In the present study, we determined whether G-CSF can improve mitochondrial damage in hydrogen peroxide (H_2O_2)-induced oxidative stress in isolated cardiac mitochondria. In the present study, isolated mitochondria from rat hearts were randomly assigned into eight treatment groups: H_2O_2 (2mM), 50 or 200 ng/ml G-CSF, H_2O_2 pre-treated with G-CSF (25, 50, 100 or 200 ng/ml) and control (n=6 each). All isolated mitochondria were measured for mitochondrial swelling, mitochondrial membrane potential changes ($\Delta\Psi_m$) and ROS production.

Our results demonstrated that in H_2O_2 pre-treated with G-CSF groups, G-CSF significantly reduced mitochondrial swelling, $\Delta\Psi_m$ and ROS production under H_2O_2 -induced oxidative stress condition in isolated cardiac mitochondria. However, the dose-dependent effect was not observed in this study. G-CSF alone did not cause any changes in isolated cardiac mitochondria. In conclusion, G-CSF can directly protect mitochondrial damage under H_2O_2 -induced oxidative stress condition in isolated cardiac mitochondria.

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- 1.3 ได้ทำการศึกษาถึงผลของยารักษาเบาหวาน Rosiglitazone ต่อการทำงานทางไฟฟ้าในหัวใจ เนื่องจากมีรายงานว่ายาตัวนี้มีผลในทางที่ไม่ดีต่อหัวใจ การศึกษานี้จะทำการศึกษาในสุกรที่ถูกเหนี่ยวนำให้เกิดภาวะกล้ามเนื้อหัวใจขาดเลือดเฉียบพลัน สารสำคัญของงานวิจัยมีดังนี้

Rosiglitazone, a peroxisome proliferator-activated receptor gamma agonist has been used to treat type II diabetes. Despite debates regarding its cardioprotection, the effects of rosiglitazone on cardiac electrophysiology are still unclear. Our study determined the effect of rosiglitazone on ventricular fibrillation (VF) incidence, VF threshold (VFT), defibrillation threshold (DFT), and mitochondrial function during ischemia and reperfusion. Twenty-six pigs were used. In each pig, either rosiglitazone (1 mg/kg) or normal saline solution was administered intravenously within 60 minutes. Then, the left anterior descending coronary artery was ligated for 60 minutes and released to promote reperfusion for 120 minutes. The cardiac electrophysiologic parameters were determined at the beginning of the study and during ischemia and reperfusion period. The heart was removed then the area at risk and the infarct size in each heart were determined. Cardiac mitochondria were isolated for determination of mitochondrial function. Rosiglitazone did not improve the DFT and VFT during ischemia – reperfusion periods. In rosiglitazone group, VF incidence was increased (58 vs. 10 %) and time to the first occurrence of VF was decreased (3 ± 2 vs. 19 ± 1 min), as compared to the vehicle group ($P < 0.05$). However, the infarct size related to the area at risk in the rosiglitazone group was significantly decreased ($P < 0.05$). In the cardiac mitochondria, rosiglitazone did not alter the level of reactive oxygen species production and could not prevent mitochondrial membrane potential changes. Rosiglitazone increased the propensity for VF, and could neither increase defibrillation efficacy nor improve cardiac mitochondrial function.

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- 1.4 ได้ทำการศึกษาถึงผลของยารักษาเบาหวานตัวใหม่ คือยาในกลุ่ม Dipeptidyl peptidase 4 inhibitor ซึ่งพบว่ามี cardioprotective effect ในสภาวะที่เกิด acute myocardial infarction สารสำคัญของงานวิจัยมีดังนี้

Dipeptidyl peptidase-4 (DPP-4) inhibitor is a new antidiabetic drug for type-2 diabetes mellitus patients. Despite its benefits on glycemic control, the effects of DPP-4 inhibitor on the heart during ischemia-reperfusion (I/R) periods are not known. We investigated the effect of DPP-4 inhibitor on cardiac electrophysiology and infarct size in a clinically relevant I/R model in swine and its underlying

cardioprotective mechanism. Fourteen pigs were randomized to receive either DPP-4 inhibitor (vildagliptin) 50 mg or normal saline intravenously prior to a 90-min left anterior descending artery occlusion, followed by a 120-min reperfusion period. The hemodynamic, cardiac electrophysiological and arrhythmic parameters, and the infarct size were determined before and during I/R. Rat cardiac mitochondria were used to study the protective effects of DPP-4 inhibitor on cardiac mitochondrial dysfunction caused by severe oxidative stress induced by H_2O_2 to mimic the I/R condition. Compared to the saline group, DPP-4 inhibitor attenuated the shortening of the effective refractory period (ERP), decreased the number of PVCs, increased the ventricular fibrillation threshold (VFT) during the ischemic period, and also decreased the infarct size. In cardiac mitochondria, DPP-4 inhibitor decreased the reactive oxygen species (ROS) production and prevented cardiac mitochondrial depolarization caused by severe oxidative stress. During I/R, DPP-4 inhibitor stabilized the cardiac electrophysiology by preventing the ERP shortening, decreasing the number of PVCs, increasing the VFT, and decreasing the infarct size. This cardioprotective effect could be due to its prevention of cardiac mitochondrial dysfunction caused by severe oxidative stress during I/R.

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- 1.5 ได้ทำการศึกษาถึงผลของสารเคมีซึ่งมีฤทธิ์ยับยั้งการทำงานของ p38 ซึ่งเป็น intracellular signaling molecule ในเซลล์กล้ามเนื้อหัวใจ ซึ่งมีหลักฐานรายงานว่าอาจจะมี cardioprotective effect ได้ แต่ผลของยาต่อหัวใจในสภาวะที่เกิด acute myocardial infarction ยังขาดความชัดเจนอยู่มาก โดยเฉพาะอย่างยิ่งฤทธิ์ของสารตัวนี้ในระยะเวลาการให้สารในช่วงเวลาต่าง ๆ ขณะที่กล้ามเนื้อหัวใจขาดเลือด สารสำคัญของงานวิจัยมีดังนี้

p38 mitogen-activated protein kinase (p38) has been shown to play an important role in facilitating myocardial infarction process in ischemia/reperfusion (I/R) injury. Inhibition of p38 prior to ischemia has been shown as cardioprotective. However, the benefit of inhibition of p38 after ischemia or during reperfusion is unknown. We tested the hypothesis that inhibition of p38 at different times during I/R can attenuate ventricular fibrillation (VF) incidence and reduce the infarct size.

Adult Wistar rats were subject to 30-min left anterior descending coronary artery (LAD) occlusion, followed by 120-min reperfusion. A p38 inhibitor, SB203580, was given intravenously (2-mg/kg) either at 15 minutes before ischemia (pretreatment group), 15 minutes after LAD occlusion (ischemia group),

or at onset of reperfusion. Saline was used as a vehicle in each group. Electrocardiogram was recorded and the arrhythmia score was analyzed based on the frequency and duration of arrhythmias detected (1 is lowest and 5 was highest arrhythmia incidence). The infarct size was determined in each heart.

SB203580 given before LAD occlusion and during ischemia significantly decreased the overall arrhythmia score and the incidence of VT/VF, compared with the vehicle-treated group (Figure). However, SB203580 given at onset of reperfusion did not show this benefit. The time to VT/VF onset was not changed for SB203580 treated at any time, compared to the vehicle. Unlike arrhythmia incidence, the infarct size was markedly decreased in SB203580-treated rats in all groups. In summary, Although SB203580 could markedly decrease the infarct size when administered before or after LAD occlusion, as well as during reperfusion, it could only effectively attenuate the fatal arrhythmias when given only prior to LAD occlusion and during ischemia. These findings indicate the crucial role of the timing of p38 inhibition in preventing fatal arrhythmia in an I/R model.

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- 1.6 ได้ทำการศึกษาถึงผลของ KP extract ต่อ nitric oxide signaling pathway ในหัวใจของหนูขาวในระดับหนึ่งแล้ว KP extract มี positive effect ต่อ nitric oxide signaling pathway และมีผลต่อการเพิ่มขึ้นของระดับ cGMP ในหัวใจของหนู รวมทั้งมีผลในการออกฤทธิ์ใกล้เคียงกับ sildenafil citrate สารสำคัญของงานวิจัยมีดังนี้

The rhizomes of Krachai-dam (*Kaempferia parviflora*, KP) is one of the Thai traditional medicine that has been used for rejuvenation in elderly people, relieving digestive disorders, gastric ulcer, diuresis, and has been used as anti-allergy and tonic. The macerated KP rhizome in alcoholic drinking or KP tea are believed to improve erectile dysfunction. However, this effect of KP has not been elucidated by scientific research. Recently, the ethanolic extract of KP has been shown to increase the endothelial nitric oxide (NO) synthase (eNOS) mRNA and protein expression in primary cell culture of human umbilical vein endothelial cells. Moreover, the effect of KP extract has shown to vasodilate rat aortic rings in an *in vitro* study and decreased mean arterial pressure in an *in vivo* model. This vasorelaxation mechanism of KP has been found to regulate via the positive effect on NO signaling pathway. Despite that, the effect of KP extract on NO signaling in the heart has never been investigated.

In the heart, NO signaling has been shown to play an important role in regulating the cellular ionic concentration, especially Ca^{2+} . It has been shown that NO can modulate the function of L-type Ca^{2+} channel by the indirect mechanism via the production of cGMP and phosphorylation of protein kinase G (PKG). Moreover, NO also increase the Ca^{2+} reuptake rate into sarcoplasmic reticulum via the increased the phosphorylation of phospholamban.

In traditional medicine, KP is believed to improve erectile dysfunction (ED) similar to the effect of sildenafil citrate, the phosphodiesterase type 5 (PDE-5) inhibitor. However, there is no scientific study to support this statement. Since KP has been shown to have positive effect on the NO signaling, which may resulting in the increased of intracellular cGMP level similar to that of sildenafil citrate, we further investigated effect of KP extract on PDE-5 in the heart.

We found that KP extract increased cGMP level in the rat hearts, but failed to increase eNOS and neuronal NOS. In swine study, KP extract at high concentration significantly decreased defibrillation efficacy and increased vulnerability to arrhythmia similar to that of the supratherapeutic concentration of sildenafil citrate. These findings suggest that KP extract could have the PDE-5 inhibitory effect similar to that of sildenafil citrate. Future studies are needed to clarify the mechanism by which KP extract has an effect on the NO signaling pathway in addition to the PDE-5 inhibitory effect.

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ใน thalassemic hearts

- 1.7 ได้ทำการศึกษาถึงกลไกในการนำเหล็กเข้าสู่เซลล์ของ cultured cardiomyocytes ที่ได้จากหัวใจของหนูที่เป็น thalassemia โดยอาศัยการใช้ blockers ต่อ L-type calcium channels, Transferin Receptor และ DMT-1 เพื่อศึกษาการเปลี่ยนแปลงการนำเหล็กเข้าสู่เซลล์และในระหว่างนี้อยู่ระหว่างรอการทดสอบเพิ่มเติม โดยการใช้ T-type calcium channel blocker ทั้งนี้พบว่า T-type calcium channel blocker สามารถป้องกันการนำเข้าของเหล็ก ใน thalassemic cardiomyocytes ได้ งานวิจัยนี้ทำให้มีการรายงานเป็นครั้งแรกถึงบทบาทของ T-type calcium channel ต่อการนำเข้าของเหล็กเข้าสู่ cardiac cells ซึ่งอาจนำไปสู่การพัฒนาการรักษาที่ดีและมีประสิทธิภาพสูงขึ้นในอนาคต

Iron-overload condition can be found in beta-thalassemic patients with regular blood transfusion, leading to iron deposition in various organs including the heart. Elevated cardiac iron causes iron-overload cardiomyopathy, a condition

which provokes mortality due to heart failure in thalassemic patients. Previous studies demonstrated that myocardial iron uptake may occur via L-type calcium channels (LTCCs). However, direct evidence regarding the claimed pathway in thalassemic cardiomyocytes has never been investigated. Hearts from genetic-altered beta-thalassemic mice and adult wild-type mice were used for cultured ventricular cardiomyocytes. Blockers for LTCC, T-type calcium channel (TTCC), transferrin receptor1 (TfR1) and divalent metal transporter1 (DMT1) were used and quantification of cellular iron uptake under various iron loading conditions was performed by Calcein-AM fluorescence assay. Microarray analysis was performed to investigate gene expressions in the hearts of these mice. This study demonstrated that iron uptake under iron-overload conditions in the cultured ventricular myocytes of thalassemic mice was greater than that of wild type cells ($p < 0.01$). TTCC blocker, efonidipine, and an iron chelator, deferoxamine, could prevent iron uptake into cultured cardiomyocytes, whereas blockers of TfR1, DMT1, and LTCC could not. Microarray analysis from thalassemic hearts demonstrated highly up-regulated genes of TTCC, zinc transporter and transferrin receptor2. Our findings indicated that iron uptake mechanisms in cultured thalassemic cardiomyocytes are mainly mediated by TTCC, suggesting that TTCC is the important pathway for iron uptake in this cultured thalassemic cardiomyocyte model.

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- 1.8 ได้ทำการศึกษาผลของการให้เหล็กในขนาดต่าง ๆ ผสมในอาหารให้กับหนูธาลัสซีเมียและหนูปกติเป็นเวลา 2 เดือน เพื่อตรวจวัดระดับของเหล็ก NTBI ในเลือดรวมถึงการเปลี่ยนแปลงทางไฟฟ้าและการทำงานของหัวใจ เพื่อที่จะศึกษาถึงการตอบสนองของการให้เหล็กต่อระดับเหล็กในอวัยวะต่าง ๆ ระหว่างหนูที่เป็นธาลัสซีเมียกับหนูปกติ และได้ทำการศึกษาถึงผลของ blocker ชนิดต่าง ๆ รวมถึง T-type calcium channel blocker ต่อการป้องกันการสะสมของเหล็ก NTBI ในหัวใจและอวัยวะต่าง ๆ รวมถึงการศึกษาถึงการเปลี่ยนแปลงทางไฟฟ้าในหัวใจของหนูที่เป็นธาลัสซีเมียและหนูปกติเพื่อศึกษาดูว่ายาชนิดต่าง ๆ ที่ให้เข้าไปสามารถป้องกันการสะสมของเหล็กในหัวใจและช่วยป้องกันการเปลี่ยนแปลงสมดุลของระบบประสาทอัตโนมัติในหัวใจได้หรือไม่ โดยที่การศึกษานี้จะเป็นการยืนยันว่าผลการศึกษาที่ได้จาก cell culture สามารถนำไปใช้ได้ในตัวสัตว์ทดลองได้หรือไม่ สารสำคัญของงานวิจัยมีดังนี้

Excess plasma iron can lead to iron deposition in many organs including the heart. In thalassemia, excess cardiac iron deposition can cause cardiac dysfunction and depressed heart rate variability (HRV). Recently, T-type calcium channel (TTCC) has been shown as a possible gateway for iron entry in thalassemic cardiomyocytes. However, the role of TTCC in "in vivo" with iron overload has never been investigated. We tested the hypothesis that TTCC blocker can improve impaired cardiac function and attenuate the depressed HRV, leading to decreased mortality in iron-overloaded mice. C57/BL6 adult mice were fed with either normal diet (control group) or diet supplemented with dicyclopentadienyl iron (FE group) for 90 days to induce iron overload condition. Then, mice in each group were divided into subgroups being treated with L-type calcium channel (LTCC) blocker, verapamil and nifedipine; TTCC blocker, efonidipine; or iron chelator deferoxamine (DFO) for 30 days. Pressure-volume (P-V) conductance catheter system was used for cardiac function assessment. HRV was determined at baseline and at the end of treatment in all mice. Iron-overloaded mice demonstrated impaired cardiac function as shown by decreased stroke volume (SV), cardiac output (CO), ejection fraction (EF), impaired HRV as indicated by increased LF/HF ratio, with high mortality rate (Figure). Efonidipine effectively improved cardiac function impairment, whereas LTCC blockers and DFO did not. Efonidipine and DFO also significantly improved HRV parameter, whereas LTCC blockers could improve it at a lower extent. Both efonidipine and DFO markedly decreased mortality, while LTCC blocker did not, in these mice with iron overload. Impaired cardiac function and depressed HRV caused by iron overload were improved by efonidipine, but not by verapamil and nifedipine, suggesting that TTCC but not LTCC, plays an important role for iron uptake in the heart of iron-overloaded mice.

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2. การผลิตนักศึกษาบัณฑิต

ขณะนี้เมื่อนักศึกษาปริญญาโท ที่ทำงานวิจัยในโครงการนี้ จำนวน 7 คน จบการศึกษาแล้ว จำนวน 4 คน ที่กำลังจะสอบป้องกันวิทยานิพนธ์ภายในเดือนตุลาคม พ.ศ. 2555 นี้ จำนวน 3 คน และมีนักศึกษาปริญญาเอก ที่ทำงานวิจัยในโครงการนี้ จำนวน 11 คน ซึ่งจบการศึกษาไปแล้ว จำนวน 2 คน และส่งได้ส่งไปฝึกปฏิบัติงานด้านงานวิจัย ณ ต่างประเทศอีกจำนวน 2 คน

3. ผลงานที่เกิดจากทุนส่งเสริมวิจัยนี้

3.1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน 34 เรื่อง

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3.2 ความก้าวหน้าในการสร้างทีมวิจัย

ในขณะนี้ได้ทำการสนับสนุนนักวิจัยรุ่นใหม่ ได้ทำวิจัยในสาขาที่เกี่ยวข้องกับโครงการ พร้อมทั้งได้สนับสนุนให้ขอรับทุนวิจัยสำหรับนักวิจัยรุ่นใหม่จาก สกว. ซึ่งขณะนี้ มีผู้ที่ได้รับทุนไปแล้ว จำนวน 6 คน โดยได้ทำการศึกษาวิจัยในด้านของโรคหัวใจและ กลไกการเกิดโรคเป็นหลัก นอกจากนี้ยังได้ร่วมมือในการทำวิจัยกับหน่วยงานต่าง ๆ ในการให้การฝึกอบรมนักวิจัยในสาขา Cardiac electrophysiology ด้วย นอกจากนี้ยัง ได้รับนักวิจัยในระดับปริญญาโท เข้ามาฝึกการทำวิจัยเพิ่มอีก 2 คน ภายใต้โครงการนี้

3.3 การนำผลจากโครงการไปใช้ประโยชน์

ได้มีการตีพิมพ์เผยแพร่แล้ว 34 เรื่อง (ดังรายละเอียดข้างต้น) และการบรรยายให้ความรู้ต่อสาธารณชน ดังรายละเอียดในข้อ 3 และข้อ 5.4

4. กิจกรรมอื่น ๆ ที่เกี่ยวข้อง ได้แก่

4.1 ผลงานอื่น ๆ เช่น การไปเสนอผลงาน การได้รับเชิญไปเป็นวิทยากร การได้รับรางวัล

4.1.1 การได้รับเชิญเป็นวิทยากร

ปี พ.ศ. 2555

- 1) อาจารย์พิเศษบรรยายในหัวข้อ “Vital sign” ทำการสอนนักศึกษาทันตแพทยศาสตร์ กระบวนวิชาพยาธิเคระห์โรคช่องปาก (DDIA 481) ประจำปีการศึกษา 2555 ณ คณะทันตแพทยศาสตร์
จัดโดย คณะทันตแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
ในวันที่ 16 กรกฎาคม พ.ศ. 2555 ใช้เวลา 13.00-14.00 น.
- 2) ประธาน (Chairperson) ในการบรรยายของนักศึกษาโครงการปริญญาเอก กายจนภิเชก ครั้งที่ 13 (RGJ-Ph.D. Congress XIII) ใน Section S4: Viral infection and immunology ในงานประชุมวิชาการ โครงการปริญญาเอกกายจนภิเชก ครั้งที่ 13 (RGJ-Ph.D. Congress XIII) ณ โรงแรมจอมเทียน ปาล์มบีช รีสอร์ท พัทยา จังหวัดชลบุรี
จัดโดย โครงการปริญญาเอกกายจนภิเชก (คปก.) สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
ในวันที่ 7 เมษายน พ.ศ. 2555 ใช้เวลา 08.30-10.45 น.
- 3) วิทยากรรับเชิญ บรรยายในหัวข้อ “การตอบคำถามจาก reviewer ในการส่ง manuscript ตีพิมพ์ในวารสารนานาชาติ” ในโครงการอบรมเรื่อง การตอบคำถามจาก reviewer ในการส่ง manuscript ตีพิมพ์ในวารสารนานาชาติ ณ ห้องประชุมศาสตราจารย์เกียรติคุณ นายแพทย์ชัยโรจน์ แสงอุดม ชั้น 12 อาคาร 12 ชั้น
จัดโดย งานวิจัยและวิเทศสัมพันธ์ คณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่
ในวันที่ 3 เมษายน พ.ศ. 2555 ใช้เวลา 13.30-15.00 น.
- 4) วิทยากรรับเชิญ บรรยายในหัวข้อ “Heart rate variability and its significance” ในงานประชุม International conference the oxidative stress in congenital and acquired hemolytic anemia” ณ โรงแรมการ์เดน คลิฟ รีสอร์ท แอนด์ สปา จ. ชลบุรี
จัดโดย ภาควิชาชีวเคมี คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล
ในวันที่ 22 มีนาคม พ.ศ. 2555 ใช้เวลา 15.05-15.25 น.

- 5) วิทยาการรับเชิญ บรรยายในหัวข้อ Symposium: Up-to-date in cellular and molecular biology in heart failure” ในงานประชุม The 6th Asian pacific congress of heart failure 2012 (APCHF 2012) ณ โรงแรมเลอเมเรเดียนเชียงใหม่ จ. เชียงใหม่
จัดโดย ชมรมหัวใจล้มเหลวแห่งประเทศไทย ร่วมกับ ASEAN federation of cardiology ร่วมกับ Asian pacific of society of cardiology และ สมาคมแพทย์โรคหัวใจแห่งประเทศไทย
ในวันที่ 5 กุมภาพันธ์ พ.ศ. 2555 ใช้เวลา 08.00-09.00 น.
- 6) วิทยาการรับเชิญ บรรยายในหัวข้อ “Cardiac Electrophysiology and Biomedical Engineering” ในงานประชุม The Biomedical Engineering International Conference (BME icon) 2012 ณ โรงแรม คันทรีฮิลล์ จ. เชียงใหม่
จัดโดย Thai Biomedical Engineering Society (ThaiBME)
ในวันที่ 29-31 มกราคม พ.ศ. 2555
- 7) วิทยาการรับเชิญ บรรยายในหัวข้อ “คุณภาพวิทยานิพนธ์/การค้นคว้าอิสระ/แลกเปลี่ยนประสบการณ์จากอาจารย์ที่ปรึกษาวิทยานิพนธ์” ณ โรงแรม เชียงใหม่ฮิลล์ จ. เชียงใหม่ ในงานสัมมนาเรื่อง บทบาทอาจารย์ที่ปรึกษาวิทยานิพนธ์/การค้นคว้าแบบอิสระกับคุณภาพวิทยานิพนธ์/การค้นคว้าแบบอิสระ จัดโดย งานบริหารทั่วไป บัณฑิตวิทยาลัย มหาวิทยาลัยเชียงใหม่
ในวันที่ 25 มกราคม พ.ศ. 2555

ปี พ.ศ. 2554

- 8) วิทยาการรับเชิญ บรรยายในหัวข้อ “Why Literature review?” ในงานประชุม วิชาการคลินิกวิจัย ครั้งที่ 8 ณ ชั้น 15 อาคารสุจินโณ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดย ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 6 มกราคม พ.ศ. 2554
- 9) วิทยาการรับเชิญ บรรยายในหัวข้อ “บทบาทหน้าที่ของอาจารย์ที่ปรึกษาวิทยานิพนธ์ที่ดีสำหรับนักศึกษาบัณฑิตศึกษา” ในการอบรม “อาจารย์ที่ปรึกษาวิทยานิพนธ์” ณ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดย งานบริการการศึกษา และพัฒนาคุณภาพนักศึกษา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 24 มีนาคม พ.ศ. 2554

- 10) วิทยาการรับเชิญ บรรยายในหัวข้อ “แรงจูงใจในการสร้างสรรค์งานวิจัย”
ในการสัมมนาเรื่อง “เสวนาวิชาการกับคณะกรรมการส่งเสริม พัฒนางานวิจัย
และดำเนินการเกี่ยวกับงบประมาณโรคเอดส์” ณ ห้องประชุมดอกสีก ชั้น 2
คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดย คณะเทคนิคการแพทย์
มหาวิทยาลัยเชียงใหม่ ในวันที่ 2 มิถุนายน พ.ศ. 2554
- 11) วิทยาการรับเชิญ บรรยายในหัวข้อ “การตั้งวัตถุประสงค์การเรียนรู้”
ในการสัมมนาเรื่อง “ปฐมนิเทศนักศึกษาบัณฑิต” ณ ชั้น 2 อาคารเรียนรวม ราช
นครินทร์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดย หน่วยบริหาร
หลักสูตร คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 3 มิถุนายน พ.ศ.
2554
- 12) วิทยาการรับเชิญ บรรยายในหัวข้อ “กลยุทธ์สร้างแรงบันดาลใจในการทำวิจัย”
ในการสัมมนาวิจัย (Research club) ครั้งที่ 5/2554 ณ ห้องประชุม
กรรมการคณะฯ ชั้น 2 อาคาร 7 คณะทันตแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
จัดโดย งานบริการการศึกษาบริหารงานวิจัยและบริการวิชาการ คณะทันต
แพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
ในวันที่ 29 มิถุนายน พ.ศ. 2554
- 13) วิทยาการรับเชิญ บรรยายในหัวข้อ “ทำอย่างไรให้ประสบความสำเร็จในการ
ทำงานวิจัย” ในการประชุมสัมมนาวิจัย ประจำปี 2554 ณ สวนพฤษภาดา
ทวิชล อ. ดอยสะเก็ด จ. เชียงใหม่ จัดโดย ภาควิชาออร์โทปิดิกส์ คณะ
แพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 10 กรกฎาคม พ.ศ. 2554
- 14) อาจารย์พิเศษ บรรยายในหัวข้อ “Vital sign” ทำการสอนนักศึกษาทันต-
แพทยศาสตร์ กระบวนวิชาพีเคาระห์โรคช่องปาก (DDIA 481) ประจำปีการศึกษา
2554 ณ คณะทันตแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดยคณะทันต
แพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
ในวันที่ 13 กรกฎาคม พ.ศ. 2554
- 15) วิทยาการรับเชิญ บรรยายในหัวข้อ “การเขียนบทความทางวิชาการเพื่อตีพิมพ์”
ณ ห้องพุทธชาต ชั้น 3 อาคารเฉลิมพระเกียรติ คณะเภสัชศาสตร์ จัดโดย คณะ
เภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 29 กรกฎาคม พ.ศ. 2554

- 16) วิทยากรรับเชิญ บรรยายในหัวข้อ “Overview & Pathophysiology” จัดโดย สถาบันสุขภาพเด็กแห่งชาติมหาราชินี กรมการแพทย์ ณ โรงแรมรามาคาร์-เด้น ถนนวิภาวดี กรุงเทพมหานคร ในวันที่ 8 กันยายน พ.ศ. 2554

ปี พ.ศ. 2553

- 17) วิทยากรรับเชิญบรรยายในหัวข้อ “Translational Research in Thailand” ในงานประชุมวิชาการสรีรวิทยาสมาคมแห่งประเทศไทย ครั้งที่ 39 ณ โรงแรมอมาริโอเคดริสอร์ทแอนด์ทาวเวอร์พทยา จ. ชลบุรี จัดโดยภาควิชา สรีรวิทยา มหาวิทยาลัยมหิดล ร่วมกับ สรีรวิทยาสมาคมแห่งประเทศไทย ในวันที่ 5 พฤษภาคม พ.ศ. 2553
- 18) วิทยากรรับเชิญ บรรยายในหัวข้อ “Update in Cardiovascular Research” ในงานกิจกรรม Journal Club ณ คณะสหเวชศาสตร์ มหาวิทยาลัยนเรศวร จ. พิษณุโลก จัดโดย คณะสหเวชศาสตร์ มหาวิทยาลัยนเรศวร จ. พิษณุโลก ในวันที่ 11 มิถุนายน พ.ศ. 2553
- 19) วิทยากรรับเชิญ บรรยายในหัวข้อ “Cardiac Research Update” ในงานประชุม RGJ seminar series XLVIII: Medical Science: Molecular Medicine: Today and Tomorrow ณ โรงแรมศิรินาถการ์เด้น จัดโดย ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ร่วมกับโครงการปริญญาเอกกาญจนาภิเษก สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ในวันที่ 2 กรกฎาคม พ.ศ. 2553
- 20) อาจารย์พิเศษ บรรยายในหัวข้อ “Vital sign” ทำการสอนนักศึกษาทันตแพทยศาสตร์ กระบวนวิชาวิเคราะห์โรคช่องปาก (DDIA 481) ประจำปีการศึกษา 2553 ณ คณะทันตแพทยศาสตร์ จัดโดย คณะทันตแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 7 กรกฎาคม พ.ศ. 2553
- 21) อาจารย์พิเศษ บรรยายในหัวข้อ “Cardiology (Arrhythmia)” แก่นักศึกษาสัตวแพทย์ ชั้นปีที่ 5 คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ กระบวนวิชาโรคของสุนัขและแมว (651531) ประจำปีการศึกษา 2553 ณ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดย ภาควิชาสัตวเล็ก คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 4 สิงหาคม พ.ศ. 2553

- 22) วิทยากรรับเชิญ บรรยายในหัวข้อ “Writing result and discussion” และหัวข้อ “Dealing with the Editors and Reviewer’s Comment” ในโครงการอบรมเชิงปฏิบัติการ เรื่อง “การเขียนบทความวิชาการทางการแพทย์” ณ ห้องบอลรูมอัลไพน์กอล์ฟรีสอร์ทเชียงใหม่ จัดโดย งานวิจัยและวิเทศสัมพันธ์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 9-10 กันยายน พ.ศ. 2553

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- 23) วิทยากรรับเชิญในหัวข้อ “How to perform non-invasive central arterial pressure measurement” ในงานประชุมวิชาการ Chiang Mai Cardiology Conference (CMCC) 11th จัดโดย หน่วยระบบหัวใจและหลอดเลือด ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 12 กันยายน พ.ศ. 2552
- 24) วิทยากรรับเชิญ บรรยายในหัวข้อ “From mice to men: A translation research from the heart” ในงานประชุมโครงการกาญจนาภิเษกครั้งที่ 74 (RGJ Seminar Series LXXIV) เรื่อง From Basic Biomedical Research to Sustainable Development จัดโดย ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ร่วมกับโครงการปริญญาเอกกาญจนาภิเษก สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ในวันที่ 16 กันยายน พ.ศ. 2552
- 25) วิทยากรรับเชิญ บรรยายในหัวข้อ “เกณฑ์มาตรฐานและตัวชี้วัดคุณภาพงานวิจัยและนักวิจัย: มุมมองของ Review, (Journal), Reader (ตำแหน่งทางวิชาการ) และผู้ให้ทุน” ในงานประชุมวิชาการ “Quest for Excellence” ครบรอบ 50 ปี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จัดโดย คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในวันที่ 3 พฤศจิกายน พ.ศ. 2552
- 26) วิทยากรรับเชิญ บรรยายในหัวข้อ “Defibrillation Threshold Testing” ในงานประชุมวิชาการ Cardiac Electrophysiology Club Meeting ณ โรงแรมภูเก็ต จังหวัดกรุงเทพฯ จัดโดยชมรมช่างไฟฟ้าหัวใจ (Cardiac Electrophysiology) ในวันที่ 21 พฤศจิกายน พ.ศ. 2552
- 27) วิทยากรรับเชิญ บรรยายในหัวข้อ “L-type calcium channel blocker fails to inhibit iron uptake into cardiomyocytes of β -thalassemia mice” ในงานประชุมวิชาการ Asia Pacific Iron Academy Conference 2009 ณ โรงแรมแชงกีล่า จังหวัดเชียงใหม่ จัดโดย Siriraj-Thalassemia Research Program,

Department of Research & Development ในวันที่ 28 พฤศจิกายน พ.ศ.
2552

**4.1.2 สรุปรางวัลที่ได้รับตลอดระยะเวลาที่ได้รับทุนเมธีวิจัยอาวุโส สกว. ตั้งแต่ พ.ศ.
2552-2555**

ประจำปี พ.ศ. 2553

1. นักศึกษาปริญญาโทและปริญญาเอก จากศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ ภายใต้การเป็นอาจารย์ที่ปรึกษาวิทยานิพนธ์ ได้รับรางวัลในการนำเสนอผลงานวิจัยของงานประชุมสรีรวิทยาสมาคมแห่งประเทศไทย ครั้งที่ 39 ซึ่งจัดขึ้นในวันที่ 5-7 พ.ค. 2553 ณ โรงแรม อมารีออคิดริสอร์ท แอนด์ทาวเวอร์ พัทยา จ. ชลบุรี รายละเอียด ดังนี้

1.1 การนำเสนองานวิจัยยอดเยี่ยมระดับปริญญาเอกด้วยวาจา

“รางวัลศาสตราจารย์ นายแพทย์ดิถี จิ่งเจริญ”

ผู้ได้รับรางวัลที่ 1 คือ อ. วาสนา ปรัชญาสกุล

ผลงานวิจัยเรื่อง “The defect of neuronal function of insulin receptor in rat's hippocampus following 12-week high-fat consumption”

ผู้ได้รับรางวัลที่ 2 คือ อ. ภูเนตร วีรธีรางกูร

ผลงานวิจัยเรื่อง “Effects of Kaempferia parviflora extract on the expression of nitric oxide synthase and cGMP level in rat hearts”

1.2 การนำเสนองานวิจัยยอดเยี่ยมระดับปริญญาเอกด้วยโปสเตอร์

“รางวัลศาสตราจารย์ ดร. เลียงชัย ลี้มล่อมวงศ์”

ผู้ได้รับรางวัลที่ 1 คือ น.ส. ศิรินาฏ คำฟู

ผลงานวิจัยเรื่อง “Mechanism of iron entry in cultured thalassemic cardiomyocytes”

1.3 การนำเสนองานวิจัยยอดเยี่ยมระดับปริญญาโทด้วยวาจา

รางวัลศาสตราจารย์ นายแพทย์ประสพ รัตนากร”

ผู้ได้รับรางวัลที่ 1 คือ นางสาวสาวิตรี ธรรมสอน

ผลงานวิจัยเรื่อง “Cardioprotective effects of granulocyte-colony stimulating factor against mitochondrial damage under oxidative stress in isolated cardiac mitochondria”

ประจำปี พ.ศ. 2554

2. พญ. ชลธิดา ยาระณะ ตำแหน่งแพทย์ใช้ทุน จากศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ ภายใต้การเป็นอาจารย์ที่ปรึกษา ได้รับรางวัลทุนนักวิจัยรุ่นใหม่ ประจำปี พ.ศ. 2554 จากโครงการวิจัย เรื่อง “การเปรียบเทียบผลของแคลเซียมต่อ mitochondrial permeability transition และ oxidative stress ใน isolated mitochondria จากหัวใจและสมองหนู”

ประจำปี พ.ศ. 2555

3. นักศึกษาปริญญาปริญญาเอก จากศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ ภายใต้การเป็นอาจารย์ที่ปรึกษาวิทยานิพนธ์ ได้รับรางวัลในการนำเสนอผลงานวิจัย ในงานประชุมวิชาการโครงการปริญญาเอกกาญจนาภิเษก ครั้งที่ 13 (RGJ-Ph.D. Congress XIII) ซึ่งจัดขึ้นในระหว่างวันที่ 6-8 เมษายน พ.ศ. 2555 ณ โรงแรมจอมเทียน ปาล์ม บีช รีสอร์ท เมืองพัทยา จ. ชลบุรี

ผู้ได้รับรางวัลการนำเสนอผลงานวิจัยยอดเยี่ยมระดับปริญญาเอกด้วยวาจา รายละเอียดดังนี้

3.1 อ. ดร. วาสนา ปรัชญาสกุล

ผลงานวิจัยเรื่อง “Effect of estrogen on neuronal insulin receptor function in long term high fat-fed rats”

3.2 น.ส. ศิริรัตน์ สุรินทร์แก้ว

ผลงานวิจัยเรื่อง “Inhibition of p38 activation during ischemia, but not reperfusion, effectively attenuated fatal arrhythmia incidence in rats with ischemia/reperfusion injury”

3.3 น.ส. ศิรินาฏ คำฟู

ผลงานวิจัยเรื่อง “Effect of calcium channel and divalent metal transporter1 blockers on cardiac functions, iron deposition and mortality of iron-loaded thalassemia”

4. นักศึกษาปริญญาปริญญาเอก จากศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ ภายใต้การเป็นอาจารย์ที่ปรึกษาร่วมวิทยานิพนธ์ ได้รับรางวัลในการนำเสนอผลงานวิจัย ได้รับรางวัลในการนำเสนอผลงานวิจัย ของงานประชุมสรีรวิทยาสมาคมแห่งประเทศไทย ครั้งที่ 41 ซึ่งจัดขึ้นในวันที่ 2-4 พ.ค. 2555 ณ อาคารศรีสวรินทิรา คณะแพทยศาสตร์ศิริราชพยาบาล

ผู้ได้รับรางวัลการนำเสนอผลงานวิจัยยอดเยี่ยมระดับปริญญาเอกด้วยวาจา รายละเอียดดังนี้

4.1 น.ส. นพมาศ พิพัฒน์พิบูลย์

ผลงานวิจัยเรื่อง “DPP-4 inhibitor improves neuronal insulin receptor function and brain mitochondrial function caused by high-fat diet consumption”

ผู้ได้รับรางวัลการนำเสนอผลงานวิจัยยอดเยี่ยมระดับปริญญาเอกด้วยโปสเตอร์ รายละเอียดดังนี้

4.2 ภก. จิรภาส ศรีเพ็ชรวันดี

ผลงานวิจัยเรื่อง “Mitochondrial calcium uniporter blocker effectively prevents brain mitochondrial dysfunction caused by iron overload.”

5. น.สพ. เกริกเกียรติ จินดา นักศึกษาปริญญาปริญญาเอก จากศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ ภายใต้การเป็นอาจารย์ที่ปรึกษาวิทยานิพนธ์ ได้รับรางวัลเป็นรางวัลระดับนานาชาติ รางวัล “Frontier in Cardio Vascular Biology 2012 Travel Grant Award” ในการประชุม Frontier in CardioVascular Biology 2012 Meeting ซึ่งจัดขึ้นที่กรุง London ประเทศอังกฤษ จากผลงานวิจัยเรื่อง “Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia-reperfusion injury is via prevention of cardiac mitochondrial dysfunction”

6. นักศึกษาระดับปริญญาโท จากศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ภายใต้การเป็นอาจารย์ที่ปรึกษาวิทยานิพนธ์ ได้รับรางวัล Excellent Oral Research Presentation จากการประชุม The 1st ASEAN Plus Three Graduate Research Congress (AGRC 2012) ซึ่งจัดขึ้นระหว่างวันที่ 1-2 มีนาคม พ.ศ. 2555 ณ โรงแรมดิเอ็มเพรสเชียงใหม่

ผู้ได้รับรางวัลการนำเสนอผลงานวิจัยยอดเยี่ยมระดับปริญญาเอกด้วยวาจา รายละเอียดดังนี้

6.1 น.ส. ณัฐยาภรณ์ อภัยใจ

ผลงานวิจัยเรื่อง “Effects of metformin on cardiac function in high-fat diet induced insulin resistant rats”

6.2 น.ส. หิรัญญา ปินตานา

ผลงานวิจัยเรื่อง “Effects of metformin on learning and memory behaviors in high-fat diet-induced insulin resistant rats”

4.1.3 การจัดงานประชุมวิชาการประจำปี

ครั้งที่ 1 งานประชุมวิชาการ “เมธีวิจัยอาวุโส สกว. ประจำปี พ.ศ. 2553 ศาสตราจารย์ ดร. นายแพทย์ นิพนธ์ จักรทิพากร” เรื่อง “Focused Update on Cardiac Research: From Cell to Bedside” ในวันศุกร์ ที่ 14 พฤษภาคม พ.ศ. 2553 ณ ห้องประชุม 153 ชั้น 15 อาคารสุจินโณ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

ครั้งที่ 2 งานประชุมวิชาการ “เมธีวิจัยอาวุโส สกว. ประจำปี พ.ศ. 2554 ศาสตราจารย์ ดร. นายแพทย์นิพนธ์ จักรทิพากร” เรื่อง “Cardioneural Research Network: Bridging Bench to Bedside Care” ในวันศุกร์ ที่ 17 มิถุนายน พ.ศ. 2554 ณ ห้องประชุมเอือนกัตต์ สวนพฤกษศาสตร์ทวีชล อ. ดอยสะเก็ด จ. เชียงใหม่

ครั้งที่ 3 งานประชุมวิชาการ “เมธีวิจัยอาวุโส สกว. ประจำปี พ.ศ. 2555 ศาสตราจารย์ ดร. นายแพทย์นิพนธ์ จักรทิพากร” เรื่อง “The Emerging Frontier of Neurocardiology: Autonomic Regulation of Cardiovascular Function” ในวันที่ 25-26 มิถุนายน พ.ศ. 2555 ณ รวีวารี่รีสอร์ท อ. แม่แตง จ. เชียงใหม่

4.2 กิจกรรมที่เกี่ยวข้องกับการนำผลจากโครงการไปใช้ประโยชน์

การนำความรู้ที่ได้จากงานวิจัยไปบรรยายเผยแพร่ในงานประชุมวิชาการต่าง ๆ

ระดับนานาชาติ

1. Chattipakorn S, Pipatpiboon N, Pintana H, Pratchayasakul W, Chattipakorn N. DDP-4 inhibitor prevents neuronal insulin resistance, brain mitochondrial dysfunction, and impaired learning and memory caused by high-fat diet consumption. *Diabetes* 2012 (In press, Impact Factor = 8.889)
2. Apaijai N, Chattipakorn S, **Chattipakorn N.** Dipeptidyl peptidase-4 (DPP-4) inhibitor preserves cardiac function and heart rate variability and prevents cardiac mitochondrial dysfunction in high fat-induced insulin resistant rats. *Cardiovasc Res* *Cardiovasc Res* 2012;93:1 (suppl):S44. (Impact Factor = 6.051)
3. Chinda K, Palee S, Surinkaew S, Phornphutkul M, Chattipakorn S, **Chattipakorn N.** Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia-reperfusion injury is via prevention of cardiac

mitochondrial dysfunction. *Cardiovasc Res* 2012;93:1 (suppl):S22. (Impact Factor =6.051)

4. Palee S, Chattipakorn S, **Chattipakorn N**. PPAR-gamma agonist rosiglitazone facilitated fatal arrhythmia in ischemic-reperfusion rat hearts by decreased cardiac connexin43 phosphorylation. *Cardiovasc Res* 2012;93:1 (suppl):S28-S29. (Impact Factor = 6.051)
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- 4.3 การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ ทั้งในและต่างประเทศ
1. Professor Andrew Armour
Universite De Montreal, Montreal, CANADA
 2. Professor Jeffrey L. Ardell
East Tennessee State University, Johnson City, Tennessee
 3. Professor Kenji Sunagawa
Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan
 4. Professor Inder Anand
University of Minnesota and VA Medical Center, Minneapolis, MN

5. Professor Sanjay Mittal
Medanta THE MEDICITY, Sector-38, Gurgaon, Haryana-122001, India
6. Professor Bruce H. KenKnight
The Institute of Technology, University of Minnesota, Minneapolis, MN
7. Professor R. Coronel
Academic Medical Center, Rm K2-112, Meibergdreef 9, 1105 AZ
Amsterdam the Netherlands
8. Professor Ofer Binah
Ruth & Bruce Rappaport Faculty of Medicine, Technion–Israel Institute of
Technology, Israel
9. Professor T-J Chen
Department of Physiology, College of Medicine, National Taiwan
University, Taipei, Taiwan
10. Professor Pamela A. Lucchesi
Louisiana State University, Department of Pharmacology, 1901 Perdido
Street P7-1, New Orleans, LA 70112, USA.
11. Professor Henry Krum
Head of Clinical Pharmacology Unit, Director, NHMRC Centre of Clinical
Research Excellence in Therapeutics, Faculty of Medicine, Monash
University, Victoria 3800, Australia
12. Professor Peng-Sheng Chen
Medtronic Zipes Professor of Cardiology
Kranert Institute, School of Medicine, Indiana University, Indianapolis,
USA.
13. Assoc. Professor Lori L. McMahon
Director, Neuroscience Graduate Program, Department of Physiology and
Biophysics 1918 University Blvd, MCLM 964, Birmingham AL 35294-0005
USA.
14. ศาสตราจารย์ นพ. สุทัศน์ ฟูเจริญ
โครงการวิจัยธาลัสซีเมีย สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี,
มหาวิทยาลัยมหิดล วิทยาเขตศาลายา
15. รศ. พญ. นภาพร ตนานุวัฒน์
ภาควิชาจักษุวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่
16. รศ. ดร. พัชรวิทย์ ปั่นแห้งเพชร
ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

17. รศ. ดร. รัชนิกร กัลล์ประวิทย์
ภาควิชาชีวเคมี คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล
18. อ. ดร. สราวุธ คำปาน
คณะสหเวชศาสตร์ มหาวิทยาลัยนเรศวร
19. อ. ดร. ภก. ศุภโชค มั่งมุล
คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล
20. อ. ดร. น.สพ. อนุศักดิ์ กิจถาวรรัตน์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
21. อ. ดร. สพ.ญ. สุนทรี เพ็ชรดี
คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
22. อ. ดร. สุคนธา งามประมวญ
ศูนย์วิจัยประสาทวิทยาศาสตร์, สถาบันวิทยาศาสตร์โมเลกุล
มหาวิทยาลัยมหิดล
23. อ. ดร. น. สพ. เต็มพงศ์ วงศ์ตะวัน
คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล

4.4 การได้รับทุนอื่น ๆ จากทั้งในและต่างประเทศ

1. ชื่อโครงการวิจัย: Effects of rosiglitazone on the cardiac mitochondrial function and molecular aspects of the cardioprotective effects in ischemic/reperfusion rat heart model. (PI)
แหล่งทุน: National Research Council of Thailand
จำนวนเงิน 879,400 บาท
ช่วงของวัน เดือน ปี ที่ได้รับทุน: 10/2011-09/2012 รวมเวลา 1 ปี
2. ชื่อโครงการวิจัย: Mitochondrial calcium uniporter blocker prevents isolated brain mitochondrial dysfunctions caused by iron overload. (PI)
แหล่งทุน: Faculty of Medicine Endowment Fund, Chiang Mai University
จำนวนเงิน 200,000 บาท
ช่วงของวัน เดือน ปี ที่ได้รับทุน: 03/2012-09/2013 รวมเวลา 1.6 ปี
3. ชื่อโครงการวิจัย: Effect of left vagal nerve stimulation on cardiac ischemic-reperfusion injury (PI)
แหล่งทุน: บริษัท Cyberonics ประเทศสหรัฐอเมริกา
จำนวนเงิน 3,708,750 บาท
ช่วงของวัน เดือน ปี ที่ได้รับทุน: 10/2011-09/2012 รวมเวลา 1 ปี

4. ชื่อโครงการวิจัย: ผลของยาพีพีเออาร์แกมมาอะโกนิสต์ (PPAR-gamma agonist) และเอสโตเจน ต่อภาวะการดื้อต่ออินซูลินของเซลล์สมองต่อการทำงานของไมโทคอนเดรียของเซลล์สมองในหนูที่กินอาหารไขมันสูงเป็นเวลานาน ๆ (Co-PI)
แหล่งทุน: สำนักงานกองทุนสนับสนุนการวิจัย (สกว.)
จำนวนเงิน. 1,500,000 บาท
ช่วงของวัน เดือน ปี ที่ได้รับทุน: 05/2011-05/2014 รวมเวลา 3 ปี
5. ชื่อโครงการวิจัย: Effects of Anti-Diabetic Drugs: Vildagliptin in Rats with Neuronal Insulin Resistance Induced by 12- Week High Fat Diet Consumption. (Co-PI)
แหล่งทุน: Faculty of Medicine Endowment Fund, Chiang Mai University
จำนวนเงิน. 200,000 บาท
ช่วงของวัน เดือน ปี ที่ได้รับทุน: 04/2012-10/2013 รวมเวลา 1 ปี

5. Output

Output:	ปี 2552- 2553	ปี 2553- 2554	ปี 2554- 2555	จำนวน รวม ทั้งหมด
1. ด้านงานวิจัย				
1.1 จำนวนผลงานตีพิมพ์ที่ list อยู่ใน pubmed	4	4	26	34
1.2 จำนวนผลงานวิจัยที่นำเสนอในระดับชาติ	5	2	15	22
1.3 จำนวนผลงานวิจัยที่นำเสนอในระดับนานาชาติ	4	2	14	20
1.4 รางวัลด้านการวิจัยที่ได้รับ				
➤ รางวัลการนำเสนอผลงานวิจัยดีเด่น จากงานประชุมวิชาการประจำปีของ สรีรวิทยาสมาคมแห่งประเทศไทย ครั้งที่ 39 ประจำปี พ.ศ. 2553	4			4
➤ รางวัลการนำเสนอผลงานวิจัยดีเด่น จากงานวันมหิดล คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ประจำปี 2554		1		1
➤ The Travel Award Fellowship for ESC Working Group on Cardiovascular Pharmacology and Drug Therapy. Meeting of The Frontiers in CardioVascular Biology 2012			1	1
➤ รางวัลการนำเสนอผลงานวิจัยดีเด่น จากงานประชุมวิชาการประจำปีของ สรีรวิทยาสมาคมแห่งประเทศไทย ครั้งที่ 41 ปี ประจำปี พ.ศ. 2555			2	2
➤ รางวัลการนำเสนอผลงานวิจัยดีเด่น จากงานประชุมวิชาการ งานประชุม วิชาการโครงการปริญญาเอกกาญจนาภิ- เษก ครั้งที่ 13 (RGJ-Ph.D. Congress XIII) ประจำปี พ.ศ. 2555			3	3
➤ รางวัลการนำเสนอผลงานวิจัยดีเด่น จาก			2	2

งานประชุม The 1st ASEAN Plus Three Graduate Research Congress (AGRC 2012)				
1.5 จำนวนทุนวิจัยที่ได้รับสนับสนุนจากองค์กรอื่น	2	1	3	5
2. ด้านบริการวิชาการและการฝึกอบรม				
2.1 จำนวนการเป็นอาจารย์ที่ปรึกษาหรือผู้ทรงคุณวุฒิที่ปรึกษาด้านการวิจัยทุน สกว.	4	2	1	7
2.2 จำนวนนักวิจัยที่ได้รับการฝึกฝนด้านทักษะการวิจัยระดับสูง (Post-doc)	2	2	2	2
2.3 จำนวนการจัดประชุมวิชาการ / การฝึกอบรม	1	1	1	3
3. ด้านการเรียนการสอน				
3.1 จำนวนนักศึกษาระดับบัณฑิตศึกษาที่จบการศึกษา				
- ระดับปริญญาโท	1	1	3	5
- ระดับปริญญาเอก	-	1	1	2
3.2 การเป็นอาจารย์เพื่อวิทยานิพนธ์หลักของนักศึกษาระดับปริญญาโท	2	3	1	6
3.3 การเป็นอาจารย์เพื่อวิทยานิพนธ์หลักของนักศึกษาระดับปริญญาเอก	4	4	2	6

6. ภาคผนวก

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน 34 เรื่อง

- 1 Apaijai N, Pintana H, Chattipakorn SC, **Chattipakorn N**. Effects of metformin and vildagliptin on cardiac and mitochondrial function in high-fat diet-induced insulin resistant rats. *Endocrinology* 2012 (in press) (Impact Factor = 4.99)
(เอกสารแนบหมายเลข 1)
- 2 Wongcharoen W, Jai-aue S, Phrommintikul A, Nawarawong W, Woragidpoonpol S, Tepsuwan T, Sukonthasarn A, Apaijai N, **Chattipakorn N**. Effects of Curcuminoids on Frequency of Acute Myocardial Infarction After Coronary Artery Bypass Grafting. *Am J Cardiol* 2012 (in press) (Impact Factor = 3.680)
(เอกสารแนบหมายเลข 2)
- 3 Yarana C, Sripetchwandee J, Sanit J, Chattipakorn S, **Chattipakorn N**. Calcium-induced cardiac mitochondrial dysfunction is predominantly mediated by cyclosporine A-dependent mitochondrial permeability transition pore, but not mitochondrial calcium uniporter. *Arch Med Res* 2012 (in press) (Impact factor = 1.986)
(เอกสารแนบหมายเลข 3)
- 4 Yarana C, Sanit J, **Chattipakorn N**, Chattipakorn S. Synaptic and nonsynaptic mitochondria demonstrate a different degree of calcium-induced mitochondrial dysfunction. *Life Sci* 2012;90:808-814. (Impact Factor = 2.56)
(เอกสารแนบหมายเลข 4)
- 5 Kumfu S, Chattipakorn S, Chinda K, Fucharoen S, **Chattipakorn N**. T-type calcium channel blockade improves survival and cardiovascular function in thalassemic mice. *Eur J Haematol* 2012;88:535-548. (Impact Factor = 2.785)
(เอกสารแนบหมายเลข 5)
- 6 Chinda K, Palee S, Surinkaew S, Phornphutkul M, Chattipakorn S, **Chattipakorn N**. Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia–reperfusion injury. *Int J Cardiol* 2012 (in press) (Impact Factor = 6.802)
(เอกสารแนบหมายเลข 6)

- 7 Khumpune S, Chattipakorn S, **Chattipakorn N**. Role of p38 inhibition on cardiac ischemia/reperfusion injury. *Eur J Clin Pharmacol* 2012 (in press) (Impact Factor = 3.088)
(เอกสารแนบหมายเลข 7)

- 8 Suwanchai A, Theerapiboon U, **Chattipakorn N**, Chattipakorn S. Na_v1.8, but not Na_v1.9, is up-regulated in the inflamed dental pulp tissue of human primary teeth. *Int Endod J* 2012;45(4):372-8. (Impact Factor = 2.383)
(เอกสารแนบหมายเลข 8)

- 9 Chinda K, Chattipakorn S, **Chattipakorn N**. Cardioprotective effects of incretin during ischemia-reperfusion. *Diabetes Vasc Dis Res* 2012 (in press) (Impact factor = 2.468)
(เอกสารแนบหมายเลข 9)

- 10 Weerateerangkul P, Surinkaew S, Chattipakorn S, **Chattipakorn N**. Effects of *Kaempferia parviflora* Wall. Ex. Baker on electrophysiology of the swine hearts. *Indian J Med Res* 2012 (in press) (Impact Factor = 1.826)
(เอกสารแนบหมายเลข 10)

- 11 Pramojanee S, Pratchayasakul W, **Chattipakorn N**, Chattipakorn S. Low dose dental radiation decreases oxidative stress in osteoblastic cells. *Arch Oral Biol* 2012;57(3):252-6. (Impact Factor = 1.463)
(เอกสารแนบหมายเลข 11)

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ข้อมูลและประวัติย่อของผู้ได้รับทุนวิจัยระดับปริญญาโท/เอก จากโครงการ
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หัวหน้าโครงการผู้รับทุน

Cardioprotective Effects of Metformin and Vildagliptin in Adult Rats with Insulin Resistance Induced by a High-Fat Diet

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Insulin resistance has been shown to be associated with cardiac sympathovagal imbalance, myocardial dysfunction, and cardiac mitochondrial dysfunction. Whereas metformin is a widely used antidiabetic drug to improve insulin resistance, vildagliptin is a novel oral antidiabetic drug in a group of dipeptidyl peptidase-4 inhibitors in which its cardiac effect is unclear. This study aimed to determine the cardiovascular effects of metformin and vildagliptin in rats with insulin resistance induced by high-fat diet. Male Wistar rats were fed with either a normal diet or high-fat diet ($n = 24$ each) for 12 wk. Rats in each group were divided into three subgroups to receive the vehicle, metformin (30 mg/kg, twice daily), or vildagliptin (3 mg/kg, once daily) for another 21 d. Heart rate variability (HRV), cardiac function, and cardiac mitochondrial function were determined and compared among these treatment groups. Rats exposed to a high-fat diet developed increased body weight, visceral fat, plasma insulin, cholesterol, oxidative stress, depressed HRV, and cardiac mitochondrial dysfunction. Metformin and vildagliptin did not alter body weight and plasma glucose levels but decreased the plasma insulin, total cholesterol, and oxidative stress levels. Although both metformin and vildagliptin attenuated the depressed HRV, cardiac dysfunction, and cardiac mitochondrial dysfunction, vildagliptin was more effective in this prevention. Furthermore, only vildagliptin prevented cardiac mitochondrial membrane depolarization caused by consumption of a high-fat diet. We concluded that vildagliptin is more effective in preventing cardiac sympathovagal imbalance and cardiac dysfunction, as well as cardiac mitochondrial dysfunction, than metformin in rats with insulin resistance induced by high-fat diet. (*Endocrinology* 153: 0000–0000, 2012)

Ingestion of food laden with animal fat is the major cause of obesity and can lead to an insulin-resistant condition, a state in which insulin receptor function is impaired, and is characterized by hyperinsulinemia with euglycemia (1–3). It has been shown previously that insulin resistance was associated with impaired cardiac function (4). In rats with insulin resistance induced by a high-fat diet, systolic and diastolic dysfunction (5), as well as cardiac sympathovagal imbalance indicated by depressed heart rate variability (HRV) (6), was also reported. In the past decades, several drugs used to improve insulin sensitivity have been shown

to cause serious adverse cardiac effects (7, 8). Therefore, drugs with effective glycemic control and without harmful effects to the heart are needed for use in diabetic patients.

Metformin is an oral antidiabetic drug that has been used for decades to reduce plasma glucose, improve insulin sensitivity, increase peripheral glucose uptake, and inhibit hepatic glucose production (9). Previous studies reported that metformin could improve cardiac performance in diabetic rats (10, 11). Furthermore, metformin also improved cardiac mitochondrial respiration and increased ATP synthesis in a rat model of heart failure (12). Despite these beneficial

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Abbreviations: ECG, Electrocardiogram; EDP, end diastolic pressure; ESP, end systolic pressure; HF, high frequency; HOMA, Homeostasis Model Assessment; HR, heart rate; HRV, heart rate variation; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetra ethylbenzimidazolcarbocyanine iodide; LF, low frequency; MDA, malondialdehyde; ROS, reactive oxygen species; SV, stroke volume; TBA, thiobarbituric acid.

effects, the cardioprotective effect of metformin in rats with insulin resistance induced by high-fat diet remains unknown.

Vildagliptin is a novel oral antidiabetic drug that inhibits the action of the dipeptidyl peptidase-4 enzyme, resulting in an increased level of glucagon-like peptide 1. Glucagon-like peptide 1 is an incretin hormone released from intestinal L-cells that causes an increased insulin secretion, decreased glucagon secretion, and improved insulin sensitivity and has been shown to exert direct cardiovascular effects in both experimental and clinical studies with or without insulin resistance (13, 14). Although the glycemic control effects of dipeptidyl peptidase-4 inhibitor have been extensively studied, the roles of vildagliptin on the heart are still unclear (15, 16). Furthermore, the effects of vildagliptin on the heart of rats with insulin resistance induced by high-fat diet are unknown.

The present study investigated the effects of metformin and vildagliptin on the heart of rats with insulin resistance induced by a high-fat diet. Because high-fat diet-induced insulin-resistant rats are known to have depressed HRV (6) and mitochondrial and cardiac dysfunction (17), we hypothesized that metformin and vildagliptin can improve the insulin-resistant condition, preserve cardiac sympathovagal balance, improve cardiac function, and prevent cardiac mitochondrial dysfunction in rats with insulin resistance induced by high-fat diet.

Materials and Methods

Animals and diet

All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with National Institutes of Health guidelines. Male Wistar rats weighing 180–200 g. were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. Rats were housed in a temperature control with a 12-h dark, 12-h light cycle. After 7 d of acclimatization, the rats were divided into two groups to receive either a normal diet or a high-fat diet ($n = 24/\text{group}$). In the normal diet group, rats were fed with standard laboratory chow that contained 19.77% energy from fat, whereas rats in the high-fat diet group were fed with a diet containing 59.28% energy from fat for 12 wk (3). Then, rats in each diet group were divided into three treatment groups ($n = 8/\text{group}$). The first group received 15-mg/kg metformin (Glucophage, Merck Serono, Bangkok, Thailand) twice daily (18). The second group received 3 mg/kg vildagliptin (Gulvus, Novartis, Bangkok, Thailand) once daily (19). The third group (*i.e.* control group) received normal saline in an equal volume. All rats were treated by intragastric gavage for 21 d. The body weight was recorded weekly. Blood samples were collected from the tail vein at week zero, wk 12, and at the end of 21-d treatment. The plasma was separated and stored at -85°C until

use. Heart rate variability (HRV) analysis was performed at week zero, wk 4, wk 8, wk 12, and at the end of a 21-d treatment. After being treated with either drugs or saline for 21 d, rats were anesthetized, and the cardiac function was determined using the pressure-volume catheter (Scisense, London, Ontario, Canada) (20). At the end of the study, the heart was rapidly removed and myocardial tissues were used to determine the cardiac malondialdehyde (MDA) level and cardiac mitochondrial function.

Plasma glucose, cholesterol, and insulin level determination

Plasma glucose and total cholesterol levels were measured by colorimetric assay using a commercial kit (Biotech, Bangkok, Thailand) (2). Plasma insulin levels were measured by a sandwich ELISA kit (Linco Research, St. Charles, MO) (2, 3). Insulin resistance was assessed by Homeostasis Model Assessment (HOMA) as a mathematical model describing the degree of insulin resistance, calculated from fasting plasma insulin and fasting plasma glucose concentration. A higher HOMA index indicates a higher degree of insulin resistance (2).

Plasma and cardiac MDA level determination

Plasma and cardiac MDA levels were measured using HPLC based assay (21). Cardiac tissue was homogenized in phosphate buffer, pH 2.8. Plasma and cardiac tissue were mixed with H_3PO_4 and thiobarbituric acid (TBA) to create TBA-reactive substances. The plasma and cardiac TBA-reactive substances concentration was determined directly from a standard curve and reported as MDA equivalent concentration (22).

Heart rate variability (HRV) analysis

The electrocardiogram (ECG) lead II was recorded in each rat using a PowerLab (ADInstruments, Sydney, Australia) and a Chart 5.0 program (6). During ECG recording, rats were placed in a restraint and prohibited from movement (6, 23, 24). The high frequency (HF, 0.6–3 Hz) component representing cardiac parasympathetic activity and low frequency (LF, 0.2–0.6 Hz) component representing cardiac sympathetic and parasympathetic activity were determined using a MATLAB program (6). The LF/HF ratio was considered as an indicator of cardiac sympathetic/parasympathetic tone balance (25). Increased LF/HF ratio (*i.e.* depressed HRV) indicates the cardiac sympathovagal imbalance (23).

Cardiac function measurement

Rats were anesthetized with Zoletil (50 mg/kg, Vibbac Laboratories, Carros, France) and Xylazine (0.15 mg/kg, Laboratorios Calier, S.A., Barcelona, Spain) im injection after which ventral midline incision of the neck was performed for tracheostomy, and rats were ventilated with room air. The right carotid artery was identified, and a pressure-volume loop catheter was inserted into the carotid artery and advanced into the left ventricle. Rats were stabilized for 5 min, after which the pressure-volume (P-V) loop (Scisense, Ontario, Canada) was recorded for 20 min. Cardiac function parameters including heart rate, end-systolic and end-diastolic pressure, maximum and minimum dP/dt , and stroke volume were determined using the analytical software program (Labscribe, Dover, NH).

TABLE 1. Metabolic parameters of normal diet-fed and high-fat diet-fed rats at baseline and wk 12

Metabolic parameters	Baseline		Wk 12	
	ND	HF	ND	HF
Body weight (g)	191 ± 10	192 ± 9	462 ± 6 ^a	567 ± 13 ^{a,b}
Food intake (g)	21 ± 1	20 ± 5	27 ± 3	24 ± 1
Plasma insulin (ng/ml)	2.15 ± 0.29	2.14 ± 0.26	2.19 ± 0.19	3.50 ± 0.50 ^{a,b}
Plasma glucose (mg/dl)	131.69 ± 8.79	140.11 ± 7.52	138.00 ± 9.95	139.08 ± 4.46
HOMA index	16.80 ± 2.26	16.76 ± 2.03	17.95 ± 1.56	24.26 ± 4.65 ^{a,b}
Plasma total cholesterol (mg/dl)	82.75 ± 5.63	83.15 ± 7.48	83.07 ± 7.14	132.18 ± 8.72 ^{a,b}
Plasma MDA (μmol/ml)	2.28 ± 0.10	2.13 ± 0.03	2.77 ± 0.19	6.79 ± 0.07 ^{a,b}

ND, Normal diet; HF, high-fat diet.

^a, $P < 0.05$ vs. baseline; ^b, $P < 0.05$ vs. ND wk 12.

Cardiac mitochondrial isolation and mitochondrial function determination

Cardiac mitochondrial isolation was performed as previously described (26). In brief, the heart of each rat was perfused with normal saline solution and removed rapidly after which the heart was minced and homogenized in ice-cold buffer containing sucrose (300 mmol/liter), N-(Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid sodium salt (5 mmol/liter), and EGTA (0.2 mmol/liter). Then, the homogenates were centrifuged at $800 \times g$ for 5 min, and a supernatant was collected and centrifuged at $8800 \times g$ for 5 min. Protein concentration was determined using the bicinchoninic acid assay (26). In the present study, cardiac mitochondrial function was determined by measuring the mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential changes, and mitochondrial swelling (26).

To determine cardiac mitochondrial ROS production, cardiac mitochondria were incubated with 2 μM DCFH-DA dye at 25°C for 20 min. ROS production was detected by fluorescent microplate reader with $\lambda_{\text{emission}}$ at 485 nm and $\lambda_{\text{excitation}}$ at 530 nm (BioTek, Winooski, VT) (26). To determine cardiac mitochondrial membrane potential changes, cardiac mitochondria were incubated with 5 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) dye at 37°C for 30 min. Mitochondrial membrane potential changes were measured by fluorescent microplate reader. JC-1 monomer form (green fluorescent) was detected with $\lambda_{\text{emission}}$ at 485 nm and $\lambda_{\text{excitation}}$ at 590 nm. JC-1 aggregate form (red fluorescent) was detected with $\lambda_{\text{emission}}$ at 485 nm and $\lambda_{\text{excitation}}$ at 530 nm. Mitochondrial membrane potential changes were calculated as the red/green fluorescent intensity ratio (26). Decreased red/green fluorescent intensity ratio indicated cardiac mitochondrial membrane depolarization (26).

To determine cardiac mitochondrial swelling, cardiac mitochondria were incubated with 1.5 mM respiration buffer containing 100 mM KCl, 10 mM HEPES, 5 mM KH_2PO_4 , and the absorbance was measured using a spectrophotometer. Mitochondrial swelling was indicated when the absorbance of the suspension decreased (26).

Statistical analysis

All data were expressed as mean ± SE. One-way ANOVA followed by LSD *post hoc* test was used to determine the difference between groups. $P < 0.05$ was considered statistically significant.

Results

Effects of high-fat diet consumption, metformin, and vildagliptin on metabolic parameters

At the baseline, the body weight, food intake, plasma glucose, insulin, total cholesterol, and MDA level did not differ between the normal diet and the high-fat diet groups (Table 1). After 12 wk of high-fat diet consumption, rats in this group had increased body weight and plasma cholesterol. High-fat diet-fed rats also developed insulin resistance that was characterized by an increased plasma insulin level without an alteration in the plasma glucose level, and an increase in the HOMA index. The plasma MDA level, which is an index of oxidative stress, was also increased in the high-fat group (Table 1). Unlike the high-fat fed rats, rats fed with a normal diet had no change in those metabolic parameters, except the increased body weight (Table 1).

After 21 d of metformin and vildagliptin treatment in normal diet-fed rats, the metabolic parameters including body weight, food intake, visceral fat, plasma insulin, glucose, cholesterol, MDA, cardiac MDA level, and HOMA index were not different from the vehicle-treated rats (Table 2). In high-fat-fed rats, metformin and vildagliptin could significantly decrease the plasma insulin, HOMA index, plasma cholesterol, plasma MDA, and cardiac MDA levels, compared with the vehicle-treated rats in the high-fat-fed group. Metformin and vildagliptin did not alter the body weight, food intake, visceral fat, and plasma glucose, compared with the vehicle-treated rats in the high-fat diet group (Table 2).

Effects of high-fat diet consumption, metformin, and vildagliptin on HRV

At the baseline, the LF/HF ratio was not different between the normal diet and high-fat diet groups (Fig. 1). High-fat diet consumption caused an increased LF/HF ratio beginning at wk 8 and was markedly different at wk 12 (Fig. 1). After 21 d of treatment with metformin and vilda-

TABLE 2. Effects of metformin and vildagliptin on metabolic parameters in normal diet and high-fat diet rats

Metabolic parameters	NDV	NDM	NDVil	HFV	HFM	HFVil
Body weight (g)	464 ± 8	440 ± 17	448 ± 11	544 ± 14 ^a	534 ± 5 ^a	564 ± 11 ^a
Food intake (g)	22 ± 1	21 ± 2	22 ± 2	22 ± 2	22 ± 2	23 ± 2
Visceral fat (g)	25.86 ± 2.37	21.93 ± 3.46	24.12 ± 2.25	54.12 ± 3.17 ^a	49.20 ± 5.28 ^a	50.34 ± 4.87 ^a
Plasma insulin (ng/ml)	2.73 ± 0.30	2.22 ± 0.58	2.4 ± 0.67	3.87 ± 0.5 ^a	2.69 ± 0.57 ^b	2.93 ± 0.63 ^b
Plasma glucose (mg/dl)	140.83 ± 4.94	137.72 ± 7.52	131.69 ± 8.79	143.11 ± 7.52	138.00 ± 9.95	139.08 ± 4.46
HOMA index	16.17 ± 8.12	13.58 ± 8.1	14.05 ± 5.89	25.82 ± 3.76 ^a	16.50 ± 5.67	17.11 ± 2.81
Plasma total cholesterol (mg/dl)	83.41 ± 5.55	83.43 ± 5.47	81.31 ± 6.90	160.86 ± 6.5 ^a	105.6 ± 5.60 ^b	103.47 ± 3.52 ^b
Plasma MDA (μmol/ml)	2.47 ± 0.13	2.62 ± 0.16	2.74 ± 0.06	7.08 ± 0.12 ^a	6.48 ± 0.18 ^{a,b}	6.41 ± 0.19 ^{a,b}
Cardiac MDA (μmol/mg protein)	5.39 ± 1.89	5.51 ± 1.22	5.47 ± 2.46	11.44 ± 2.15 ^a	7.05 ± 1.27 ^{a,b}	7.80 ± 1.43 ^{a,b}

NDV, Normal diet + vehicle; NDM, normal diet + metformin; NDVil, normal diet + vildagliptin; HFV, high-fat diet + vehicle; HFM, high-fat diet + metformin; and HFVil, high-fat diet + vildagliptin.

^a, $P < 0.05$ vs. NDV; ^b, $P < 0.05$ vs. HFV.

gliptin in the high-fat diet group, a significantly decreased LF/HF ratio could be seen, compared with the vehicle-treated group (Fig. 2). Although both metformin and vildagliptin could decrease the LF/HF ratio, it was vildagliptin that brought the ratio back to the baseline level (Fig. 2).

Effects of high-fat diet consumption, metformin, and vildagliptin on cardiac function

In the normal-diet group, cardiac function parameters, including heart rate (HR), end systolic pressure (ESP), end diastolic pressure (EDP), +dP/dt, −dP/dt, and stroke volume (SV) were not different among the vehicle-, metformin-, and vildagliptin-treated rats (Table 3). In high-fat diet-fed rats treated with the vehicle, heart rate (HR), EDP, and −dP/dt were increased, whereas the ESP, +dP/dt, and SV were decreased, compared with the normal-diet group. In high-fat diet rats treated with metformin and vildagliptin, the EDP and −dP/dt were decreased, whereas the ESP,

+dP/dt, and SV were increased, compared with the vehicle-treated rats fed with high-fat diet. However, only vildagliptin could restore EDP, whereas metformin could only partially improve the EDP in these high-fat diet rats. Furthermore, decreased HR was observed only in the vildagliptin-treated rats in the high-fat diet group. Metformin did not decrease the HR in these high-fat fed rats (Table 3).

Effects of high-fat diet consumption, metformin, and vildagliptin on cardiac mitochondrial function

Cardiac mitochondrial ROS production

In normal diet-fed rats treated with metformin and vildagliptin, the levels of ROS production were not different from those of the vehicle-treated rats (Fig. 3). How-

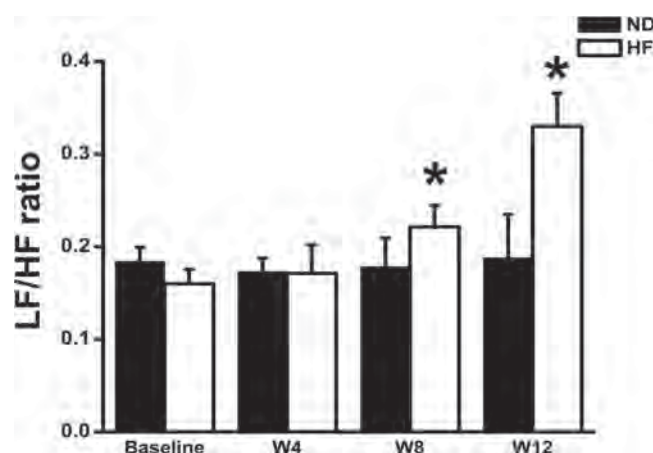


FIG. 1. LF/HF ratio in normal diet fed- and high-fat diet-fed rats. LF/HF ratio significantly increased during wk 8 (W8) of high-fat diet consumption, compared with baseline. *, $P < 0.05$ vs. baseline. ND, normal diet; HF, high-fat diet; black bar, ND; white bar, HF.

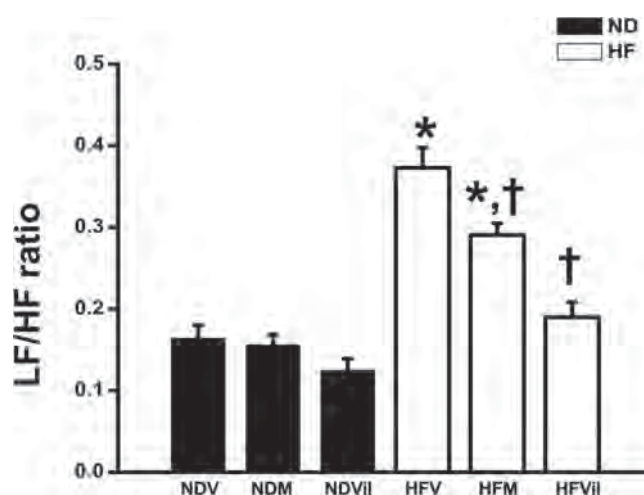


FIG. 2. LF/HF ratio in normal diet and high-fat diet rats treated with vehicle, metformin, and vildagliptin. Vildagliptin completely preserved cardiac sympathovagal imbalance, compared with vehicle. *, $P < 0.05$ vs. NDV; †, $P < 0.05$ vs. HFV. NDV, normal diet + vehicle; NMF, normal diet + metformin; NDVil, normal diet + vildagliptin; HFV, high-fat diet + vehicle; HFM, high-fat diet + metformin; and HFVil, high-fat diet + vildagliptin; black bar, ND; white bar, HF.

TABLE 3. Effects of metformin and vildagliptin on cardiac function in normal diet and high-fat diet rats

Cardiac function	NDV	NDM	NDVil	HFV	HFM	HFVil
HR (beats/min)	327 ± 27	306 ± 24	331 ± 30	416 ± 13 ^a	400 ± 18 ^b	347 ± 27 ^c
SBP (mm Hg)	130 ± 1	130 ± 1	130 ± 2	133 ± 1	132 ± 3	131 ± 2
DBP (mm Hg)	106 ± 1	106 ± 1	107 ± 2	110 ± 1	110 ± 3	108 ± 2
ESP (mm Hg)	131 ± 7	138 ± 7	137 ± 4	117 ± 15 ^a	126 ± 3 ^c	131 ± 11 ^c
EDP (mm Hg)	17 ± 1	16 ± 1	16 ± 1	37 ± 2 ^a	24 ± 4 ^{b,c}	21 ± 4 ^c
+dP/dt (mm Hg/sec)	8829 ± 401	8622 ± 128	8760 ± 231	6896 ± 277 ^a	8077 ± 334 ^{b,c}	8092 ± 282 ^{c,d}
−dP/dt (mm Hg/sec)	−5532 ± 316	−6654 ± 683	−5751 ± 101	−3940 ± 499 ^a	−5511 ± 556 ^{b,c}	−5666 ± 858 ^c
SV (μl/g)	1.04 ± 0.03	1.04 ± 0.03	1.02 ± 0.07	0.81 ± 0.06 ^a	0.96 ± 0.04 ^c	0.98 ± 0.05 ^c

NDV, Normal diet + vehicle; NDM, normal diet + metformin; NDVil, normal diet + vildagliptin; HFV, high-fat diet + vehicle; HFM, high-fat diet + metformin; and HFVil, high-fat diet + vildagliptin; DBP, diastolic blood pressure; SBP, systolic blood pressure.

^a, $P < 0.05$ vs. NDV; ^b, $P < 0.05$ vs. NDM; ^c, $P < 0.05$ vs. HFV; ^d, $P < 0.05$ vs. NDVil.

ever, the ROS level was significantly increased in the high-fat diet-fed rats treated with vehicle. Both metformin and vildagliptin could decrease the ROS level, compared with the vehicle-treated high-fat diet-fed rats. However, ROS reduction in the vildagliptin-treated group was greater than that in the metformin-treated group and was not different from that in the normal-diet group (Fig. 3).

Cardiac mitochondrial membrane potential changes ($\Delta\psi_m$)

Similar to the ROS level, the vehicle-treated rats in the high-fat diet group had mitochondrial depolarization as indicated by a decreased red/green fluorescent intensity ratio, compared with the vehicle-treated rats in the normal-diet group (Fig. 4). Both metformin and vildagliptin could attenuate the cardiac mitochondrial depolarization in high-fat diet-fed rats, compared with the vehicle-treated

rats of the high-fat diet group. However, only vildagliptin could completely prevent cardiac mitochondrial depolarization in the high-fat diet-fed rats (Fig. 4).

Cardiac mitochondrial swelling

In the normal-diet group, no cardiac mitochondrial swelling was observed in all treated groups (Fig. 5). In high-fat diet-fed rats treated with the vehicle, the absorbance was significantly decreased indicating cardiac mitochondrial swelling. After treatment with metformin or vildagliptin for 21 d, no cardiac mitochondrial swelling was observed in either treatment group (Fig. 5).

Discussion

The major findings of this study are as follows. First, metformin and vildagliptin improved the metabolic parameters of the insulin-resistant condition and oxidative stress

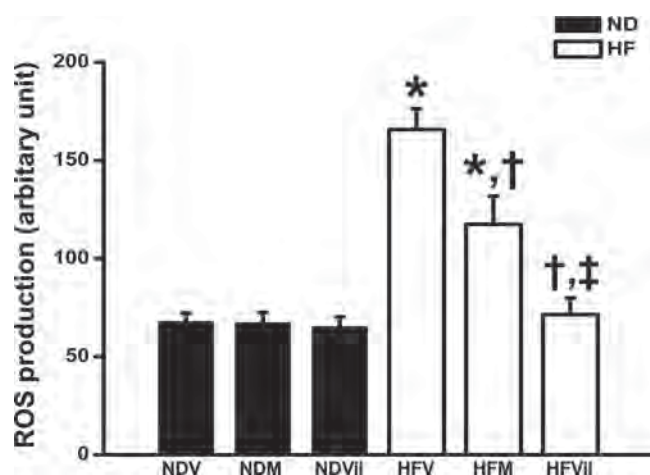


FIG. 3. Cardiac mitochondrial ROS production of normal diet and high-fat diet rats treated with vehicle, metformin, and vildagliptin. Vildagliptin markedly reduced ROS production, compared with vehicle and high-fat diet rats treated with metformin. *, $P < 0.05$ vs. NDV; †, $P < 0.05$ vs. HFV; ‡, $P < 0.05$ vs. HFM. ROS = Reactive oxygen species; NDV, Normal diet + vehicle; NDM, normal diet + metformin; NDVil, normal diet + vildagliptin; HFV, high-fat diet + vehicle; HFM, high-fat diet + metformin; and HFVil, high-fat diet + vildagliptin; black bar, ND; white bar, HF.

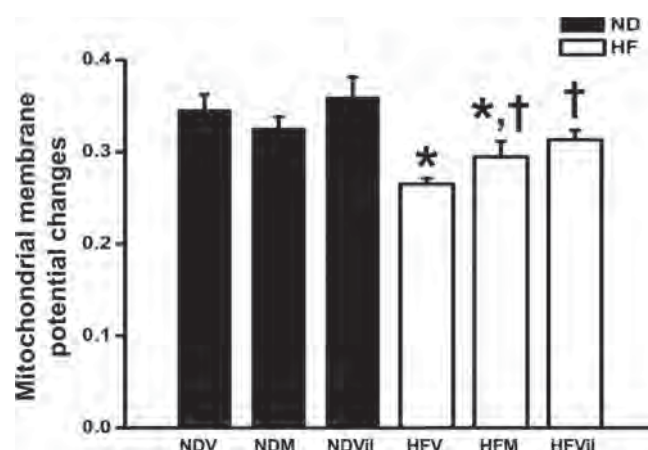


FIG. 4. Cardiac mitochondrial membrane potential changes ($\Delta\psi_m$) in normal diet- and high-fat diet-fed rats treated with vehicle, metformin, and vildagliptin. Vildagliptin significantly preserved $\Delta\psi_m$, compared with vehicle. *, $P < 0.05$ vs. NDV; †, $P < 0.05$ vs. HFV. NDV, Normal diet + vehicle; NDM, normal diet + metformin; NDVil, normal diet + vildagliptin; HFV, high-fat diet + vehicle; HFM, high-fat diet + metformin; and HFVil, high-fat diet + vildagliptin.

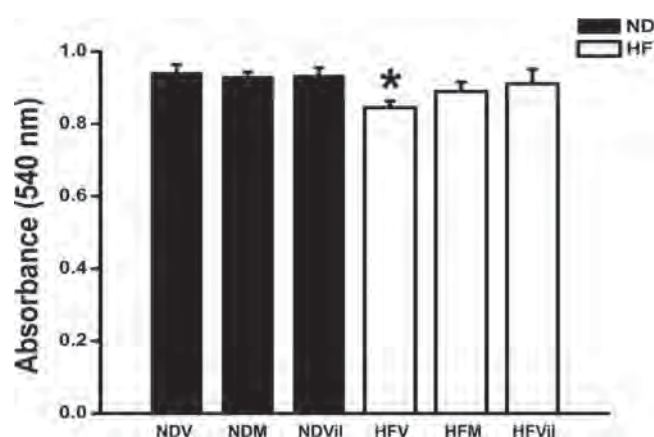


FIG. 5. Cardiac mitochondrial swelling of normal diet-fed and high-fat diet-fed rats treated with vehicle, metformin, and vildagliptin. Metformin and vildagliptin completely prevented cardiac mitochondrial swelling, compared with HFV. *, $P < 0.05$ vs. NDV; †, $P < 0.05$ vs. HFV. NDV, Normal diet + vehicle; NDM, normal diet + metformin; NDVil, normal diet + vildagliptin; HFV, high-fat diet + vehicle; HFM, high-fat diet + metformin; HFVil, high-fat diet + vildagliptin.

caused by long-term high-fat diet consumption. Second, both metformin and vildagliptin improved cardiac sympathovagal tone imbalance; however, only vildagliptin restored HRV to normal levels. Third, metformin and vildagliptin attenuated cardiac contractile dysfunction. Fourth, metformin and vildagliptin improved cardiac mitochondrial dysfunction caused by consumption of a high-fat diet. However, only vildagliptin completely restored cardiac mitochondrial function.

Long term high-fat diet consumption is known to cause an insulin-resistant condition (3). In this study, rats fed with 12 wk of high-fat diet developed an insulin resistance, characterized by increased insulin and cholesterol levels, but normal plasma glucose levels (2, 3). Previous studies reported that both metformin and vildagliptin have beneficial effects on metabolic parameters in type 2 diabetes patients (27, 28). In this study, metformin and vildagliptin improved the insulin-resistant condition in high-fat diet-fed rats by reducing plasma insulin and cholesterol levels as well as the HOMA index.

Metformin is a widely used antidiabetic drug, which can reduce plasma insulin and cholesterol levels in nonobese insulin-resistant patients (29) and high-fructose diet-induced diabetic rats (30). However, the effect of vildagliptin, a novel antidiabetic drug, on plasma insulin levels is still debated. Although Mari *et al.* (31) reported that vildagliptin reduced plasma insulin levels in patients with type 2 diabetes, Ahren *et al.* (32) found that vildagliptin did not alter plasma insulin levels in these diabetic patients. An experimental study demonstrated that the iv administration of vildagliptin increased the plasma insulin level in conscious dogs (33). In our study, the plasma insulin level in high-fat diet-fed rats was increased, and

vildagliptin could reduce plasma insulin as well as plasma cholesterol levels without altering the plasma glucose levels in these insulin-resistant rats. This finding is consistent with a previous clinical study showing that vildagliptin could reduce plasma cholesterol level in diabetic patients (34, 35). The insulin-resistant condition has also been shown to be associated with an increase of oxidative stress levels (36). In this study, we found that plasma and cardiac MDA levels, a marker of oxidative stress, were increased in high-fat diet-fed rats. Treatment with metformin and vildagliptin could attenuate MDA levels in both plasma and heart tissue in insulin-resistant rats, indicating their antioxidative effect.

In past decades, HRV has been used to determine cardiac sympathovagal balance (37). It is well accepted that the LF/HF ratio is the important indicator of cardiac autonomic balance (37, 38) and that an increased LF/HF ratio represents depressed HRV or imbalanced cardiac autonomic tone (23). In this study, depressed HRV was initially observed at wk 8 of high-fat diet-fed rats and markedly depressed in wk 12 after high-fat diet consumption. Previous studies in both human and animal models reported that depressed HRV is associated with the insulin-resistant condition (6, 39). In the present study, metformin and vildagliptin improved not only insulin resistance, but also HRV in insulin-resistant rats. Because insulin resistance and oxidative stress are known to influence the depressed HRV, our findings that the reduction of plasma insulin, as well as reduced plasma and cardiac MDA, could play a crucial role in the protection of the sympathovagal tone imbalance. Although metformin has been shown to improve HRV in type 2 diabetes patients (40), our study demonstrated, for the first time, that vildagliptin had higher efficacy than metformin in preventing the depressed HRV because it could completely prevent cardiac sympathovagal imbalance caused by high-fat diet consumption. One limitation in this HRV study was that the ECG was recorded while the animal was restrained, and this could have affected cardiac autonomic control. However, because all animals were under the same condition during ECG recording, the findings on the effects of high-fat diet consumption as well as the effects of both metformin and vildagliptin on the HRV could still be directly compared.

Previous studies demonstrated that insulin resistance could raise an incidence of coronary artery disease and deteriorate cardiac function (41), and that cardiac systolic dysfunction could be developed after 7 wk of high-fat diet consumption (42). Consistent with those studies, our results showed that both cardiac systolic and diastolic dysfunction were observed in high-fat diet-fed rats treated with the vehicle. Although metformin has been shown pre-

viously to protect cardiac contractile dysfunction in diabetic mice, diabetic rats, and nondiabetic rats (10, 43–45), the cardiac effect of vildagliptin is still unclear. In the present study, we demonstrated that although both metformin and vildagliptin could attenuate the impairment of cardiac function in these obese high-fat diet-induced insulin-resistant rats, only vildagliptin that could restore the EDP and decrease the heart rate in these rats.

Cardiac mitochondria are known as a power house to supply energy for the heart to maintain its daily electrical and mechanical events. Insulin resistance has been shown to be associated with impaired cardiac mitochondrial function, leading to cardiac contractile dysfunction (46). Oxidative stress is known as an important factor to cause cardiac mitochondrial dysfunction. Cardiac mitochondrial dysfunction can be characterized by increased cardiac mitochondrial ROS production, mitochondrial membrane depolarization, and mitochondrial swelling (26, 47, 48). Consistent with a previous report (2), high-fat diet consumption for 12 wk in our study caused cardiac mitochondrial dysfunction. Both metformin and vildagliptin were also effective in attenuating cardiac mitochondrial dysfunction caused by long-term high-fat diet consumption. Although both metformin and vildagliptin could attenuate cardiac mitochondrial ROS production, only vildagliptin that could completely prevent ROS production caused by high-fat diet consumption. Furthermore, despite the fact that both metformin and vildagliptin could attenuate cardiac mitochondrial membrane depolarization, vildagliptin was more effective in preventing mitochondrial depolarization in rats exposed to a high-fat diet. These benefits of vildagliptin in preventing cardiac mitochondrial dysfunction as well as oxidative stress could be responsible for the improved cardiac function and HRV observed in this study.

In summary, our study demonstrates that long-term high-fat diet-fed rats could develop insulin resistance, depressed HRV, cardiac contractile dysfunction, and cardiac mitochondrial dysfunction. Although both metformin and vildagliptin could attenuate these impairments, vildagliptin demonstrated a better efficacy in preventing cardiac dysfunction, depressed HRV, and impaired cardiac mitochondrial function caused by consumption of food containing a high proportion of fat.

Acknowledgments

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Effects of Curcuminoids on Frequency of Acute Myocardial Infarction After Coronary Artery Bypass Grafting

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It is well established that myocardial infarction (MI) associated with coronary artery bypass grafting (CABG) predicts a poor outcome. Nevertheless, cardioprotective therapies to limit myocardial injury after CABG are lacking. Previous studies have shown that curcuminoids decrease proinflammatory cytokines during cardiopulmonary bypass surgery and decrease the occurrence of cardiomyocytic apoptosis after cardiac ischemia/reperfusion injury in animal models. We aimed to evaluate whether curcuminoids prevent MI after CABG compared to placebo. The 121 consecutive patients undergoing CABG were randomly allocated to receive placebo or curcuminoids 4 g/day beginning 3 days before the scheduled surgery and continued until 5 days after surgery. The primary end point was incidence of in-hospital MI. The secondary end point was the effect of curcuminoids on C-reactive protein, plasma malondialdehyde, and N-terminal pro-B-type natriuretic peptide levels. Baseline characteristics were comparable between the curcuminoid and placebo groups. Mean age was 61 ± 9 years. On-pump CABG procedures were performed in 51.2% of patients. Incidence of in-hospital MI was decreased from 30.0% in the placebo group to 13.1% in the curcuminoid group (adjusted hazard ratio 0.35, 0.13 to 0.95, $p = 0.038$). Postoperative C-reactive protein, malondialdehyde, and N-terminal pro-B-type natriuretic peptide levels were also lower in the curcuminoid than in the placebo group. In conclusion, we demonstrated that curcuminoids significantly decreased MI associated with CABG. The antioxidant and anti-inflammatory effects of curcuminoids may account for their cardioprotective effects shown in this study. © 2012 Elsevier Inc. All rights reserved. (Am J Cardiol 2012;xx:xxx)

Curcuminoids, the polyphenols responsible for the yellow color of the curry spice turmeric, have been used to treat a variety of diseases in traditional Chinese and Indian medicine. The major curcuminoids present in turmeric are curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III).¹ Modern scientific research has confirmed the good therapeutic effects of the curcuminoid complex and its pharmacologic safety has been well established.² A previous study has shown that curcuminoids suppress proinflammatory cytokines during cardiopulmonary bypass surgery and decrease the occurrence of cardiomyocytic apoptosis after cardiac ischemia/reperfusion

injury in an animal model.³ Furthermore, a histopathologic study has demonstrated that curcuminoid treatment decreases the degree of myocardial necrosis in isoproterenol-administered rats.⁴ The well-known anti-inflammatory, antioxidant, and membrane-stabilizing effects of curcuminoids may help preserve cellular viability during cardiopulmonary bypass surgery.^{5,6} Therefore, curcuminoids may have a potential role in the limitation of myocardial ischemia/reperfusion injury after coronary artery bypass grafting (CABG). The present study evaluated whether curcuminoids prevent myocardial infarction (MI) after CABG compared to placebo.

Methods

The present study was a randomized, prospective, double-blinded, placebo-controlled trial performed at Maharak Nakorn Chiang Mai Hospital, Chiang Mai University. We prospectively studied 121 consecutive patients undergoing CABG without valve surgery from September 2009 to December 2011. Informed consent was obtained from each patient to participate in the study. Patients were excluded if they had emergency cardiac surgery or any increase in creatine kinase-MB above the upper limit of the normal range at time of randomization. Because curcuminoids are mainly metabolized by the liver, patients with cholestatic jaundice (total bilirubin higher than twofold the upper normal limit) or severe liver disease (aspartate aminotransferase or alanine amino-

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Table 1
Demographic data and clinical features

Characteristics	Curcuminoid (n = 61)	Placebo (n = 60)	p Value
Age (years)	61.0 ± 9.1	61.1 ± 8.2	0.966
Men	34 (55.7%)	35 (58.3%)	0.917
Body mass index (kg/m ²)	24.1 ± 3.4	24.8 ± 4.8	0.290
New York Heart Association class	1.9 ± 0.5	2.0 ± 0.5	0.224
Canadian Cardiovascular Society class	1.9 ± 0.6	2.0 ± 0.5	0.194
Serum creatinine (mg/dl)	1.3 ± 0.4	1.4 ± 0.6	0.380
Preoperative creatine kinase-MB (ng/ml)	4.5 ± 4.1	5.5 ± 6.8	0.308
Preoperative C-reactive protein (mg/dl)	0.4 ± 0.5	0.5 ± 0.9	0.313
Preoperative malondialdehyde (mmol/ml)	7.4 ± 1.4	7.4 ± 1.1	0.908
Preoperative N-terminal pro-B-type natriuretic peptide (pg/ml)*	410.9 ± 577.2	533.4 ± 1,529.7	0.219
Diabetes mellitus	23 (37.7%)	30 (50.0%)	0.238
Hypertension†	55 (90.2%)	54 (90.0%)	1.000
Dyslipidemia‡	55 (90.2%)	52 (86.7%)	0.751
Previous myocardial infarction	17 (27.9%)	15 (25.0%)	0.879
Current smoker	8 (13.1%)	4 (6.7%)	0.378
Heart failure	5 (8.3%)	6 (10.0%)	1.000
Previous coronary angioplasty	4 (6.6%)	1 (1.7%)	0.371
Left ventricular ejection fraction (%)	54.8 ± 14.4	51.6 ± 15.1	0.483
Preoperative medications			
Aspirin or clopidogrel	55 (90.2%)	59 (98.3%)	0.125
β blocker	48 (78.7%)	48 (80.0%)	0.891
Statin	56 (91.8%)	56 (93.3%)	1.000
Angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker	42 (68.9%)	44 (73.3%)	0.732

* Median ± interquartile range.

† Blood pressure ≥140/90 mm Hg or currently treated with antihypertensive drugs.

‡ Low-density lipoprotein cholesterol >100 mg/dl, high-density lipoprotein cholesterol <40 mg/dl, or triglyceride >150 mg/dl.

Table 2
Perioperative features of patients in curcuminoid and placebo groups

Characteristics	Curcuminoid (n = 61)	Placebo (n = 60)	p Value
Vessel involvement			
Left main coronary artery stenosis	12 (20.3%)	17 (28.3%)	0.422
3-Vessel disease	45 (76.3%)	49 (81.7%)	0.619
Off-pump coronary artery bypass grafting	32 (52.5%)	27 (45.0%)	0.523
On-pump coronary artery bypass grafting	29 (47.5%)	33 (55.0%)	
On-pump with beating heart	14 (23.3%)	17 (28.3%)	
On-pump with cardioplegic arrest	15 (24.6%)	16 (26.7%)	
Cardiopulmonary bypass duration (minutes)	108.5 ± 48.1	106.6 ± 43.0	0.872
Cross-clamp duration (minutes)	80.0 ± 27.7	71.4 ± 26.3	0.379
Temporary ventricular pacing	24 (39.3%)	19 (31.8%)	0.231
Number of bypass grafts	3.5 ± 1.3	3.7 ± 1.0	0.374
Myocardial infarction after coronary artery bypass grafting	8 (13.1%)	18 (30.0%)	0.028
Non-Q-wave myocardial infarction	8 (13.1%)	15 (25.0%)	
Q-wave myocardial infarction	0 (0%)	3 (5.0%)	

transferase higher than threefold the upper normal limit) were not included in the study.

Curcuminoids and placebo used in the present study were provided in caplet form by the Research and Development Institute, the Government Pharmaceutical Organization, Bangkok, Thailand. One curcuminoid capsule contained curcuminoids 250 mg, which consisted of curcumin, demethoxycurcumin, and bisdemethoxycurcumin, in a ratio of 1.0:0.6:0.3, respectively, confirmed by high-performance liquid chromatography/mass spectrometry.

Enrolled patients were randomly allocated to receive

placebo or curcuminoids 4 capsules 4 times/day (4 g/day) in addition to standard therapy beginning 3 days before the scheduled surgery and patients continued to receive the assigned treatment until 5 days after surgery. To assign patients to curcuminoids or placebo, a block randomization sequence was obtained by a statistical consultant who was not involved in the study. Assigned therapy was fully blinded; surgeons and investigators performing postoperative assessment were not aware of the randomization assignment.

All patients undergoing CABG were treated with standard therapy according to their physicians. Three surgeons

performed CABG in the present study. The surgical techniques were determined at the discretion of the individual surgeons. On-pump CABG procedures were performed in 62 patients (51.2%), of whom 31 (25.6%) underwent on-pump CABG with the heart beating and 31 (25.6%) underwent on-pump CABG with conventional cardioplegic arrest. Myocardial protection was done with antegrade and retrograde cold blood cardioplegia. Off-pump CABG was performed in the remaining patients. The conduits used in patients in this study included the internal mammary arteries, radial arteries, and saphenous veins.

Twelve-lead electrocardiograms were recorded before surgery, 24, 48, and 72 hours after surgery, and 30-day follow-up visit. Serial creatine kinase-MB levels were assessed before surgery, at 8, 16, 24, 48, and 72 hours after intensive care unit arrival, and whenever an ischemic event was suspected. N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) level was assessed before surgery and on the 5th postoperative day.

To examine the effects of curcuminoids on inflammatory response and oxidative stress after surgery, C-reactive protein (CRP) level was assessed before surgery and on postoperative days 3 and 5. In addition, plasma malondialdehyde (MDA) level, a marker for oxidative stress, was assessed before surgery and on postoperative day 5 using the high-performance liquid chromatographic method.

The primary end point of the study was to demonstrate that curcuminoids decrease the incidence of in-hospital MI compared to placebo. The secondary end point was to examine the effects of curcuminoids on CRP, MDA, and NT-pro-BNP levels after surgery.

The diagnosis of Q-wave MI was determined by the presence of new pathologic Q waves according to Minnesota Code criteria or new-onset left bundle branch block and creatine kinase-MB increase more than fivefold the upper normal limit of the investigators' local laboratory within 24 hours of CABG. In the absence of the aforementioned electrocardiographic findings, creatine kinase-MB increase >10-fold the upper normal limit within 24 hours of CABG was considered indicative of non-Q-wave MI.⁷ If MI was suspected >24 hours after CABG, a creatine kinase-MB increase >2 times the upper normal limit with chest pain or an increase >3 times the upper normal limit was considered indicative of MI.⁸

All analyses were done on an intention-to-treat basis. Demographic and perioperative variables were compared between groups with *t* test for normally distributed values; otherwise the Mann-Whitney *U* test was used. Proportions were compared by chi-square test or Fisher's exact test when appropriate. Continuous variables are presented as mean $\pm$ SD or median $\pm$ interquartile range when appropriate. Categorical variables are displayed as percentages. Hazard ratios and 95% confidence intervals to assess the risk of the primary end point according to potential confounding variables were determined by logistic regression. Multivariate analyses were performed for variables with a *p* value <0.1 in univariate analysis using the logistic regression procedure. A *p* value <0.05 (2-tailed) was considered statistically significant.

Table 3

Multivariable logistic regression for myocardial infarction after coronary artery bypass grafting

Risk Factor	OR (95% CI)	<i>p</i> Value
Curcuminoid therapy	0.35 (0.13–0.95)	0.038
On-pump coronary artery bypass grafting	5.23 (1.92–14.28)	0.001

CI = confidence interval; OR = odds ratio.

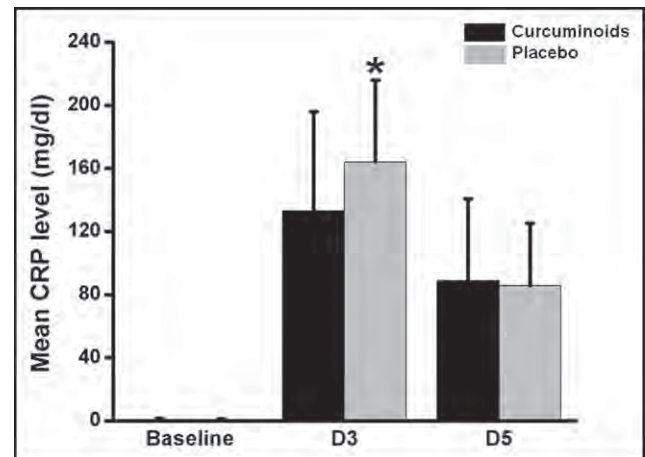


Figure 1. C-reactive protein levels before and after coronary artery bypass grafting in curcuminoid and placebo groups. C-reactive protein level on postoperative day 3 (D3) was significantly lower in the curcuminoid group compared to the placebo group. **p* <0.05 versus placebo. D5 = postoperative day 5.

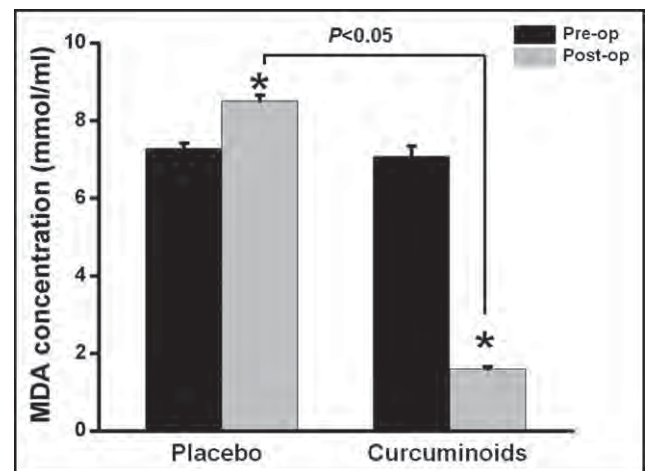


Figure 2. Levels of plasma malondialdehyde before (Pre-op) and after (Post-op) coronary artery bypass grafting in curcuminoid and placebo groups. Plasma malondialdehyde level increased after coronary artery bypass grafting in the placebo group but decreased significantly after coronary artery bypass grafting in the curcuminoid group. **p* <0.05 versus preoperatively.

Results

Demographic and perioperative variables are presented in Tables 1 and 2, respectively. From September 2009 to December 2011, 121 consecutive patients who met the inclusion criteria were randomly divided to a curcuminoid group (*n* = 61) or a control group (*n* = 60). Baseline characteristics of patients in the 2 treatment groups were

Table 4
Adverse events and study drug discontinuation

Characteristics	Curcuminoid (n = 61)	Placebo (n = 60)	p Value
Adverse events			
Nausea	8 (13.1%)	5 (8.3%)	0.559
Diarrhea	2 (3.3%)	2 (3.3%)	1.000
Abdominal pain	3 (4.9%)	1 (1.7%)	0.619
Dizziness	2 (3.3%)	1 (1.7%)	1.000
Sore throat	1 (1.6%)	1 (1.7%)	1.000
Serious adverse events of special interest			
Serum creatinine increase*	4 (6.6%)	2 (3.3%)	0.691
Liver function			
Alanine aminotransferase or aspartate aminotransferase >3× upper limit of normal range	0 (0%)	2 (3.3%)	0.469
Alanine aminotransferase or aspartate aminotransferase >3× upper limit of normal range with concurrent bilirubin >2× upper limit of normal range	0 (0%)	1 (1.7%)	0.993
Inotrope requirement	43 (70.5%)	44 (73.3%)	0.884
Intra-aortic balloon pump usage	0 (0%)	4 (6.7%)	0.057
Severe postoperative hemorrhage required reoperation to stop bleeding	1 (1.6%)	1 (1.7%)	1.000
Stroke/transient ischemic attack	2 (3.3%)	1 (1.7%)	1.000
Death	1 (1.6%)	1 (1.7%)	1.000
Premature study drug discontinuation			
Overall	14 (22.9%)	11 (18.3%)	0.654
Owing to adverse drug events	6 (9.8%)	4 (6.7%)	0.743
By subject's request	7 (11.5%)	6 (10.0%)	1.000
For other reasons	1 (1.6%)	1 (1.7%)	1.000

* Increase in serum creatinine of $\geq 50\%$.

comparable including age, gender, co-morbidities, and previous percutaneous coronary revascularization (Table 1). Perioperative features were not different between the curcuminoid and placebo groups (Table 2).

Incidence of the primary outcome (in-hospital MI) was decreased from 30.0% in the placebo group to 13.1% in the curcuminoid group (unadjusted hazard ratio 0.35, 0.14 to 0.89, $p = 0.028$). Most MI events were non-Q-wave MI (Table 2). Apart from curcuminoid treatment, other predictors of in-hospital MI were identified. We found that on-pump CABG was significantly associated with a higher incidence of MI compared to off-pump surgery (35.5%, 22 of 62, vs 6.8%, 4 of 59, respectively, $p < 0.001$). After multivariate analysis, we found that curcuminoid therapy remained the independent protective factor of in-hospital MI and that on-pump CABG was the independent predictive factor of in-hospital MI (Table 3). Of 121 patients, 57 patients underwent echocardiography 1 month after surgery. Incidence of postoperative left ventricular dysfunction (left ventricular ejection fraction $< 40\%$) was significantly higher in the placebo group than in the curcuminoid group (25.9%, 7 of 27, vs 3.3%, 1 of 30, respectively, $p = 0.021$).

Baseline preoperative CRP, MDA, and NT-pro-BNP levels were not different between the curcuminoid and placebo groups. However, mean increase in CRP level on postoperative day 3 compared to baseline level was significantly greater in the placebo group than in the curcuminoid group (difference $+161.8 \pm 54.1$ vs $+128.6 \pm 60.5$ mg/dl, respectively, $p = 0.031$; Figure 1). Plasma MDA level was increased after CABG in the placebo group but was decreased significantly after CABG in the curcuminoid group (difference $+0.8 \pm 1.4$ vs -5.7 ± 1.5 mmol/ml, respectively, $p < 0.001$; Figure 2). Furthermore, mean increase in

postoperative NT-pro-BNP level compared to preoperative level was greater in the placebo group than in the curcuminoid group (difference $+2,542.2 \pm 2,631.2$ vs $+1,822.1 \pm 2,102.9$ pg/ml, respectively, $p = 0.015$).

Incidence of drug-related adverse events was not different between the curcuminoid and placebo groups (Table 4). The main drug-related adverse events were gastrointestinal symptoms. Incidences of serious adverse events and drug discontinuation did not differ between the 2 groups.

Discussion

Adequate myocardial protection during CABG is crucial in preventing myocardial injury after surgery.^{7,9} Previous studies have shown that an increase of cardiac enzymes after CABG is associated with increased long-term mortality.¹⁰ Nevertheless, some interventions reported to be cardioprotective in experimental models of ischemia/reperfusion injury have failed to translate their protective effects into clinical studies.¹¹ Until recently, only few clinical studies have shown promising results.^{7,12–14} Mangano et al¹⁴ recently examined the efficacy of the adenosine-regulating agent acadesine in patients undergoing CABG. They demonstrated that acadesine improved long-term survival in this group of patients. Furthermore, cariporide, the sodium-hydrogen exchange inhibitor, has been shown to decrease the incidence of MI associated with CABG, although the neurologic complications observed with the high dose preclude its clinical use.⁷

Preclinical data have shown that curcuminoids have cardiovascular protective effects in experimental models of various cardiac conditions.¹⁵ In the present study, we demonstrated that curcuminoids decreased the incidence of in-

hospital MI after CABG significantly. In addition, curcuminoids attenuated postoperative NT-pro-BNP levels and decreased the incidence of postoperative left ventricular dysfunction. Accumulating evidence has suggested that curcuminoids have a diverse range of molecular targets and influence numerous biochemical and molecular cascades.² We propose that the beneficial effects of curcuminoids in the decrease of MI may be exerted by several mechanisms. First, it has been suggested that oxidative stress and systemic inflammatory response during cardiopulmonary bypass may account for ischemia/reperfusion injury occurring in patients receiving CABG. Curcuminoids have been shown to possess striking antioxidant and anti-inflammatory properties and to inhibit such mediators of inflammation as nuclear factor- κ B, cyclooxygenase-2, lipoxygenase, and inducible nitric oxide synthase.¹⁶ Correspondingly, in the present study, we demonstrated that curcuminoids decreased postoperative CRP and MDA levels significantly. Therefore, the anti-inflammatory and antioxidative effects of curcuminoids may attenuate myocardial injury associated with cardiac surgery. Second, curcuminoids may protect against cardiac injury through a membrane-stabilizing effect.^{17–20} Nirmala and Puvanakrishnan^{4,21} demonstrated that curcuminoids significantly attenuated increased lysosomal hydrolase activity in serum and myocardial tissue in isoproterenol-induced MI in rats. Histopathologic findings also showed that the curcuminoid treatment decreased the degree of myocardial necrosis in isoproterenol-administered rats.⁴ The membrane-stabilizing effect of curcuminoids may protect cells from autolytic and heterolytic damage and may attenuate the tissue damage owing to myocardial ischemia. Third, additional evidence from in vitro studied has shown that curcuminoids inhibit human platelet activation.^{21,22} The antiplatelet property of curcuminoids may potentially decrease the occurrence of myocardial ischemia.

Because of the relatively small studied population, our results need to be confirmed in larger studies. Apart from the anti-inflammatory and antioxidant effects of curcuminoids shown in this study, other mechanisms of cardioprotective effects of curcuminoids are not clearly elucidated. Furthermore, the effect of curcuminoids on long-term outcome after CABG is unknown. Future studies are warranted to clarify this issue.

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ORIGINAL ARTICLE

Calcium-induced Cardiac Mitochondrial Dysfunction Is Predominantly Mediated by Cyclosporine A-dependent Mitochondrial Permeability Transition Pore But Not Mitochondrial Calcium Uniporter

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Background and Aims. Cardiac mitochondrial Ca^{2+} overload plays a critical role in mechanical and electrical dysfunction leading to cardiac cell death and fatal arrhythmia. Because Ca^{2+} overload is related to mitochondrial permeability transition, reactive oxygen species (ROS) production and membrane potential ($\Delta\Psi_m$) dissipation, we probed the mechanistic association between Ca^{2+} overload, oxidative stress, mitochondrial permeability transition pore (mPTP) and mitochondrial calcium uniporter (MCU) in isolated cardiac mitochondria.

Methods. Various concentrations of Ca^{2+} (5–200 μM) were used to induce mitochondrial dysfunction. Cyclosporin A (CsA, an mPTP blocker) and Ru360 (an MCU blocker) were used to test its protective effects on Ca^{2+} -induced mitochondrial dysfunction.

Results. High concentrations of Ca^{2+} ($\geq 100 \mu\text{M}$) caused overt mitochondrial swelling and $\Delta\Psi_m$ collapse. However, only slight increases in ROS production were detected. Blocking the MCU by Ru360 is less effective in protecting mitochondrial dysfunction.

Conclusions. A dominant cause of Ca^{2+} -induced cardiac mitochondrial dysfunction was mediated through the mPTP rather than MCU. Therefore, CsA could be more effective than Ru360 in preventing Ca^{2+} -induced cardiac mitochondrial dysfunction. © 2012 IMSS. Published by Elsevier Inc.

Key Words: Cardiac mitochondria, Calcium, Permeability transition, Reactive oxygen species, Membrane potential.

Introduction

Intracellular Ca^{2+} overload of cardiomyocytes can lead to both electrical and mechanical dysfunction (1). Mitochondria play a crucial role in buffering cytosolic Ca^{2+} for maintaining physiological Ca^{2+} signals (2). When mitochondrial Ca^{2+} uptake exceeds a certain threshold level, the mitochondria can no longer regulate the intramitochondrial Ca^{2+} , resulting in the opening of mitochondrial permeability transition pore (mPTP) (3,4). Opening of mPTP allows the influx of water and solutes ($< 1500 \text{ Da}$)

into the matrix causing mitochondrial swelling, mitochondrial membrane potential ($\Delta\Psi_m$) collapse, and eventually cell death (2). However, the mechanism by which Ca^{2+} mediates the mPTP opening in cardiac cells is still controversial and varies in different models (5). Ca^{2+} overload also leads to the increase in reactive oxygen species (ROS) production (6–8). However, the relationship between Ca^{2+} overload, mPTP opening, $\Delta\Psi_m$ change and oxidative stress is still elusive.

Cyclosporin A (CsA) is regarded as a potent inhibitor of mPTP by preventing the interaction of cyclophilin D and adenine nucleotide translocator (ANT). It has long been used to identify mPTP activity in various tissues (9,10) and has been proven to be a cardioprotective agent (11). Therefore, in this study we investigated the mechanistic association between Ca^{2+} overload, mitochondrial

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dysfunction, oxidative stress and the mPTP opening in cardiac mitochondria. Moreover, because the mitochondrial calcium uniporter (MCU) has been proposed as a principal portal for Ca^{2+} influx (12–14), we also investigated the effect of Ru360, a selective MCU blocker, against mitochondrial dysfunction. In the present study we tested the hypotheses that the mechanism of Ca^{2+} -induced cardiac mitochondrial dysfunction is mainly via the mPTP and the MCU.

Materials and Methods

Ethics Approval

This study was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University.

Animals

Male Wistar rats ($n = 18$, 350–400 g) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. All animals were housed in a room with controlled temperature maintaining between 22 and 25°C with a constant 12 h light/dark cycle. Rats were fed with standard rat pellet diet and water ad libitum.

Experimental Protocols

Isolated cardiac mitochondria were used in all experiments. Various doses of CaCl_2 (5, 10, 100, 200 μM) were applied to isolated cardiac mitochondria for 10 min before the measurement of mitochondrial swelling, ROS production and $\Delta\Psi\text{m}$ dissipation. In addition, the effect of the mPTP blocker (CsA) and the MCU blocker (Ru360) on attenuating Ca^{2+} -induced mitochondrial dysfunction was also investigated. Isolated cardiac mitochondria were pretreated with CsA at the concentration of 5 μM for 30 min before the application of CaCl_2 (15) or with Ru360 10 μM for 5 min (16).

Isolation of Cardiac Mitochondria

Cardiac mitochondria were isolated according to the method described previously (17,18). Male Wistar rats weighing 350–400 g were anesthetized by isoflurane and thiopental (80 mg/kg), and the hearts were perfused with ice-cold normal saline. The heart was then removed, minced with razors, and homogenized in ice-cold isolation buffer containing sucrose 300 mM, TES 5 mM and EGTA 0.2 mM, pH 7.2 (4°C). The homogenate was centrifuged at 800 g for 5 min. The supernatant was collected and centrifuged at 8800 g for 5 min. The mitochondrial pellet was resuspended in ice-cold isolation buffer and finally centrifuged at 8800 g for 5 min. The mitochondrial pellet was suspended in 2 ml of respiration buffer containing

100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM KH_2PO_4 , pH 7.4, at 37°C. Mitochondrial protein concentration was measured using bicinchoninic acid (BCA) assay (19).

Identification of Cardiac Mitochondria with Electron Microscopy

Electron microscopy was used to identify the morphology of cardiac mitochondria (20). Isolated mitochondria were fixed overnight by mixing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C. The pellet was then rinsed in cacodylate buffer and postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature. Next, the mitochondrial pellet was dehydrated in a graded series of ethanol and embeded in Epon-Araldite. The pellet was then cut into ultrathin sections (60–80 nm thick) using a diamond knife, placed on copper grids and stained with uranyl acetate and lead citrate. Finally, mitochondria were observed with a transmission electron microscope.

Measurement of Cardiac Mitochondrial Swelling

Mitochondria suspension was added with respiration buffer to a final concentration of 0.4 mg/ml. Cardiac mitochondrial swelling was determined by the decrease of light absorbance at 540 nm using a microplate reader (18–21).

Measurement of ROS Production

Cardiac mitochondria (0.4 mg/mL) were incubated with 2 μM dichlorohydro-fluorescein diacetate (DCFDA) at 25°C for 20 min. In the presence of ROS, DCFDA was oxidized to DCF and the fluorescence increased. Fluorescence was determined at 485 nm for excitation and 530 nm for emission. The ROS level was expressed in arbitrary units of fluorescence intensity of DCF (18).

Measurement of Mitochondrial Membrane Potential Change ($\Delta\Psi\text{m}$)

The mitochondrial membrane potential change of isolated cardiac mitochondria were evaluated by using the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (22,23). JC-1 is a lipophilic, cationic dye which can pass into mitochondria. When mitochondria have high $\Delta\Psi\text{m}$, JC-1 is in the aggregate form, which shows red fluorescence. With low $\Delta\Psi\text{m}$ (i.e., depolarized state), it remains in monomeric form showing green fluorescence. The isolated mitochondria (0.4 mg/mL) were incubated with 310 nM JC-1 at 37°C for 30 min (18). Fluorescence intensity was measured using a fluorescent microplate reader. The green fluorescence of JC-1 monomer was excited at 485 nm and the emission was noticed at 530 nm. The red fluorescence of J-aggregates was excited at 485 nm and the emission was detected at 590

nm. The mitochondrial depolarization was indicated by a decrease in red/green fluorescence intensity ratio.

Statistical Analysis

Data were presented as mean $\pm$ SEM. Evaluation of the differences was made by two-way ANOVA followed by the Fisher post-hoc test. Statistical significance was considered at $p < 0.05$.

Chemicals

All reagents used in this study were purchased from Sigma (St. Louis, MO).

Results

Effects of Ca^{2+} on Cardiac Mitochondrial Swelling

When exposed with Ca^{2+} at 5 and 10 μM , cardiac mitochondrial swelling was not observed, whereas when exposed with Ca^{2+} at 100 μM and 200 μM , cardiac mitochondrial swelling was obviously seen as indicated by markedly decreased absorbance (Figure 1A). At 30 min after Ca^{2+} incubation,

only the absorbance in the cardiac mitochondria exposed to 100 and 200 μM Ca^{2+} was significantly decreased, indicating cardiac mitochondrial swelling, compared to the control (Figure 1A). Data from the decrease in absorbance were confirmed by the morphology of mitochondria taken from the electron microscope. Ca^{2+} at 200 μM caused severe morphological change of cardiac mitochondria, evidenced by matrix expansion and unfolding of the cristae (Figure 1B).

Effect of Ca^{2+} on Cardiac Mitochondrial ROS Production and $\Delta\Psi_m$ Change

At low doses of Ca^{2+} (5 and 10 μM), the ROS level was not different from the control (Figure 2A). When the cardiac mitochondria were exposed to high doses of Ca^{2+} (100 and 200 μM), a small but significant increase of ROS level was observed. However, data from $\Delta\Psi_m$ showed that 10 μM Ca^{2+} could trigger mitochondrial membrane depolarization without the change in ROS production (Figure 2B). Further, a high concentration of Ca^{2+} (100 and 200 μM) induced more $\Delta\Psi_m$ dissipation than the low concentration (Figure 2B).

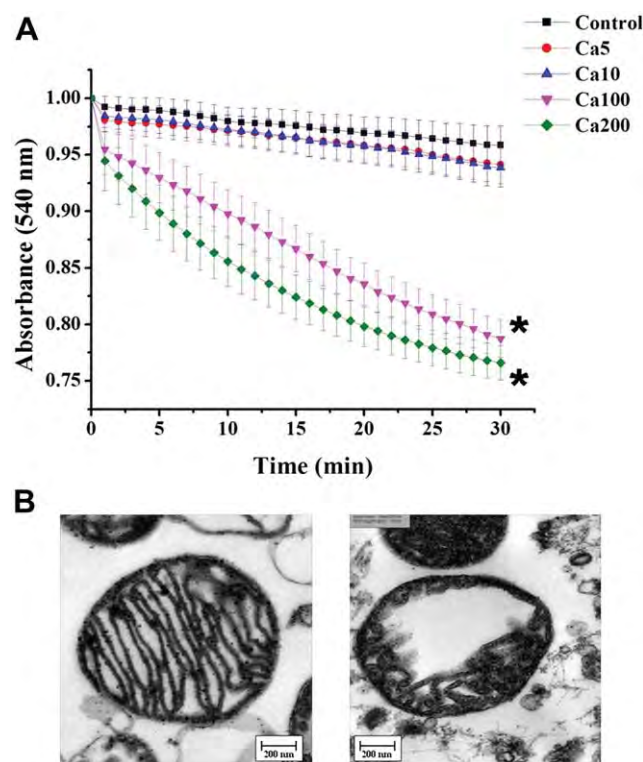


Figure 1. Cardiac mitochondrial swelling in response to increasing concentration of Ca^{2+} . (A) Kinetic study of cardiac mitochondrial swelling. (B) Effect of Ca^{2+} on morphological change of cardiac mitochondria. Isolated cardiac mitochondria without Ca^{2+} (left) and with 200 μM Ca^{2+} (right), Magnifications: 1:15,000. Control: isolated cardiac mitochondria without Ca^{2+} exposure. Ca5, Ca10, Ca100, Ca200: Ca^{2+} -treated mitochondria at 5, 10, 100, and 200 μM , respectively. * $p < 0.05$ vs. control.

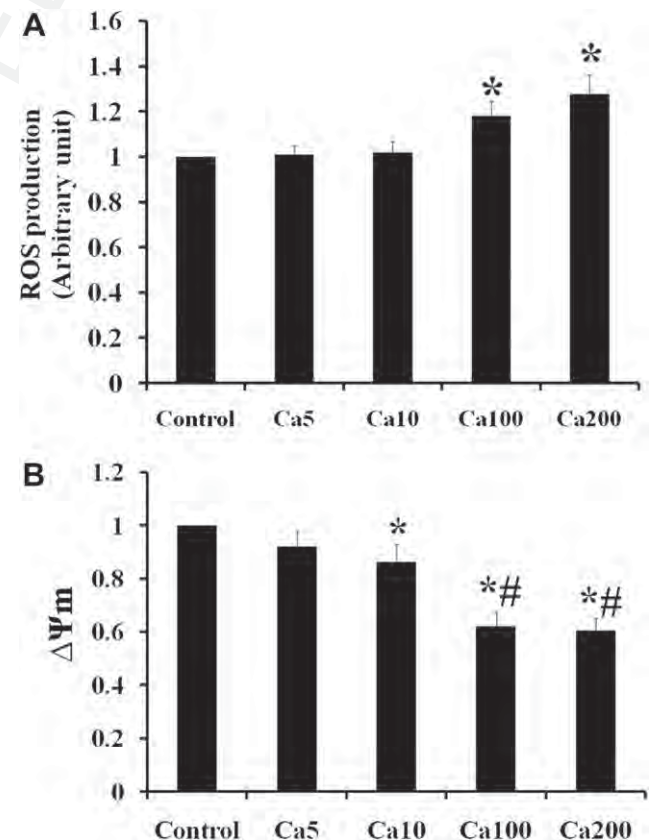


Figure 2. Effects of Ca^{2+} on cardiac mitochondrial ROS production (A) and $\Delta\Psi_m$ change (B). Control: isolated cardiac mitochondria without Ca^{2+} exposure, Ca5, Ca10, Ca100, Ca200: Ca^{2+} -treated mitochondria at 5, 10, 100, and 200 μM , respectively. * $p < 0.05$ vs. control, # $p < 0.05$ vs. Ca10.

Effects of CsA and Ru360 on Mitochondrial Dysfunction

From the kinetic curve of the absorbance, it was found that CsA could apparently protect cardiac mitochondrial swelling especially in early Ca^{2+} -overloaded time points, i.e., the first 15 min (Figure 3A). However, Ru360 could modestly protect cardiac mitochondria from Ca^{2+} -induced swelling (Figure 3A). At 15 min after 200 μM Ca^{2+} loading, CsA could completely protect cardiac mitochondrial swelling, whereas Ru360 provided only partial protection (Figure 3B). Nevertheless, at 30 min after Ca^{2+} exposure, CsA could only partially protect mitochondrial swelling and Ru360 could no longer mitigate the swelling (Figure 3C). CsA and Ru360 alone did not alter mitochondrial ROS and membrane potential (Figure 4). CsA, as well as Ru360, could completely prevent Ca^{2+} -induced ROS production of cardiac mitochondria. However, the level of ROS attenuation by CsA is larger than Ru360 (Figure 4A). For the mitochondrial membrane potential, both CsA and Ru360 could only partially attenuate $\Delta\Psi_m$ collapse in cardiac mitochondria (Figure 4B). When the concentration of CsA and Ru360 was decreased in half, it was still effective in attenuating mitochondrial dysfunction (Figure 4). However, CsA at a lower dose had lower effectiveness in preventing mitochondrial depolarization.

Discussion

In the present study the major findings are that in cardiac mitochondria overloaded with Ca^{2+} : 1) mitochondrial swelling, elevated ROS production and mitochondrial membrane depolarization were observed; 2) CsA pretreatment could completely prevent cardiac mitochondrial swelling in the first 15 min after Ca^{2+} exposure, protect ROS production, and partially alleviate $\Delta\Psi_m$ dissipation; and 3) Ru360 pretreatment could not completely avert mitochondrial swelling, although it could attenuate ROS production and partially block $\Delta\Psi_m$ collapse.

In the heart under physiological condition, Ca^{2+} plays an important part in mitochondrial oxidative-phosphorylation which enhances ATP production to support contractile activity and ion transport systems of myocardium (24). However, excessive Ca^{2+} loading occurring during cardiac ischemia/reperfusion can trigger mPTP opening and lead to apoptotic cell death (25,26). In the present study we found that Ca^{2+} at 5 μM did not cause cardiac mitochondrial swelling, ROS production, and $\Delta\Psi_m$ collapse. However, 10 μM Ca^{2+} could induce slight mitochondrial depolarization. This finding may represent the role of low-dose Ca^{2+} in physiological process. Depolarization of cardiac mitochondria following 10 μM Ca^{2+} may be due to Ca^{2+} influx down a potential gradient (27) and be related to Ca^{2+} uptake through the MCU, which occurs only when the concentration of Ca^{2+} reaches 10 μM (28). With exposure of high concentrations of Ca^{2+} (100 and 200 μM), cardiac

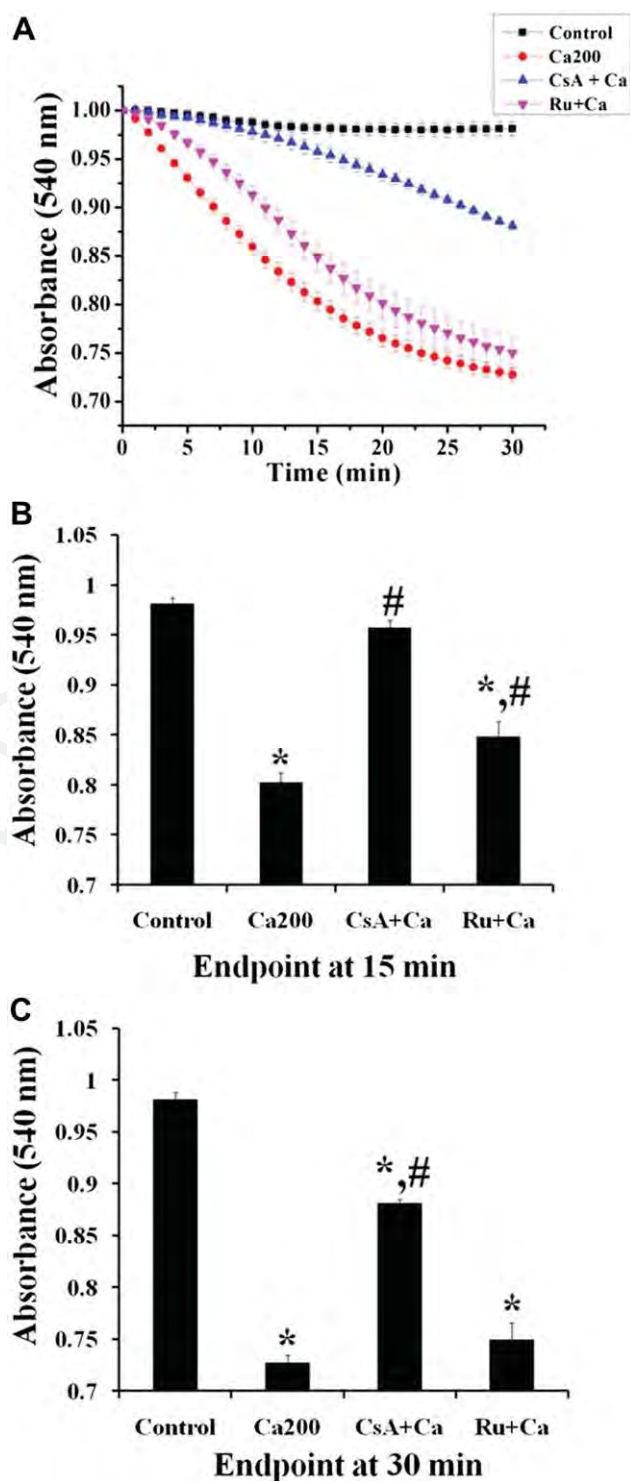


Figure 3. Effects of CsA and Ru360 on cardiac mitochondrial swelling. (A) Kinetic study of cardiac mitochondrial swelling. (B) Mitochondrial swelling at 15 min after 200 μM Ca^{2+} exposure. (C) Mitochondrial swelling at 30 min after 200 μM Ca^{2+} exposure. Control: isolated cardiac mitochondria without Ca^{2+} exposure, Ca200: Ca^{2+} -treated mitochondria at 200 μM , CsA + Ca: mitochondria pretreated with CsA followed by 200 μM Ca^{2+} application, Ru + Ca: mitochondria pretreated with Ru360 followed by 200 μM Ca^{2+} application. * $p < 0.05$ vs. control, # $p < 0.05$ vs. Ca200.

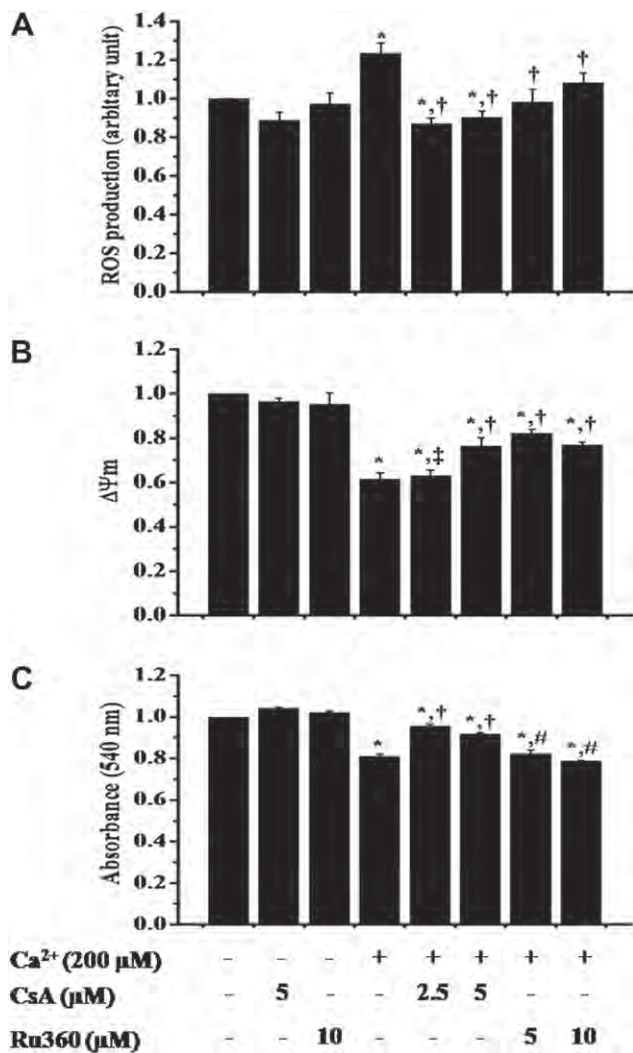


Figure 4. Effects of CsA and Ru360 on cardiac mitochondrial ROS production. (A) $\Delta\Psi_m$ dissipation (B) and mitochondrial swelling. (C) Neither CsA nor Ru360 alone altered any determined parameters. CsA at 2.5 and 5 μM , and Ru360 at 5 and 10 μM provided similar results on ROS production and mitochondrial swelling. * $p < 0.05$ vs. control, † $p < 0.05$ vs. Ca^{2+} treatment, ‡ $p < 0.05$ vs. 5 μM CsA+ Ca^{2+} , # $p < 0.05$ vs. CsA+ Ca^{2+} .

mitochondria became more depolarized and obviously swelling, observed by the decreasing of absorbance and severe morphological changes in electron microscopy. These high concentrations of Ca^{2+} could also induce oxidative stress by increasing ROS production. However, in cardiac mitochondria, Ca^{2+} -induced ROS production was observed in the condition in which complex III in the electron transport chain was inhibited (29). The increase in ROS production, which enhanced by Ca^{2+} alone, has been shown to be due to the loss of cytochrome c or glutathione-antioxidant enzymes through the mPTP (30,31). Our findings supported the hypothesis of Ca^{2+} threshold for the mPTP opening (32).

For the protective effects of CsA, our results demonstrated that within 15 min after Ca^{2+} exposure, CsA could completely prevent cardiac mitochondria from swelling,

consistent with the effect on protecting ROS production. Therefore, it could be implied that the opening of mPTP plays a critical role in cardiac mitochondrial dysfunction in conditions of early Ca^{2+} overload. However, CsA could only partially prevent $\Delta\Psi_m$ collapse because inhibition of mPTP did not block Ca^{2+} influx and, therefore, did not prevent alteration of the electron gradient across the inner membrane. The protective effect of CsA was attenuated in the next 15 min after Ca^{2+} exposure when the extent of mitochondrial swelling was so large that CsA no longer prevented mitochondrial swelling. Because the CsA could not inhibit Ca^{2+} influx into the mitochondria, high Ca^{2+} accumulation occurred in late Ca^{2+} exposure and could competitively interfere with the effect of CsA at mPTP, resulting in partial mPTP opening and later mitochondrial swelling (33,34).

As mitochondrial Ca^{2+} overload is a key factor of mPTP opening and the MCU has been proposed as a dominant portal of Ca^{2+} influx (12–14), we also further investigated the effect of Ru360, a selective MCU blocker against cardiac mitochondrial dysfunction. We found that Ru360 could decrease ROS production, although to a lesser extent than CsA. Moreover, Ru360 could not prevent mitochondrial swelling from Ca^{2+} overload in both early and late Ca^{2+} exposure. Considering the partial effect of Ru360 on inhibition of $\Delta\Psi_m$ collapse, this finding suggested that Ca^{2+} may possibly enter the matrix through alternative pathways other than the MCU. Recently, growing bodies of evidence of other mechanisms related to mitochondrial Ca^{2+} influx have been reported. These mechanisms include mitochondrial ryanodine receptor (35,36), a rapid mode of Ca^{2+} uptake (36), mitochondrial uncoupling proteins (37), and leucine zipper EF hand-containing transmembrane protein 1 (LETM1) $\text{Ca}^{2+}/\text{H}^{+}$ antiporter (38). Ca^{2+} influx through these alternative Ca^{2+} uptake channels could also trigger mPTP opening. Therefore, Ru360 may not be a promising agent for protecting cardiac mitochondrial dysfunction from calcium overload. Nevertheless, because this was an *in vitro* study performed in isolated cardiac mitochondria, there may be some limitations in that isolated mitochondria lack the effects of the cellular environment, which can interfere with the biological response. Also, direct intracellular calcium measurement, as well as calcium retention capacity, was not investigated in the present study. Future *in vivo* studies are needed to confirm the efficacy of both agents as well as to seek for the roles of those possible alternative portals for calcium entry to warrant their possible therapeutic use.

In conclusion, our finding suggested that Ca^{2+} -induced cardiac mitochondrial swelling, ROS production and $\Delta\Psi_m$ dissipation is due to mPTP opening. Moreover, MCU may not be the principal pathway for cardiac mitochondrial Ca^{2+} uptake. CsA but not Ru360, was shown to be an effective agent against calcium-induced cardiac mitochondrial dysfunction.

Acknowledgments

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Synaptic and nonsynaptic mitochondria demonstrate a different degree of calcium-induced mitochondrial dysfunction

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ABSTRACT

Aims: Since variety in response to Ca²⁺-induced mitochondrial dysfunction in different neuronal mitochondrial populations is associated with the pathogenesis of several neurological diseases, we investigated the effects of Ca²⁺ overload on synaptic (SM) and nonsynaptic mitochondrial (NM) dysfunction and probed the effects of cyclosporin A (CsA), 4'-chlorodiazepam (CDP) and Ru360 on relieving mitochondrial damage.

Main methods: SM and NM mitochondria were isolated from rats' brains (n = 5/group) and treated with various concentrations (5, 10, 100, and 200 μM) of Ca²⁺, with and without CsA (mPTP blocker), CDP (PBR/TSPO blocker) and Ru360 (MCU blocker) pretreatments. Mitochondrial function was determined by mitochondrial swelling, ROS production and mitochondrial membrane potential changes (ΔΨm).

Key findings: At 200-μM Ca²⁺, SM presented mitochondrial swelling to a greater extent than NM. At 100 and 200-μM Ca²⁺, the ROS production of SM was higher than that of NM and ΔΨm dissipation of SM was also larger. CsA, CDP and Ru360 could reduce ROS production of SM and NM with exposure to 200-μM Ca²⁺. However, only Ru360 could completely inhibit ROS generation in both SM and NM, whereas CsA and CDP could only partially reduce the ROS level in SM. Moreover, CsA and CDP pretreatments were not able to restore ΔΨm. However, Ru360 pretreatment could protect ΔΨm dissipation in both SM and NM, with complete protection observed only in NM.

Significance: Our findings suggested that mitochondrial calcium uniporter is a possible major pathway for calcium uptake in both mitochondrial populations. However, SM might have additional pathways involved in the calcium uptake.

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Introduction

Mitochondria are critical regulators of neuronal cell survival and cell death (Boitier et al., 1999; Hyder et al., 2006; Murchison and Griffith, 2007; Yuan and Yankner, 2000). The pathophysiology of numerous neurological disorders, such as ischemic-reperfusion injury (Blomgren et al., 2003; Pandya et al., 2011), traumatic brain injury (Friberg and Wieloch, 2002; Norenberg and Rao, 2007) and neurodegenerative diseases (Reddy and Reddy, 2011; Reeve et al., 2008), is related to mitochondrial dysfunction, which leads to neuronal apoptosis. The most important factor inducing mitochondrial dysfunction is Ca²⁺ overload, which primarily occurs during neuroexcitotoxicity (Duchen, 2004; Nicholls, 2009; Starkov et al., 2004). Ca²⁺ overload is involved with mitochondrial membrane potential depolarization (ΔΨm dissipation), which has been proposed as an initiator as well as a consequence of mitochondrial transition pore (mPTP) opening

(Ly et al., 2003; Wadia et al., 1998). Reactive oxygen species (ROS) are also crucial players in neuronal mitochondrial dysfunction (Wang et al., 2011). However, the relationship between Ca²⁺ overload and ROS in brain mitochondria is diverse depending on experimental conditions (Gyulkhandanyan and Pennefather, 2004; Komary et al., 2008; Panov et al., 2007; Petrosillo et al., 2004; Schonfeld and Reiser, 2007; Votyakova and Reynolds, 2005). Moreover, the correlation between Ca²⁺-induced mitochondrial damage, ΔΨm dissipation, and ROS production is still controversial (Adam-Vizi and Starkov, 2010).

Brain mitochondria are classified into two groups, the synaptic mitochondria (SM) and the nonsynaptic mitochondria (NM). The properties of these two types of mitochondria especially in Ca²⁺ handling are different (Guo et al., 2005; Li et al., 2004). SM, which are located around the synapse, are exposed to extensive Ca²⁺ fluctuations and are at high risk for oxidative stress and Ca²⁺ accumulative damages (Banachlocha et al., 1997; Martinez et al., 1996). A previous study reported that the difference in the Ca²⁺-induced mPTP opening could be due to the higher level of cyclophilin D (CypD) in SM (Naga et al., 2007). However, direct inhibition of CypD by cyclosporine A (CsA) cannot increase the Ca²⁺ accumulation capacity in SM (Brown et al., 2006), suggesting that additional mechanisms are likely

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responsible for the differences in Ca^{2+} handling in SM and NM. The main portal pathway for Ca^{2+} uptake of neuronal mitochondria proposed by previous studies is the mitochondrial calcium uniporter (MCU). However, subsequent studies of other tissues have discovered additional Ca^{2+} uptake mechanisms such as the rapid mode of Ca^{2+} uptake (RAM) (Buntinas et al., 2001; Sparagna et al., 1995), and the mitochondrial ryanodine receptor (mRyR) (Altschaffl et al., 2007; Beutner et al., 2001; Beutner et al., 2005). Moreover, the Ca^{2+} uptake mechanism of SM and NM in the Ca^{2+} overload condition has not been investigated and whether Ca^{2+} entry via MCU in the SM and NM is different is not known. Therefore, in this study, we tested the hypothesis that 1) SM respond to Ca^{2+} overload conditions in a different way from NM, and 2) the mechanisms for Ca^{2+} entry via MCU in the SM and NM are different.

Materials and methods

Reagents

All of the reagents used in this study were purchased from Sigma (St. Louis, MO., USA), except Ru360, which was purchased from Calbiochem (San Diego, CA., USA).

Bovine serum albumin (BSA) and pyruvic acid were purchased from Amresco (Solon, OH., USA). CsA and CDP were prepared in DMSO and further diluted to final concentrations by 2% DMSO. Ru360 was prepared in deionized water.

Animal preparation

This study was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University. Wistar rats (300–400 g) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. All animals were housed in a controlled room temperature maintained between 22 and 25 °C in a constant 12-h light/dark cycle. They were fed with standard pellet rat diet and water ad libitum.

Experimental protocols

Isolated mitochondria from synaptosomes and nonsynaptosomes of rat cortical brains were used as in a previous study (Chelli et al., 2001; Novalija et al., 2003; Thummasorn et al., 2011; Tong et al., 2005). The first protocol was to investigate the effect of Ca^{2+} -induced mitochondrial dysfunction on synaptic versus nonsynaptic mitochondria in the morphological aspects of mitochondrial swelling, ROS

production and $\Delta\Psi\text{m}$ dissipation. In this study, CaCl_2 at concentrations of 5, 10, 100 and 200 μM were used ($n=6/\text{group}$ for ROS and $\Delta\Psi\text{m}$ measurements). Various doses of CaCl_2 were applied for 10 min to isolated mitochondria before the assessment of all parameters. In the second protocol, the mechanism underlying the differences in Ca^{2+} responses of SM and NM was investigated by several pharmacological interventions: cyclosporine A (CsA), 4'-chlorodiazepam (CDP) and Ru360 (as shown in Fig. 1). CsA is known as a mitochondrial permeability transition pore blocker, CDP is the specific peripheral benzodiazepine receptor (or presently known as translocator protein; TP50) antagonist, and Ru360 is the mitochondrial calcium uniporter blocker. In the second protocol, both SM and NM were randomly assigned into eight groups: control (vehicle), CsA (5 μM), CDP (100 μM), Ru360 (10 μM), CaCl_2 (200 μM), CaCl_2 pretreated with CsA, CaCl_2 pretreated with CDP and CaCl_2 pretreated with Ru360 ($n=5/\text{group}$). CsA and CDP were applied to the mitochondria for 30 min prior to exposure to Ca^{2+} or vehicle for 30 min, while Ru360 was added at 3 min before Ca^{2+} exposure. Doses of all blockers were used according to those reported previously (Thummasorn et al., 2011; Zhang et al., 2006).

Isolation of nonsynaptic brain mitochondria

Nonsynaptic brain mitochondria were isolated from 300–350 g male Wistar rats using a method modified from that described in a previous study (Lai and Clark, 1979; Clark and Nicklas, 1970; Krasnikov et al., 2005). Following decapitation, brains were rapidly removed and placed in ice-cold isolation buffer containing 320 mM sucrose, 10 mM HEPES, and 0.5 mM EGTA at pH 7.4. All homogenization and centrifugation steps were carried out at 4 °C. The cortices were chopped into small pieces with razors. Then, the brains were homogenized by the homogenizer containing 10 ml of isolation buffer. The homogenate was centrifuged at 1300 g for 3 min. The supernatant was centrifuged at 1300 g for 3 min. The supernatant was collected and centrifuged at 17,000 g for 8 min. The pellets obtained from this step were suspended in 3 ml of isolation buffer and applied to the top of 3 ml of 10% (w/v) Ficoll overlaid by 3 ml of 7.5% (w/v) Ficoll. After centrifugation at 99,000 g for 20 min, aliquots were removed. The nonsynaptic mitochondria-containing pellets at the bottom of the tube were resuspended in isolation buffer and recentrifuged at 12,000 g for 8 min. The pellet was resuspended in an isolation buffer supplemented with 0.5 mg/ml BSA and centrifuged at 12,000 g for 8 min. Protein concentration was determined using a Bicinchoninic Acid (BCA) assay with bovine serum albumin used as a concentration standard (Walker, 1994).

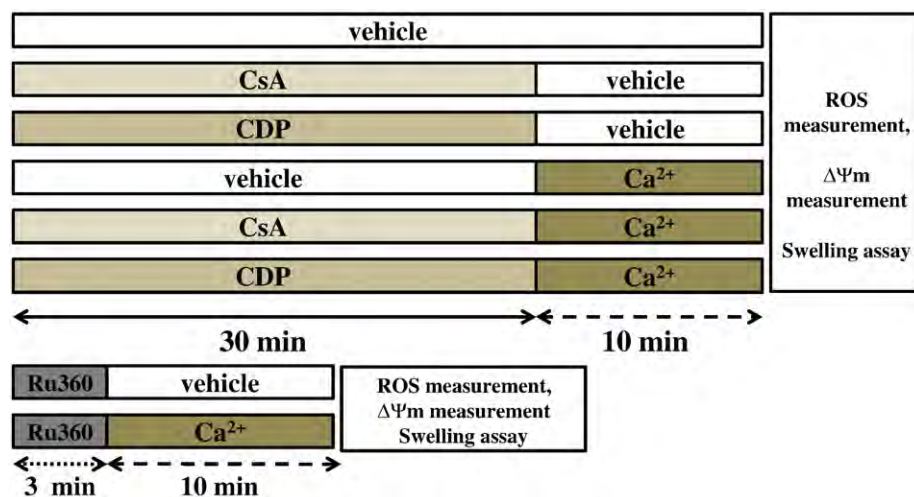


Fig. 1. Study protocol to determine the effects of cyclosporine A (CsA), 4'-chlorodiazepam (CDP) and Ru360 on Ca^{2+} -induced mitochondrial dysfunction.

Isolation of synaptic brain mitochondria

Brain synaptic mitochondria from male Wistar rats (300–350 g) were isolated according to the method described in a previous study (Kudin et al., 2004; Pallotti and Lenaz, 2007; Berman et al., 2000; Gangolf et al., 2010; Rosenthal et al., 1987) using 0.02% digitonin to free mitochondria from the synaptosomal fraction. After decapitation, the brain was transferred to ice-cold MSE solution (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, 1 mg/ml BSA, pH 7.4), minced and transferred to 10 ml of MSE solution containing 0.05% nalgase for homogenizations. Then, the homogenate was centrifuged at 2 000 g for 4 min. The supernatant was centrifuged at 12,000 g for 9 min. The synaptosome-enriched pellets were resuspended in MSE containing 0.02% digitonin and centrifuged at 12,000 g for 11 min.

Identification of mitochondria with electron microscopy

Isolated mitochondria were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4 °C, postfixed with 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature. Then, the pellets were dehydrated in a graded series of ethanol and embedded in Epon-Araldite as described in a previous study (Chelli et al., 2001).

After being cut with a diamond knife, ultrathin sections were placed on copper grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope.

Mitochondria were classified into two categories; intact mitochondria and damaged mitochondria. Intact mitochondria were characterized by condensed cristae and an uninterrupted outer membrane. Conversely, damaged mitochondria or mitochondrial swelling is described as fragmented cristae or an interrupted outer membrane and expanding matrix (Lifshitz et al., 2003).

Measurement of mitochondrial ROS production

ROS production in mitochondria was determined spectrofluorometrically, using dichlorohydro-fluorescein diacetate (DCFDA) (Novalija et al., 2003). Synaptic or nonsynaptic mitochondria (0.4 mg/ml) were incubated with 2 μ M DCFDA at 25 °C for 20 min. Fluorescence was determined at 485 nm for excitation and 530 nm for emission. The ROS level was manifested as arbitrary units of fluorescence intensity of DCF as described in a previous study (Thummasorn et al., 2011).

Measurement of mitochondrial membrane potential ($\Delta\Psi$ m)

The dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to assess the change in $\Delta\Psi$ m as described in a previous study (Tong et al., 2005). Decreased red/green fluorescent intensity ratio indicates mitochondrial depolarization (Perry et al., 2011). The isolated mitochondria (0.4 mg/ml) were incubated with 310 nM JC-1 at 37 °C for 30 min.

Fluorescence intensity was measured by a fluorescence microplate reader using 485- and 530-nm (green) wavelengths for the excitation and 485- and 590 nm (red) for the emission.

Measurement of mitochondrial swelling

Mitochondrial swelling was assessed by measuring changes in the absorbance of the suspension at the 540-nm wavelength using a microplate reader. SM or NM (0.4 mg/ml) were incubated in a 2-ml respiration buffer (containing 150 mM KCl, 5 mM HEPES, 5 mM $K_2HPO_4 \cdot 3H_2O$, 2 mM L-glutamate, 5 mM pyruvate sodium salt). Decreased absorbance indicated mitochondrial swelling (Thummasorn et al., 2011).

Data analysis

All data were expressed as means $\pm$ SEM. Comparisons were made by one-way ANOVA followed by the Fisher post-hoc test. Results were statistically significant with $P < 0.05$.

Results

Ca^{2+} -induced oxidative stress and mitochondrial membrane depolarization in SM and NM

At baseline, there was no significant difference between the ROS level in the SM (137.19 ± 21.53 au) and NM (155.48 ± 23.60 au), and the $\Delta\Psi$ m between SM (1.12 ± 0.20) and NM (0.67 ± 0.22). Ca^{2+} at 5 μ M did not cause any changes in ROS production in either SM or NM (Fig. 2). However, Ca^{2+} at 10 μ M increased the mitochondrial ROS production in NM, but not SM (Fig. 2). Ca^{2+} at 100 and 200 μ M increased ROS production in a dose-dependent manner in both SM and NM (Fig. 2). We also found that SM generated significantly higher levels of ROS in response to 200- μ M Ca^{2+} than did NM (Fig. 2). Low concentrations of Ca^{2+} (5, 10 μ M) did not change the $\Delta\Psi$ m in either SM or NM (Fig. 3). When Ca^{2+} concentration was increased to 100 and 200 μ M, the SM encountered mitochondrial depolarization in a dose dependent manner, as shown by an obvious decrease in $\Delta\Psi$ m dissipation. In NM, however, mitochondrial depolarization occurred only when treated with 200- μ M Ca^{2+} (Fig. 3). Moreover, when applied with 100 and 200- μ M Ca^{2+} , the level of SM $\Delta\Psi$ m dissipation was greater than that of NM (Fig. 3). In the swelling assay, we found that 5, 10 and 100- μ M Ca^{2+} did not cause NM swelling, while the 100- μ M Ca^{2+} started to cause SM swelling (Fig. 4). However, the 200- μ M Ca^{2+} could cause both SM and NM swelling (Fig. 4). Consistent with the morphology changes (as shown in Fig. 5), the exposure of 200- μ M Ca^{2+} in both SM and NM caused severe mitochondrial swelling. However, SM exhibited greater swelling and morphological changes than those from NM (Figs. 4 and 5).

Effects of CsA, CDP, and Ru360 on SM and NM ROS production

The levels of ROS production were not altered in either SM or NM applied with CsA, CDP or Ru360, alone (Fig. 6). The ROS levels were dramatically increased in both SM and NM treated with 200- μ M Ca^{2+}

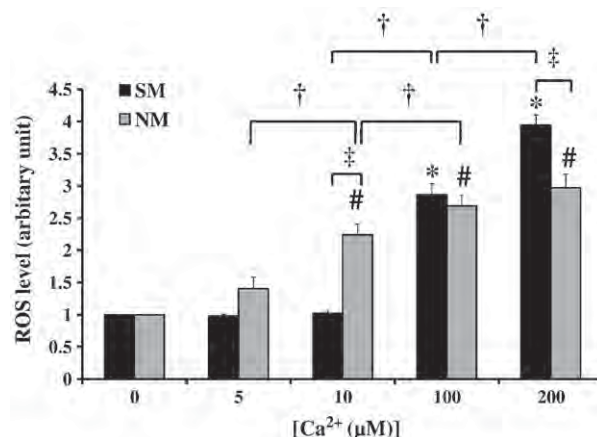


Fig. 2. ROS production in response to increasing concentration of Ca^{2+} in mitochondria isolated from synaptosomes and nonsynaptosomes ($n = 8/\text{group}$). 0, 5, 10, 100, and 200 = control group and $CaCl_2$ -treated mitochondria at 5, 10, 100, and 200 μ M, respectively. SM: synaptic mitochondria, NM: nonsynaptic mitochondria, * $P < 0.05$ compared to the control group for SM, # $P < 0.05$ compared to the control group for NM, † $P < 0.05$ differences between different calcium concentrations at the same group, ‡ $P < 0.05$ differences between different groups at the same calcium concentration.

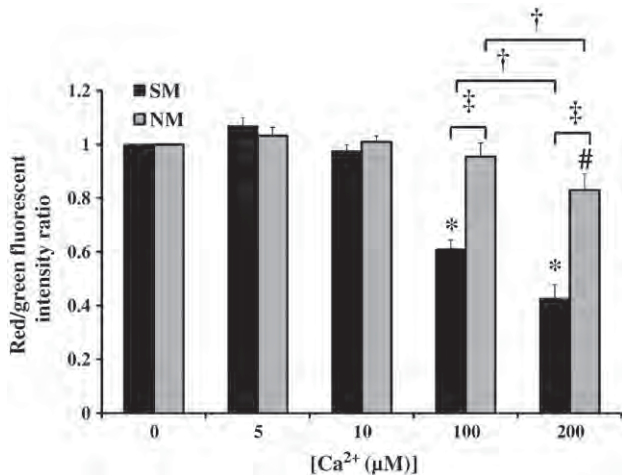


Fig. 3. Ca²⁺-induced $\Delta\Psi_m$ dissipation rates of mitochondria isolated from synaptosomes and nonsynaptosomes ($n=8/\text{group}$). SM: synaptic mitochondria, NM: nonsynaptic mitochondria, * $P<0.05$ compared to the control group for SM, # $P<0.05$ compared to the control group for NM, † $P<0.05$ differences between different calcium concentrations in the same group, ‡ $P<0.05$ differences between different groups at the same calcium concentration.

(Fig. 6). The ROS level, however, was increased to a much higher level in SM, than in NM. When CsA, CDP and Ru360 were applied to SM and NM prior to 200- μM Ca²⁺ application, the ROS levels were significantly reduced, compared to the Ca²⁺-treated group. However, the ROS level in CsA and CDP pretreatment groups of SM was still greater than that in the control group, whereas the ROS level in NM was reduced to the same levels as the control group. In addition, the ROS levels in Ru360 pretreatment of both SM and NM were at the same levels as in the control group (Fig. 6).

Effects of CsA, CDP, and Ru360 on SM and NM membrane potential dissipation ($\Delta\Psi_m$)

At the baseline, CsA and CDP caused a significant decrease of $\Delta\Psi_m$, but Ru360 did not (Fig. 7). With exposure to 200- μM Ca²⁺, both SM and NM underwent mitochondrial depolarization. As shown in Fig. 7, CsA and CDP pretreatment did not help in attenuating

the $\Delta\Psi_m$ collapse in either SM or NM. However, Ru360 pretreatment completely restored the membrane potential of NM to the normal level, whereas it only partially prevented $\Delta\Psi_m$ collapse in SM (Fig. 7).

Effect of CsA, CDP, and Ru360 on SM and NM swelling

CsA, CDP and Ru360 alone did not alter the absorbance in both SM and NM, compared to the control group. Among all pharmacological interventions, only Ru360 could completely prevent both SM and NM swelling following a 200- μM Ca²⁺ application (Fig. 8). For CsA and CDP, they could completely prevent mitochondrial swelling only in NM. In SM, both CsA and CDP could only partially prevent mitochondrial swelling (Fig. 8).

Discussion

Mitochondrial Ca²⁺ overload is a crucial pathogenesis of numerous neurological disorders including neurodegenerative diseases, ischemic brain injury, inflammatory processes and epilepsy (Zundorf and Reiser, 2010). Previous studies in isolated mitochondria have shown that excessive Ca²⁺ triggered mitochondrial permeability transition (mPT) evidenced by mitochondrial membrane potential ($\Delta\Psi_m$) collapse, ROS production, mitochondrial swelling, and outer membrane rupture leading to apoptotic cascade activation (Pivovarova and Andrews, 2010). In the CNS, differences in Ca²⁺ handling have been observed in different brain regions and neuronal mitochondrial populations. Synaptic mitochondria are at high risk for accumulative mitochondrial perturbation and consequently become more challenging to therapeutic interventions (Du et al., 2010). Mitochondrial swelling is one of the most important markers of the mPTP opening (Bernardi, 1996; Friberg and Wieloch, 2002). In the present study, we demonstrated that morphological changes in SM exposed to 200- μM Ca²⁺ were greater than those in the NM which reflected that SM underwent more mPTP opening as compared to the NM during Ca²⁺ exposure. This finding is consistent with that of a previous study which reported that SM had less capacity for Ca²⁺ accumulation than did NM prior to mitochondrial permeability transition (mPT), suggesting that Ca²⁺-induced mPT in SM is more intense than in NM (Brown et al., 2006).

Ca²⁺ overload can also give rise to oxidative stress. Several mechanisms have been proposed to be involved in Ca²⁺-induced ROS production. Under physiologic conditions, Ca²⁺ may be both a stimulator for oxidative-phosphorylation and a partial inhibitor of the electron transport chain, each of which actions leads to ROS production (Brookes et al., 2004). Another mechanism is the Ca²⁺-triggered mPTP-opening when intramitochondrial Ca²⁺ reaches a threshold level (Ow et al., 2008). Opening of the mPTP leads to cytochrome c release causing an increase in ROS production (Starkov et al., 2004). According to the critical ROS threshold hypothesis, ROS at a certain level could trigger mitochondrial membrane depolarization (Zorov et al., 2006). Hence, it is possible that Ca²⁺, which surges to the threshold level, might induce an increase of the ROS level high enough to cause $\Delta\Psi_m$ dissipation.

In this study, we demonstrated that 100- μM Ca²⁺ or higher caused ROS production, $\Delta\Psi_m$ dissipation, and mitochondrial swelling in SM. This finding implies that ROS level caused by 100- μM Ca²⁺ could reach the threshold level of SM to cause SM dysfunction. However, in NM, 10- μM and 100- μM Ca²⁺ triggered ROS production without causing $\Delta\Psi_m$ dissipation or mitochondrial swelling, while 200- μM Ca²⁺ triggered both ROS generation, $\Delta\Psi_m$ collapse, and mitochondrial swelling in NM. This finding suggests that the ROS levels caused by 10- μM and 100- μM Ca²⁺ were not high enough to induce $\Delta\Psi_m$ collapse and mitochondrial swelling. Nevertheless, the ROS level caused by 200- μM Ca²⁺ reached the critical threshold of NM to undergo NM dysfunction. Therefore, from the data of ROS, $\Delta\Psi_m$ and mitochondrial swelling, it may be concluded that the critical

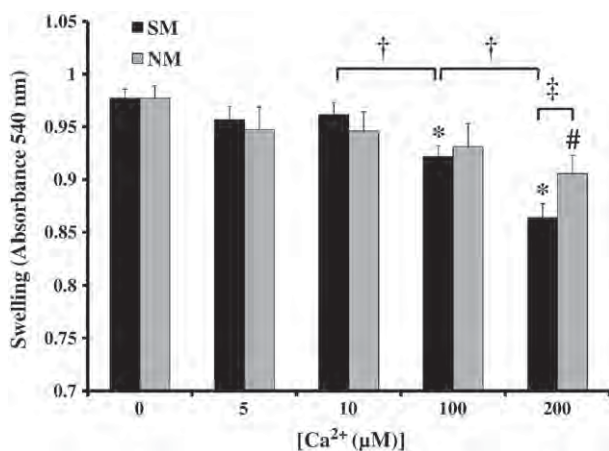


Fig. 4. Ca²⁺-induced mitochondrial swelling of SM and NM ($N=8/\text{group}$) after 10-min of Ca²⁺ application. SM: synaptic mitochondria, NM: nonsynaptic mitochondria, * $P<0.05$ compared to the control group for SM, # $P<0.05$ compared to the control group for NM, † $P<0.05$ differences between different calcium concentrations in the same group, ‡ $P<0.05$ differences between different groups at the same calcium concentration.

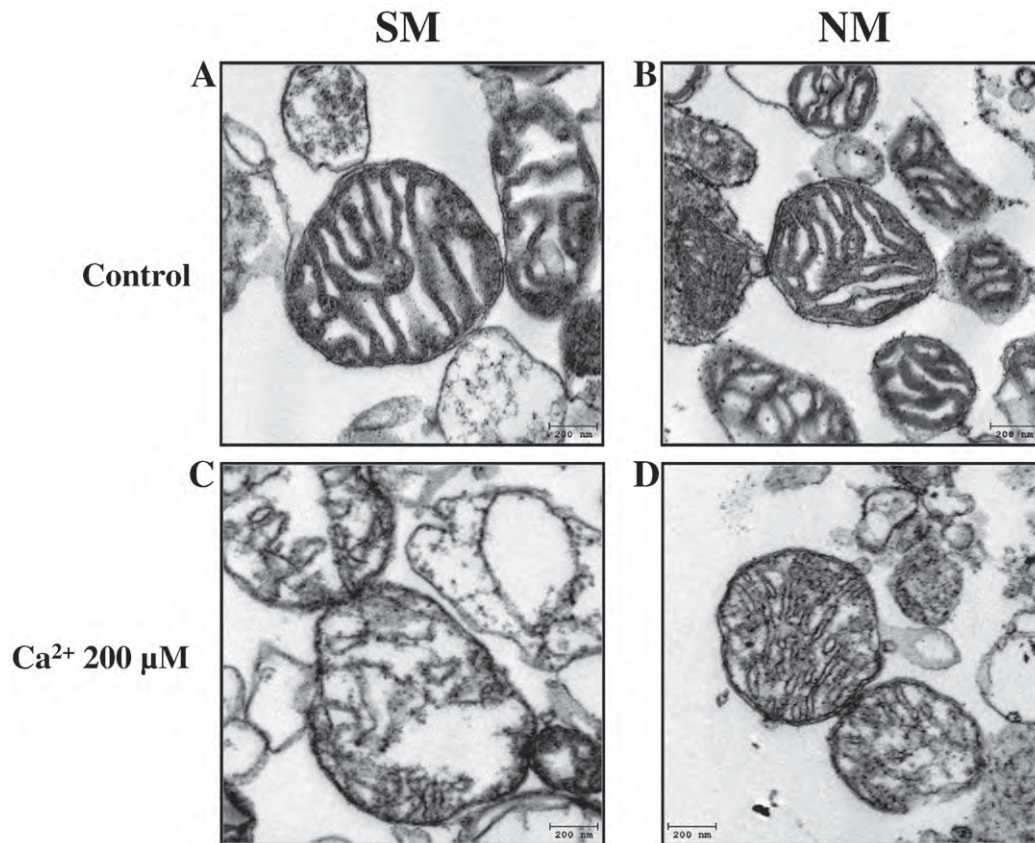


Fig. 5. Effect of Ca^{2+} overload on mitochondrial morphology of SM and NM. SM or NM (0.4 mg/ml) was incubated with respiration buffer (A, B) or with respiration buffer plus 200 μM Ca^{2+} (C, D) for 10 min before fixation. SM: synaptic mitochondria, NM: nonsynaptic mitochondria, magnification: 1:15,000 (A–D).

threshold levels of Ca^{2+} induced mitochondrial dysfunction in NM were higher than that in SM.

Since the heterogeneity of mitochondrial membrane channel properties of different mitochondrial populations might determine diverse sensitivities to Ca^{2+} insult and underlie pathogenesis of neuronal dysfunction, we examined the pharmacological effects on ROS production,

$\Delta\psi\text{m}$ dissipation, and mitochondrial swelling. In the present study, we found that pretreatment with CsA or CDP greatly reduced ROS levels of NM to the normal levels but only partially decreased ROS levels of SM. Since CsA and CDP directly interact with the mPTP components, the differences in response to these agents suggest the possibility that the quantity of both CypD and TSPO or their susceptibility to Ca^{2+} on the

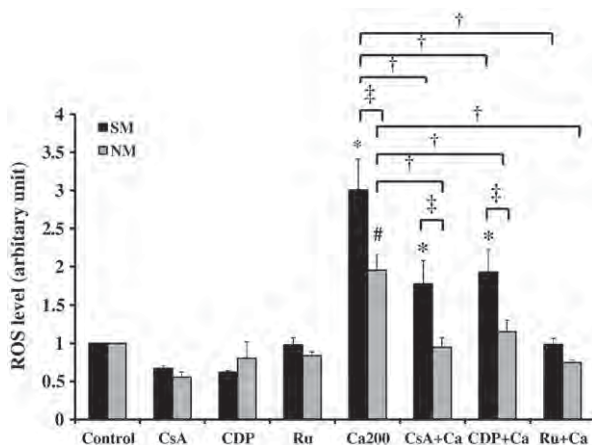


Fig. 6. Effects of CsA (5 μM), CDP (100 μM) and Ru360 (10 μM) on synaptic and nonsynaptic mitochondrial ROS production ($n = 5/\text{group}$). CsA, CDP, and Ru: mitochondria treated with 5- μM CsA, 100- μM CDP, and 10- μM Ru360, respectively. Ca200: mitochondria treated with CaCl_2 200 μM . CsA + Ca, CDP + Ca, Ru + Ca: mitochondria pretreated with 5 μM CsA, 100 μM CDP, and 10 μM Ru360 followed by CaCl_2 application, respectively. SM: synaptic mitochondria, NM: nonsynaptic mitochondria, * $P < 0.05$ compared to the control group for SM, # $P < 0.05$ compared to the control group for NM, † $P < 0.05$ compared to 200 μM Ca^{2+} in the same group, ‡ $P < 0.05$ compared to SM at the same pretreatment group.

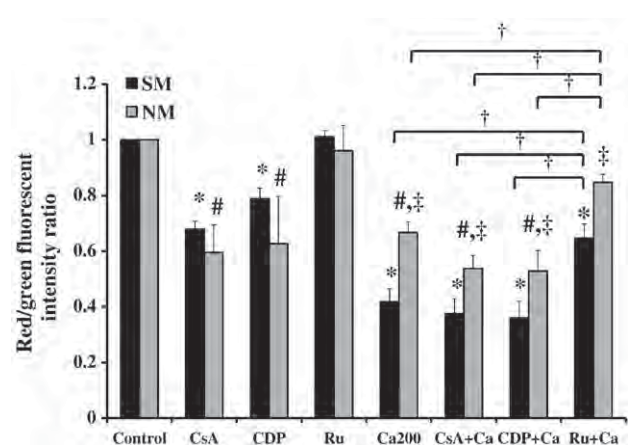


Fig. 7. Effects of CsA, CDP, and Ru360 on synaptic and nonsynaptic mitochondrial $\Delta\psi\text{m}$ dissipation ($n = 5/\text{group}$). CsA, CDP, and Ru: mitochondria treated with 5- μM CsA, 100- μM CDP, and 10- μM Ru360, respectively. Ca200: mitochondria treated with CaCl_2 200 μM . CsA + Ca, CDP + Ca, Ru + Ca: mitochondria pretreated with 5- μM CsA, 100- μM CDP, and 10- μM Ru360 followed by CaCl_2 application, respectively. SM: synaptic mitochondria, NM: nonsynaptic mitochondria, * $P < 0.05$ compared to the control group for SM, # $P < 0.05$ compared to the control group for NM, † $P < 0.05$ compared to 200 μM Ca^{2+} in the same group, ‡ $P < 0.05$ compared to SM at the same pretreatment group.

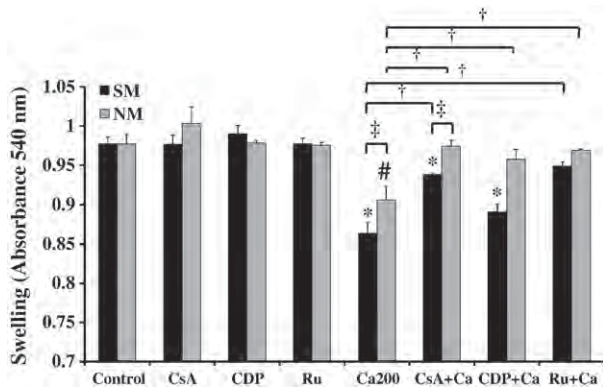


Fig. 8. Effects of CsA, CDP, and Ru360 on synaptic and nonsynaptic mitochondrial swelling ($n=5/\text{group}$). CsA, CDP, and Ru: mitochondria treated with 5- μM CsA, 100- μM CDP, and 10- μM Ru360, respectively. Ca200: mitochondria treated with 200- μM CaCl_2 . CsA + Ca, CDP + Ca, Ru + Ca: mitochondria pretreated with 5- μM CsA, 100- μM CDP, and 10- μM Ru360 followed by CaCl_2 application, respectively. SM: synaptic mitochondria, NM: nonsynaptic mitochondria, * $P<0.05$ compared to the control group for SM, # $P<0.05$ compared to the control group for NM, † $P<0.05$ compared to 200 μM Ca^{2+} in the same group, ‡ $P<0.05$ compared to SM of the same treatment group.

mitochondrial membranes of SM might be greater than on NM. This finding was consistent with that of a recent study which reported the larger amount of CypD in SM compared with NM (Naga et al., 2007). That finding and our findings may be a major factor underlying the differences in Ca^{2+} -induced mitochondrial dysfunction between SM and NM. However, pretreatment with higher concentrations of CsA and CDP did not decrease the ROS levels in SM to the normal level (data not shown), suggesting that other mechanisms besides mPTP might be responsible for ROS production in SM. The findings that CsA and CDP could completely prevent mitochondrial swelling in NM, but not SM, support this hypothesis.

In the present study, a specific MCU inhibitor Ru360 could decrease ROS production and mitochondrial swelling caused by excess Ca^{2+} in both SM and NM to normal levels, suggesting that Ca^{2+} influx could occur directly via MCU. By inhibiting MCU, extramitochondrial Ca^{2+} could not influx into the matrix, leading to the blockage of Ca^{2+} -induced ROS production processes. These findings suggested that MCU could play a key role in increased ROS production induced by Ca^{2+} in both SM and NM. Furthermore, our results on the pharmacological interventions on mitochondrial membrane potential changes demonstrated that only Ru360 can attenuate the Ca^{2+} -induced mitochondrial depolarization in both SM and NM, whereas CsA and CDP could not. Although both CsA and CDP could reduce ROS production, they did not prevent mitochondrial depolarization. Mitochondrial depolarization due to excess Ca^{2+} could also involve the mechanism unrelated to the mPTP opening. Excess Ca^{2+} influx via the MCU could cause mitochondrial depolarization directly since Ca^{2+} uptake through MCU occurs down a potential gradient, thus allowing a large amount of positive charges into the mitochondria (Gunter and Pfeiffer, 1990). Although CsA and CDP directly inhibited mPTP opening and prevented loss of cytochrome c and ROS production, they could not prevent Ca^{2+} entry via MCU. Instead, Ru360 (MCU blocker), which directly inhibited Ca^{2+} influx, could protect mitochondrial depolarization. The findings that Ru360 could reduce ROS production greater than that by CsA and CDP could be another reason for the effect of Ru360 on relieving $\Delta\Psi\text{m}$ dissipation. Since Ca^{2+} entry into the mitochondria is the main cause of increased ROS production in this case, inhibiting Ca^{2+} influx could directly prevent excess ROS production as observed in the present study. However, the effect of Ru360 on $\Delta\Psi\text{m}$ in SM and NM was not similar. Ru360 could completely prevent $\Delta\Psi\text{m}$ dissipation in NM but partially prevent $\Delta\Psi\text{m}$ dissipation in SM, suggesting that MCU can be defined as a key pathway of Ca^{2+} sequestration in NM, but the additional alternative channels such as mitochondrial RyR or RAM may also take part in the Ca^{2+} uptake mechanism of SM.

Limitation of the study

Although the methods for NM and SM isolation we used in the present study have been used previously, they were different buffer systems, and may in part affect the mitochondrial physiological function. Therefore, the responses of SM and NM to the pharmacological interventions could be similar or different if the NM and SM were isolated with the same buffer system. Future studies are needed to investigate the effects of the buffer systems on mitochondrial responses to the drugs.

Conclusion

The findings in this study established that isolated mitochondria derived from synaptosomes presented more severe mitochondrial dysfunction in response to Ca^{2+} overload than did mitochondria derived from nonsynaptosomes. This divergence is not only the result of differences in mPTP components but also the differences in the Ca^{2+} uptake mechanism. Finally, the MCU may be the major portal for Ca^{2+} entry in NM. However, both MCU and other pathways such as RyR or RAM could be portals of Ca^{2+} entry in SM.

Although alternative mechanisms are still undiscovered, our results provided additional insights and explanation for the susceptibility of synaptic mitochondria, which could be useful for further therapeutic approach in numerous neuronal disorders, particularly disorders due to the calcium overload condition.

Conflict of interest statement

The authors state that there are no conflicts of interest.

Acknowledgments

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ORIGINAL ARTICLE

T-type calcium channel blockade improves survival and cardiovascular function in thalassemic mice

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Abstract

Objectives: Iron-overload cardiomyopathy is a major cause of morbidity and mortality in patients with thalassemia. However, the precise mechanisms of iron entry and sequestration in the heart are still unclear. Our previous study showed that Fe^{2+} uptake in thalassemic cardiomyocytes are mainly mediated by T-type calcium channels (TTCC). Nevertheless, the role of TTCC as well as other transporters such as divalent metal transporter1 (DMT1) and L-type calcium channels (LTCC) as possible portals for iron entry into the heart in *in vivo* thalassemic mice under an iron-overload condition has not been investigated. **Methods:** An iron-overload condition was induced in genetically altered β -thalassemic mice and adult wild-type mice by feeding them with an iron diet (0.2% ferrocene w/w) for 3 months. Then, blockers for LTCC (verapamil and nifedipine), TTCC (efonidipine), and DMT1 (ebselen) as well as iron chelator desferoxamine (DFO) were given for 1 month with continuous iron feeding. **Results:** Treatment with LTCC, TTCC, DMT1 blockers, and DFO reduced cardiac iron deposit, cardiac malondialdehyde (MDA), plasma non-transferrin-bound iron, and improved heart rate variability and left ventricular (LV) function in thalassemic mice with iron overload. Only TTCC and DMT1 blockers and DFO reduced liver iron accumulation, liver MDA, plasma MDA, and decreased mortality rate in iron-overloaded thalassemic mice. **Conclusions:** DMT1, LTCC, and TTCC played important roles for iron entry in the thalassemic heart under an iron-overloaded condition. Unlike LTCC blocker, TTCC blocker provided all benefits including attenuating iron deposit in both the heart and liver, reduced oxidative stress, and decreased mortality in iron-overloaded mice.

Key words cardiac iron; left ventricular function; heart rate variability; calcium channel; iron overload

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Beta-thalassemia is an inherited hemoglobin disorder, resulting in chronic hemolytic anemia, which is typically required for regular blood transfusion (1, 2). It is prevalent in people of Mediterranean origin, Middle East, North India, and Southeast Asia (3, 4). The iron-overload condition in thalassemia can lead to iron accumulation in various organs, especially in the heart (5, 6), leading to iron-overload cardiomyopathy, which is the major cause of mortality in patients with thalassemia (7, 8). However, the mechanisms of iron uptake into the heart leading to abnormal left ventricular (LV) function are still unclear. A previous study showed that the L-type calcium channels (LTCC) played a role for iron

uptake into the heart of the isolated perfused rat heart (9) and in an *in vivo* rat model of iron overload (10). However, inconsistent findings were reported in cultured cardiomyocyte studies (11, 12). Recently, using the thalassemic mouse model, our study demonstrated for the first time that T-type calcium channels (TTCC) were reexpressed in thalassemic mouse hearts (11). We also demonstrated that the TTCC blocker (efonidipine), but not the LTCC blocker (verapamil), prevented Fe^{2+} uptake into thalassemic cardiomyocytes (11).

Normally, iron can enter the cell such as enterocyte, hepatocyte, and cardiomyocyte via the divalent metal transporter1 (DMT1) and transferrin receptor1 (TfR1)

(13–15). However, under iron-overload conditions, previous studies demonstrated that TfR1 and DMT1 mRNA and protein expressions in the heart were suppressed (16, 17), suggesting that they may not be major transporters for iron uptake into the hearts under this condition. Moreover, it has been shown that TfR1 and DMT1 did not play important roles in Fe^{2+} uptake in cultured thalassemic cardiomyocytes (11). Nevertheless, the role of TTCC, LTCC, and DMT1 as iron transporters in '*in vivo*' thalassemic mice under iron-overload conditions has never been investigated.

Heart rate variability (HRV) is a measure of variation in the heart rate. Variation in the beat-to-beat interval is a physiological phenomenon, involving the sympathetic and parasympathetic nervous system (18, 19). HRV has been proposed as a new risk stratifier in postmyocardial infarction and heart failure patients (20). In patients with thalassemia, several studies demonstrated that the HRV was depressed indicating cardiac autonomic imbalance in these patients (18, 19). Similar findings, that is, depressed HRV, were also demonstrated in a mouse model of β -thalassemia (21). However, the effects of TTCC, LTCC, and DMT1 blockers on HRV as well as on LV function in iron-overloaded thalassemic mice have never been investigated.

In this study, we tested the hypothesis that pharmacological interventions with TTCC, LTCC, and DMT1 blockers and iron chelator desferoxamine (DFO) attenuate cardiac iron accumulation, improve HRV and LV function, and reduce cardiac oxidative stress in iron-overloaded thalassemic mice.

Materials and methods

Animal models

Two types of adult C57/BL6 mice (3–6 months old): wild type ($\mu\beta^{+/+}$, WT) and heterozygous β^{KO} type ($\mu\beta^{\text{th-3}/+}$, HT), were used in this study (11, 21). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, Chiang Mai University, and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animals were housed in controlled temperature and humidity rooms with 12-h light–dark cycles.

Iron treatment and pharmacological interventions

Iron overload was induced by feeding WT and HT mice (FE group) with FE diet (0.2% ferrocene w/w), whereas WT and HT mice in the control group were fed with a normal diet for 90 d. Then, the mice in the FE group

were randomly divided into six subgroups ($n = 8$ each) and were treated with drugs while being fed an FE diet for another 30 d. Group I (FE) mice were given intraperitoneal (IP) injections with 0.5% DMSO daily. Group II (FE/DFO) mice were injected with deferoxamine (DFO, 42 mg/kg) in 0.5% DMSO IP daily (22). Group III (FE/verapamil) mice were injected with verapamil (10 mg/kg) in 0.5% DMSO twice daily (alternating subcutaneous and IP) (23). Group IV (FE/nifedipine) mice were injected with nifedipine (5 mg/kg) in 0.5% DMSO IP daily (24). Group V (FE/efonidipine) mice were injected with efonidipine (4 mg/kg) in 0.5% DMSO IP daily (25). Group VI (FE/ebesen) mice were injected with ebesen (5 mg/kg) in 0.5% DMSO IP daily (26). Mice in the control group were injected with 0.5% DMSO IP daily and fed with the normal diet. Each group of mice were injected with drug at 6 pm each day except Fe/verapamil group, which were injected twice daily at 6 am and 6 pm each day. At the end of treatments (i.e., 4 months after iron-loading and treatment), the LV function, HRV, and cardiac iron deposition were assessed in all mice. The mortality rate was also determined in all groups.

Quantification of plasma non-transferrin-bound iron

The non-transferrin-bound iron (NTBI) concentration was measured using the NTA chelation/HPLC method established by Singh *et al.* (27) with the aluminum blocking step. Plasma was incubated with NTA solution (a final concentration of 80 mM) pH 7.0 for 30 min at room temperature to produce an Fe^{3+} -(NTA)₂ complex. Afterward, the Fe^{3+} -(NTA)₂ was separated from the plasma proteins by spinning the plasma mixture through a membrane filter (NanoSep[®], 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI, USA). The concentration of the Fe^{3+} -(NTA)₂ representing NTBI in the ultrafiltrate was determined using a non-metallic HPLC system. The analytes were fractionated onto a glass analytical column (ChromSep-ODS1, 100 × 3.0 mm, 5 μm) and eluted with mobile-phase solvent (3 mM CP22 in 19% acetonitrile/MOPS buffer pH 7.0) at a flow rate of 1.0 mL/min. The effluents were monitored at 450 nm using a flow cell detector (SpecMonitor2300; LDC Milton-Roy Inc., Riviera Beach, FL, USA) and conducted with BDS software (BarSpec Ltd., Rehovot, Israel). The NTBI concentration was calculated from a calibration curve made with different iron concentrations Fe^{3+} -(NTA)₂ in 80 mM NTA, pH 7.0 ranging 0–16 μM .

Measurement of heart rate variability

Cardiac autonomic nervous activity was evaluated by spectral analysis of the RR interval variability. All mice

underwent measurement of HRV at month 0 (M0), three (M3), and four (M4). The lead II electrocardiogram (ECG) was recorded using needle electrodes (21) and recorded continuously using Power Lab with chart 5.0 (21). During the recording of the ECG, mice were placed in a familiar environment with unnecessary noise, put to calm, and prohibited from movement. The ECG data were analyzed using MATLAB program (21). The RR interval was determined using the peaks of QRS complex and stored as an interval tachogram. From the section of tachogram of at least 300 consecutive interval values, the standard deviation of all RR intervals (SDNN) and root mean square of successive difference (rMSSD) were calculated. The power spectra of RR interval variability were obtained using fast Fourier transform algorithm, and the high-frequency (HF: 0.6–3 Hz) and the low-frequency (LF: 0.2–0.6 Hz) components were determined (21). The power below 0.2 Hz is considered as a very low frequency (VLF). Each spectral component was calculated by determining the area under the respective part of the power spectral density function and was presented in absolute unit (ms^2). To minimize the effect of changes in total power on the LF and HF components, LF and HF were expressed as normalized units (LFnu and HFnu) by dividing the LF and HF by the total power minus VLF (19, 21). The LF/HF ratio is considered an index of autonomic balance (19, 21).

Left ventricular pressure-volume loops (P-V loops) analysis

The WT and HT mice were anesthetized and maintained under physiological conditions. Anesthesia was induced with zoletil (20 mg/kg body weight), injected intraperitoneally (28). The endotracheal tube was inserted, and respiration was maintained by the Harvard rodent ventilator model 683 (Harvard Apparatus, Holliston, MA, USA), which started immediately with room air using a volume of 200–250 μL and ventilator rate at 110–130 breaths/min to maintain PCO_2 , PO_2 , and pH parameters (29). The abdomen was opened subcostally. The diaphragm was incised by a transverse substernal approach leaving the pericardium intact. The left ventricle was entered through an apical stab with a 23 1/2 G needle, followed immediately by the pressure-volume conductance catheter (SciSense, ON, Canada) for measuring the LV pressure and volume (29). The end-systolic pressure (ESP) and end-diastolic pressure (EDP), maximum pressure (P_{max}) and minimum pressure (P_{min}), maximum and minimum dP/dt (dP/dt_{max} , dP/dt_{min}), stroke volume (SV), cardiac output (CO), and stroke work (SW) were measured using the P-V conductance catheter system (29, 30).

Percent of organ weight index

The heart, liver, spleen, and kidney were removed and weighed. The weight index was determined by using the equation: weight index (%) = (organ weight/body weight) $\times$ 100 (31). All organs were remained frozen at -80°C for further analysis.

Prussian blue staining for iron tissue

Iron deposition in HT heart and liver tissues was determined by fixing the dissected tissues in 10% neutral buffer formalin, embedding in paraffin boxes, cutting with a sliding microtome (5- μm -thick section), and staining with Prussian blue dye solution. Prussian blue-stained tissue slides were examined under a light microscope (Olympus Corporation, Philadelphia, PA, USA) (40 $\times$ magnification objective lens) and recorded with a digital camera (Sony Corp., Minato-ku, Japan).

Cardiac iron determination

At the end of the PV loop determination, the hearts were removed and homogenated in deionized water 1 : 10 (w/v). Then, 100 μL of heart tissue homogenates were precipitated in precipitation solution (1 N HCl and 10% trichloroacetic acid) and heated at 95°C for 1 h. Then, the tubes were cooled down at room temperature for 2 min, vortex mixed, and then centrifuged at 8200 g for 10 min. The supernatants (50 μL) were mixed with 50 μL of chromogen solution [0.508 mM ferrozine, 1.5 M sodium acetate and 0.1% or 1.5% (v/v) thioglycolic acid] and incubated at room temperature for 30 min. After incubation, the absorbance was measured at 562 nm, and the cardiac iron concentration was compared with the iron standard curve (32).

Hepatic iron content determination

The hepatic iron content (HIC) was determined using a colorimetric technique (33) and expressed in milligrams of non-heme iron per gram of dry weight. The liver tissue was dried at 120°C for 24 h and weighed. The tissue was digested with the acid mixture (concentrated sulfuric acid : concentrated nitric acid = 1 : 1, v/v) at room temperature and adjusted to the final volume of 10 mL with deionized water. The tissue hydrolysate (50 μL) was incubated with hydroxylamine hydrochloride solution (50 μL) to reduce the ferric ion to ferrous ion at room temperature for 10 min. The pH of the mixture was adjusted to 5.0 with 0.1 M acetate buffer pH 5.0 solution, and the Fe^{2+} present in the solution was allowed to react with a 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution to form a violet-colored product. The OD of the product was measured at 593 nm against the reagent blank. The hepatic

iron concentration was calculated from a calibration curve made from different concentrations of ferrous ammonium sulfate ranging from 12.5 to 200 μM . Data of HIC were expressed as mg/g dry weight.

Determination of malondialdehyde concentration

The heart, liver, and plasma malondialdehyde (MDA) concentrations were measured by using the HPLC method (34). The dry liver tissues (100 mg) or heart tissues (30 mg) were homogenized in the solution containing 50 mM phosphate buffer pH 2.8 (0.8 mL), methanol (0.1 mL) and butyrate hydroxytoluene (BHT) (50 ppm) in an ice bath. A 0.5-mL aliquot of the homogenate or plasma was mixed with 1.1 mL of 10% (*w/v*) trichloroacetic acid (TCA) containing BHT (50 ppm), heated at 90°C for 30 min and cooled down to room temperature. The mixture was centrifuged at 3300 *g* for 10 min to achieve a clear supernatant. The supernatant (0.5 mL) was mixed with 0.44 M H_3PO_4 (1.5 mL) and 0.6% (*w/v*) thiobarbituric acid (TBA) solution (1.0 mL) and incubated at 90°C for 30 min to produce pink-colored products called thiobarbituric acid-reactive substances (TBARS). The solution was passed through a syringe filter (polysulfone type membrane, pore size 0.45 μm ; Whatman International, Maidstone, UK) and analyzed with the HPLC system. The TBARS were fractionated on the adsorption column (Water Spherosorb ODS2 type, 250 $\times$ 4.3 mm, 5 μm), eluted with mobile-phase solvent of 50 mM KH_2PO_4 : methanol (65 : 35 *v/v*) at flow rate of 1.0 mL/min and on-line detected at 532 nm. Data were collected and analyzed with the BDS software (BarSpec Ltd.).

A standard curve was constructed from the peak height of standard 1,1,3,3-tetramethoxypropane (standard reagent for MDA) at different concentrations (0–100 μM). Tissues and plasma TBARS concentration were determined directly from the standard curve and reported as a MDA equivalent concentration (34). The MDA concentrations were expressed in μM /mg protein for heart and liver tissues and in μM for plasma samples.

RNA isolation for microarray analysis

The heart samples of WT ($n = 3$) and HT (Fe group) ($n = 3$) mice were used for RNA isolation and microarray analysis. The method was similar to that described previously (11).

Real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR)

Real-time RT-PCR was used to assess mRNA transcript levels (35–37). Total RNA was extracted from the ventricles of all HT groups ($n = 3$ –4), using purelink RNA mini purification kit (Invitrogen Corp., Grang Island,

NY, USA) according to the manufacturer's instructions. RNA samples were treated with DNaseI (Invitrogen Corp.) to eliminate genomic DNA contamination, and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Lab Ltd, Hercules, CA, USA) according to the manufacturer's instructions. The cDNA samples were prepared with SsoFast EvaGreen Supermix (Bio-Rad Lab Ltd.), and real-time PCR was performed in 96-well plates in triplicate and were cycled for 45 cycles with Chromo4 Real-time PCR detector (Bio-Rad). The cycling conditions included in a hot start at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, and 60°C for 10 s. To detect LTCC, TTCC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression, the gene-specific primers for these genes were used. Sense and antisense primers (10 nM) were forward (F) 5'-TCTGCCTCTCTAGGTCGAA-3' and reverse (R) 5'-GGGAATGTGGTAGGAGAATG-3' for LTCC($\alpha 1\text{C}$) (38), forward (F) 5'-TGTGGAAATGGTGGTGAAGA-3' and reverse (R) 5'-ACTGCGGAG-AAGCTGACATT-3' for TTCC($\alpha 1\text{G}$) (38), forward (F) 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse (R) 5'-TTGCTGTTGAAGTCGCAGGAG-3' for GAPDH (39). The fluorescent amplification curve of the product was determined, and the cycle at which the fluorescence reached a threshold was recorded (C_t) in triplicate and averaged. To control for variability in RNA quantity, the measured abundances of marker genes were normalized to that of GAPDH using the formula $\Delta C_t = C_t$ (Detected Genes) – C_t (GAPDH). GAPDH mRNA was used as an internal control.

Statistical analysis

Data were reported as the mean $\pm$ standard error of mean (SEM) and were processed using the SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) release 13.0 for Windows. One-way ANOVA analyses and Student's *t*-test were performed for group comparisons. Chi-square test was used for mortality rate comparisons between groups. *P*-value < 0.05 was considered statistically significant.

Results

Effect of iron administration on plasma non-transferrin-bound iron level

At the baseline (M0), the levels of plasma NTBI were not detected both in wild-type and in thalassemic mice (Fig. 1A). After exposing both types of mice (WT, HT) to a diet enriched with iron for 1 (M1), 2 (M2), 3 (M3), or 4 (M4) months, NTBI levels were significantly higher and followed a cumulative dose-dependent relationship (Fig. 1A).

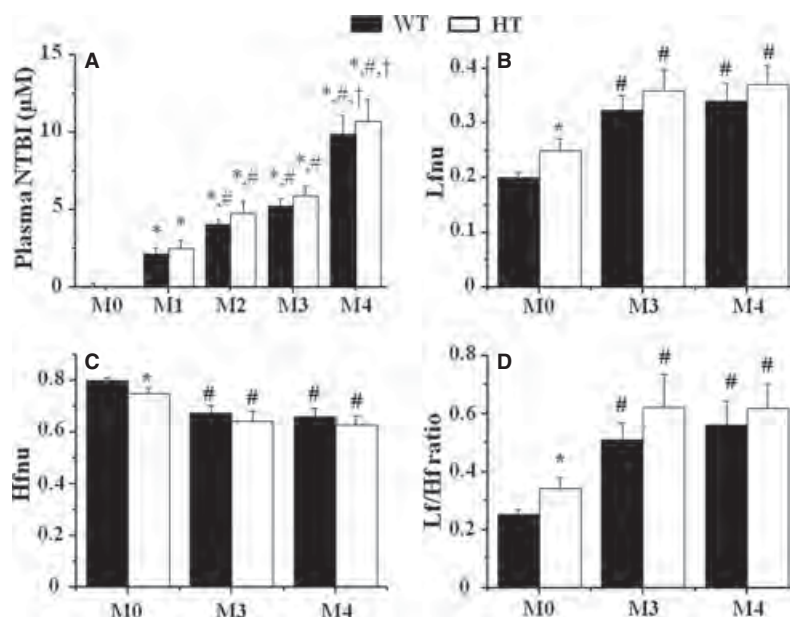


Figure 1 Effect of iron overload on plasma non-transferrin-bound iron (NTBI) level (A) and heart rate variability (HRV) (B–D) in wild-type (WT) and thalassemic mice (HT) without any pharmacological interventions at baseline (M0), 1 month (M1), 2 months (M2), 3 months (M3), and 4 months (M4) after iron feeding ($n = 8$ – 10 /group). * $P < 0.05$ vs. M0, # $P < 0.05$ vs. M1, † $P < 0.05$ vs. M3. Normalized low-frequency power (Lfnu) (B), normalized high-frequency power (Hfnu) (C) and Lf/Hf ratio (D). * $P < 0.05$ vs. WT, # $P < 0.05$ vs. M0.

Effect of iron administration on heart rate variability

At the baseline (M0), thalassemic mice had a higher Lfnu (Fig. 1B), lower Hfnu (Fig. 1C), and higher Lf/Hf ratio (Fig. 1D) than those in the WT mice. After iron was administered for 3 and 4 months, both types of mice (WT, HT) showed a significantly increased Lfnu and Lf/Hf ratio and decreased Hfnu, compared with those at month 0 (Fig. 1). The heart rate, mean RR, SDNN, and rMSSD in both the WT (Table 1) and HT (Table 2) were not significantly different throughout the experiment (M0, M3, M4).

Table 1 Effect of iron administration on heart rate variability in wild-type mice

Parameters	Month 0	Month 3	Month 4
RR _{max}	103 ± 2	105 ± 3	102 ± 2
RR _{min}	84 ± 1	85 ± 1	85 ± 1
Mean RR	92 ± 1	95 ± 1	93 ± 1
SDNN (ms)	2.92 ± 0.19	2.97 ± 0.31	3.00 ± 0.50
rMSSD (ms)	2.70 ± 0.19	2.61 ± 0.34	2.54 ± 0.57
HR _{max} (beats/min)	708 ± 4	710 ± 5	709 ± 9
HR _{min} (beats/min)	589 ± 9	586 ± 15	591 ± 14
Mean HR (beats/min)	647 ± 7	637 ± 7	644 ± 7

SDNN, standard deviation of all RR intervals; rMSSD, root mean square of successive difference of RR; HR, heart rate; Hfnu, normalized high-frequency power; Lfnu, normalized low-frequency power.

Effects of pharmacological interventions on organ weight index

In the control group, the liver weight indexes were not different between the WT and HT (Fig. 2A). After 4 months of iron feeding, both types of mice (WT, HT) showed a significantly increased liver weight index. Pharmacological interventions did not prevent elevation of liver weight index (Fig. 2A). The spleen weight index of the HT mice was significantly larger than the WT in the control group (Fig. 2B). After 4 months of iron feeding, both types of mice (WT, HT) showed a

Table 2 Effect of iron administration on heart rate variability in thalassemic mice

Parameters	Month 0	Month 3	Month 4
RR _{max}	108 ± 3	103 ± 4	102 ± 2
RR _{min}	85 ± 1	87 ± 1	85 ± 1
Mean RR	92 ± 1	95 ± 1	93 ± 1
SDNN (ms)	3.69 ± 0.30	3.04 ± 0.54	3.00 ± 0.50
rMSSD (ms)	3.39 ± 0.32	3.38 ± 0.70	2.54 ± 0.57
HR _{max} (beats/min)	701 ± 4	703 ± 9	709 ± 9
HR _{min} (beats/min)	584 ± 15	587 ± 18	591 ± 14
Mean HR (beats/min)	654 ± 6	635 ± 8	644 ± 7

SDNN, standard deviation of all RR intervals; rMSSD, root mean square of successive difference of RR; HR, heart rate; Hfnu, normalized high-frequency power; Lfnu, normalized low-frequency power.

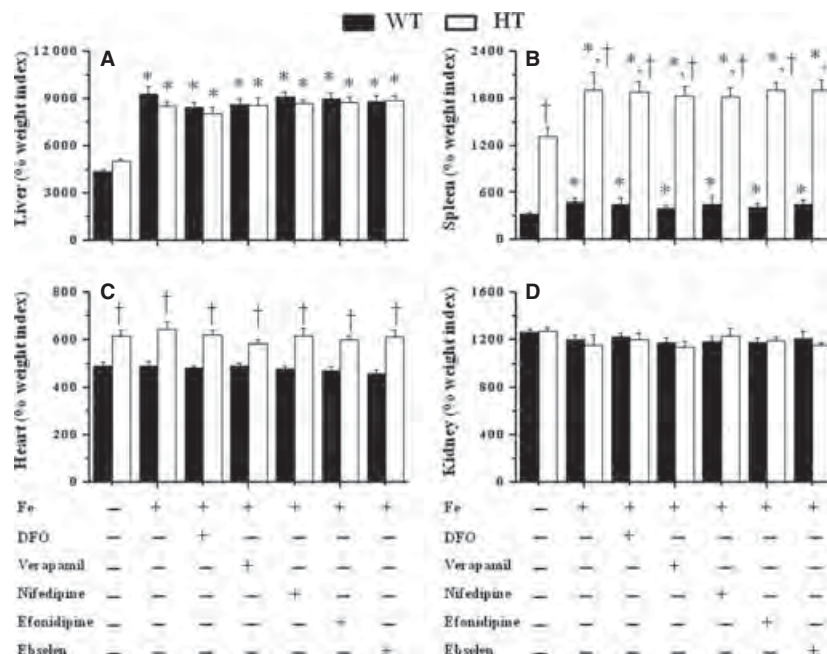


Figure 2 Effects of pharmacological interventions on percentage of organ weight index [i.e., liver (A), spleen (B), heart (C), kidney (D)] in wild-type (WT) and thalassemic mice (HT) ($n = 8-10/\text{group}$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. WT.

significantly increased spleen weight index, and none of the pharmacological interventions prevented or attenuated it (Fig. 2B). The heart weight index of the HT mice was significantly larger than the WT in the control group (Fig. 2C). After 4 months of iron feeding and pharmacological interventions, the heart weight index in both types of mice (WT, HT) was not significantly different from the control group (Fig. 2C). Kidney weight indexes were not different in both types of mice (WT, HT) and in all groups throughout the experiment (Fig. 2D).

Effect of pharmacological interventions on heart rate variability

Wild-type mice in the FE group had increased Lf/Hf ratio, Lf_{nu} and decreased Hf_{nu}, suggesting that iron-overloaded conditions impaired cardiac autonomic regulation of heart rate compared with the control group (Table 3). In the treatment groups, verapamil, nifedipine, efonidipine, DMT1 blocker and iron chelator significantly decreased the Lf/Hf ratio, Lf_{nu} and increased the Hf_{nu} (Table 3). The results of the heart rate, RR, SDNN and rMSSD in WT mice after pharmacological

Table 3 Effects of pharmacological interventions on heart rate variability in wild-type mice

Parameters	Control	FE	FE/DFO	FE/Verapamil	FE/Nifedipine	FE/Efonidipine	FE/Ebselen
RR _{max}	100 ± 3	102 ± 2	104 ± 4	101 ± 7	106 ± 3	101 ± 3	101 ± 2
RR _{min}	83 ± 1	85 ± 1	86 ± 1	83 ± 1	85 ± 1	85 ± 1	85 ± 1
Mean RR	90 ± 1	93 ± 1	93 ± 1	92 ± 2	93 ± 2	94 ± 1	92 ± 1
SDNN (ms)	2.92 ± 0.46	3.00 ± 0.50	2.74 ± 0.63	2.83 ± 0.69	2.65 ± 0.44	2.73 ± 0.61	2.13 ± 0.43
rMSSD (ms)	2.85 ± 0.56	2.54 ± 0.57	2.55 ± 0.81	2.58 ± 0.73	2.45 ± 0.56	2.53 ± 0.56	2.34 ± 0.62
HR _{max} (beats/min)	714 ± 8	709 ± 9	702 ± 9	709 ± 8	704 ± 7	709 ± 8	710 ± 10
HR _{min} (beats/min)	602 ± 15	591 ± 14	605 ± 19	601 ± 25	608 ± 12	606 ± 24	603 ± 16
Mean HR (beats/min)	662 ± 8	644 ± 7	646 ± 9	652 ± 17	648 ± 15	645 ± 7	653 ± 8
Lf/Hf ratio	0.232 ± 0.021	0.707 ± 0.049*	0.224 ± 0.030 [#]	0.294 ± 0.019 [#]	0.317 ± 0.018 [#]	0.263 ± 0.033 [#]	0.283 ± 0.036 [#]
Hf _{nu}	0.812 ± 0.014	0.585 ± 0.017*	0.821 ± 0.021 [#]	0.774 ± 0.012 [#]	0.760 ± 0.012 [#]	0.798 ± 0.020 [#]	0.783 ± 0.023 [#]
Lf _{nu}	0.188 ± 0.014	0.415 ± 0.017*	0.179 ± 0.021 [#]	0.226 ± 0.012 [#]	0.240 ± 0.012 [#]	0.202 ± 0.020 [#]	0.218 ± 0.023 [#]

SDNN, standard deviation of all RR intervals; rMSSD, root mean square of successive difference of RR; HR, heart rate; Hf_{nu}, normalized high-frequency power; Lf_{nu}, normalized low-frequency power.

* $P < 0.05$ vs. control, [#] $P < 0.05$ vs. FE.

interventions were not different from the control group throughout the experiment (Table 3).

Similar results were found in FE group of thalassemic mice. Increased Lf/Hf ratio and Lf_{nu}, and decreased Hf_{nu} were observed, suggesting that iron-overload conditions are associated with progressive deterioration of the already impaired cardiac autonomic function, compared with the control group (Table 4). Treatment with verapamil, nifedipine, efonidipine, DMT1 blocker and iron chelator decreased Lf/Hf ratio, Lf_{nu} and increase Hf_{nu}, which were back to the level observed in the control group (Table 4). The heart rate, RR, SDNN and rMSSD in thalassemic mice after pharmacological interventions were not significantly different from the control group throughout the experiment (Table 4).

Effect of pharmacological interventions on LV function

Wild-type mice in the FE group showed significantly decreased ESP, $P_{\max}$, maximum dP/dt, SV, CO and SW, compared with the control group (Table 5). Treatment

with verapamil, nifedipine, efonidipine, DMT1 blocker and iron chelator showed significantly increased ESP, $P_{\max}$, SV, CO and SW, which were back to the level in the control group (Table 5). The heart rate, EDP, $P_{\min}$ and minimum dP/dt in WT mice after pharmacological interventions were not significantly different from the control group throughout the experiment (Table 5).

Similar results were found in the FE group of thalassemic mice, in which significantly decreased ESP, $P_{\max}$, maximum dP/dt, SV, CO and SW were found, compared with the control group (Table 6). Treatment with verapamil, nifedipine, efonidipine, DMT1 blocker and iron chelator showed significantly increased ESP, $P_{\max}$, SV, CO and SW, which were back to the level in the control group (Table 6). Moreover, in thalassemic mice with iron overload, treatment with efonidipine significantly increased the maximum dP/dt compared with the FE group (Table 6). The heart rate, EDP, $P_{\min}$ and minimum dP/dt in thalassemic mice after pharmacological interventions were not significantly different from the control group throughout the experiment (Table 6). The

Table 4 Effects of pharmacological interventions on heart rate variability in thalassemic mice

Parameters	Control	FE	FE/DFO	FE/Verapamil	FE/Nifedipine	FE/Efonidipine	FE/Ebselen
RR _{max}	103 ± 3	110 ± 3	105 ± 5	110 ± 7	103 ± 6	106 ± 6	108 ± 7
RR _{min}	84 ± 1	87 ± 1	86 ± 2	87 ± 1	85 ± 1	86 ± 1	87 ± 1
Mean RR	91 ± 1	94 ± 2	94 ± 2	94 ± 1	95 ± 2	93 ± 1	93 ± 1
SDNN (ms)	3.48 ± 0.51	3.53 ± 0.51	3.44 ± 0.77	3.55 ± 0.56	3.03 ± 0.70	3.13 ± 0.77	3.11 ± 0.51
rMSSD (ms)	3.37 ± 0.72	3.03 ± 0.85	3.89 ± 1.27	3.64 ± 0.84	3.68 ± 0.97	3.59 ± 1.09	3.36 ± 0.79
HR _{max} (beats/min)	693 ± 7	677 ± 13	689 ± 8	688 ± 7	693 ± 6	696 ± 8	686 ± 9
HR _{min} (beats/min)	580 ± 18	570 ± 7	576 ± 27	562 ± 19	571 ± 37	580 ± 15	593 ± 15
Mean HR (beats/min)	645 ± 11	615 ± 5	626 ± 12	646 ± 8	638 ± 7	641 ± 7	647 ± 7
Lf/Hf ratio	0.366 ± 0.027	0.673 ± 0.078*	0.324 ± 0.068 [#]	0.316 ± 0.037 [#]	0.355 ± 0.059 [#]	0.346 ± 0.044 [#]	0.334 ± 0.055 [#]
Hf _{nu}	0.734 ± 0.015	0.605 ± 0.027*	0.766 ± 0.035 [#]	0.763 ± 0.021 [#]	0.749 ± 0.028 [#]	0.751 ± 0.024 [#]	0.759 ± 0.031 [#]
Lf _{nu}	0.266 ± 0.015	0.395 ± 0.027*	0.234 ± 0.035 [#]	0.237 ± 0.021 [#]	0.251 ± 0.028 [#]	0.249 ± 0.024 [#]	0.241 ± 0.031 [#]

SDNN, standard deviation of all RR intervals; rMSSD, root mean square of successive difference of RR; HR, heart rate; Hf_{nu}, normalized high-frequency power; Lf_{nu}, normalized low-frequency power.

* $P < 0.05$ vs. control, [#] $P < 0.05$ vs. FE.

Table 5 Effects of pharmacological interventions on left ventricular function in wild-type mice

Hemodynamic parameters	Control	FE	FE/DFO	FE/Verapamil	FE/Nifedipine	FE/Efonidipine	FE/Ebselen
HR (beats/min)	328 ± 21	327 ± 26	321 ± 15	334 ± 16	335 ± 12	329 ± 16	332 ± 24
ESP (mmHg)	112 ± 5	82 ± 3*	118 ± 9 [#]	111 ± 8 [#]	121 ± 10 [#]	114 ± 6 [#]	116 ± 6 [#]
EDP (mmHg)	47 ± 4	41 ± 3	48 ± 3	45 ± 3	38 ± 3	42 ± 4	41 ± 4
$P_{\max}$ (mmHg)	118 ± 5	90 ± 4*	123 ± 8 [#]	117 ± 8 [#]	130 ± 6 [#]	117 ± 6 [#]	121 ± 5 [#]
$P_{\min}$ (mmHg)	45 ± 4	38 ± 3	44 ± 4	41 ± 3	35 ± 3	40 ± 4	38 ± 4
dP/dt _{max} (mmHg/s)	7957 ± 2160	5282 ± 1893*	8112 ± 2156 [#]	7154 ± 1433	6217 ± 2197	6996 ± 2196	6701 ± 2386
dP/dt _{min} (mmHg/s)	-4740 ± 176	-4972 ± 448	-5484 ± 736	-5908 ± 824	-6312 ± 688	-5944 ± 362	-5142 ± 906
Stroke volume (μL)	16 ± 1	10 ± 1*	19 ± 3 [#]	18 ± 1 [#]	17 ± 1 [#]	19 ± 2 [#]	21 ± 3 [#]
Cardiac output (μL/min)	5.07 ± 0.38	3.15 ± 0.20*	5.42 ± 0.96 [#]	6.22 ± 0.39 [#]	5.43 ± 0.27 [#]	5.64 ± 0.56 [#]	5.97 ± 0.69 [#]
Stroke work (mmHg/μL)	1721 ± 211	683 ± 82*	1480 ± 145 [#]	1287 ± 93 [#]	1671 ± 334 [#]	1314 ± 153 [#]	1699 ± 287 [#]

HR, heart rate; ESP, EDP, end-systolic and end-diastolic pressure; $P_{\max}$, $P_{\min}$, maximum and minimum pressure; dP/dt_{max}, dP/dt_{min}, maximum and minimum dP/dt.

* $P < 0.05$ vs. control, [#] $P < 0.05$ vs. FE.

Table 6 Effects of pharmacological interventions on left ventricular function in thalassemic mice

Hemodynamic parameters	Control	FE	FE/DFO	FE/Verapamil	FE/Nifedipine	FE/Efonidipine	FE/Ebselen
HR (beats/min)	334 ± 13	322 ± 21	336 ± 13	320 ± 20	318 ± 18	323 ± 11	335 ± 26
ESP (mmHg)	122 ± 6	82 ± 6*	118 ± 6 [#]	111 ± 2 [#]	116 ± 6 [#]	115 ± 3 [#]	112 ± 6 [#]
EDP (mmHg)	42 ± 1	39 ± 1	36 ± 1	38 ± 1	40 ± 2	41 ± 1	39 ± 2
$P_{\max}$ (mmHg)	125 ± 6	88 ± 6*	122 ± 6 [#]	115 ± 1 [#]	119 ± 6 [#]	120 ± 3 [#]	122 ± 6 [#]
$P_{\min}$ (mmHg)	38 ± 1	36 ± 1	33 ± 1	34 ± 1	36 ± 2	37 ± 1	35 ± 1
$dP/dt_{\max}$ (mmHg/s)	8335 ± 2229	5957 ± 1156*	6158 ± 2267*	5105 ± 1840*	6516 ± 1080*	7753 ± 1999 [#]	5456 ± 1523*
$dP/dt_{\min}$ (mmHg/s)	-6552 ± 822	-6878 ± 1362	-7536 ± 394	-6938 ± 464	-6176 ± 414	-5976 ± 506	-7594 ± 638
Stroke volume (μL)	36 ± 9	14 ± 2*	33 ± 5 [#]	31 ± 4 [#]	32 ± 5 [#]	31 ± 3 [#]	31 ± 6 [#]
Cardiac output (μL/min)	9.85 ± 0.68	3.93 ± 0.35*	9.66 ± 1.58 [#]	7.77 ± 0.62 [#]	9.42 ± 0.93 [#]	8.52 ± 0.64 [#]	9.88 ± 2.01 [#]
Stroke work (mmHg/μL)	2432 ± 363	1061 ± 142*	2010 ± 284 [#]	2069 ± 283 [#]	2315 ± 366 [#]	2098 ± 247 [#]	2792 ± 406 [#]

HR, heart rate; ESP, EDP, end-systolic and end-diastolic pressure; $P_{\max}$, $P_{\min}$, maximum and minimum pressure; $dP/dt_{\max}$, $dP/dt_{\min}$, maximum and minimum dP/dt .

* $P < 0.05$ vs. control, [#] $P < 0.05$ vs. FE.

CO in HT mice was higher than that in WT mice, indicating the high-output state in HT mice, which is similar to that found in patients with thalassemia (3).

Prussian blue iron staining in the heart and liver of thalassemic mice

Prussian blue iron staining showed increased iron accumulation in heart and liver tissues in HT/Fe group, compared with the control HT group (Fig. 3B). DFO, verapamil, nifedipine, efonidipine and ebselen decreased the cardiac iron accumulation in HT heart under iron-overload condition (Fig. 3). Only DFO, efonidipine and ebselen decreased the liver iron accumulation in HT under iron-overload condition (Fig. 3).

Effects of pharmacological interventions on cardiac iron concentration and cardiac MDA content

At 4 months of iron administration, increased cardiac iron was significantly higher in the FE group (WT, HT), compared with the control group (Fig. 4A,B). DFO, verapamil, nifedipine, efonidipine and ebselen decreased the cardiac iron concentration in both types of mice, compared with the FE group, and were not different among groups of treatment (Fig. 4A,B). Similar results were found in cardiac MDA content, in which both types of mice in the FE group (WT, HT) had a significantly increased cardiac MDA content, compared with the control group (Fig. 4C,D). All of pharmacological interventions decreased the cardiac MDA concentration in both types of mice, compared with the FE group, and were not different among groups of treatment (Fig. 4C,D).

Effects of pharmacological interventions on liver iron concentration and liver MDA content

In the control group, the liver iron (0.74 ± 0.05 vs. 0.28 ± 0.04 mg/g dry weight) and liver MDA

(8.87 ± 1.04 vs. 5.46 ± 0.47 μM/mg protein) of HT mice were significantly higher than WT mice ($P < 0.01$) (Fig. 5A,B). At 4 months of iron administration, increased liver iron in the FE group (WT, HT) was demonstrated, compared with the control group (Fig. 5A,B). Only DFO, efonidipine and ebselen decreased the liver iron concentration in both types of mice, compared with the FE group (Fig. 5A,B). Similar results were found in the liver MDA content, in which mice in the FE group (WT, HT) had significantly increased liver MDA content, compared with the control group (Fig. 5C,D). Only DFO, efonidipine and ebselen decreased the liver MDA concentration in both types of mice, compared with the FE group (Fig. 5C,D).

Effects of pharmacological interventions on plasma non-transferrin-bound iron and plasma MDA level

In the control group, the levels of plasma NTBI were not detected both in wild-type and in thalassemic mice (Fig. 6A,B). At 4 months of iron administration, both types of mice in the FE group (WT, HT) had significantly increased plasma NTBI, compared with the control group (Fig. 6A,B). DFO, verapamil, nifedipine, efonidipine and ebselen decreased plasma NTBI in both types of mice, compared with the FE group, and was not different among groups of treatment (Fig. 6A,B). At 4 months of iron administration, increased plasma MDA in the FE group (WT, HT) was demonstrated compared with the control group (Fig. 6C,D). In both types of mice (WT, HT), only DFO, efonidipine and ebselen decreased the plasma MDA, compared with the FE group (Fig. 6C,D).

Effect of pharmacological intervention on mortality rate

Iron-overloaded wild-type mice had an increased mortality rate, compared with the control group (Fig. 6E).

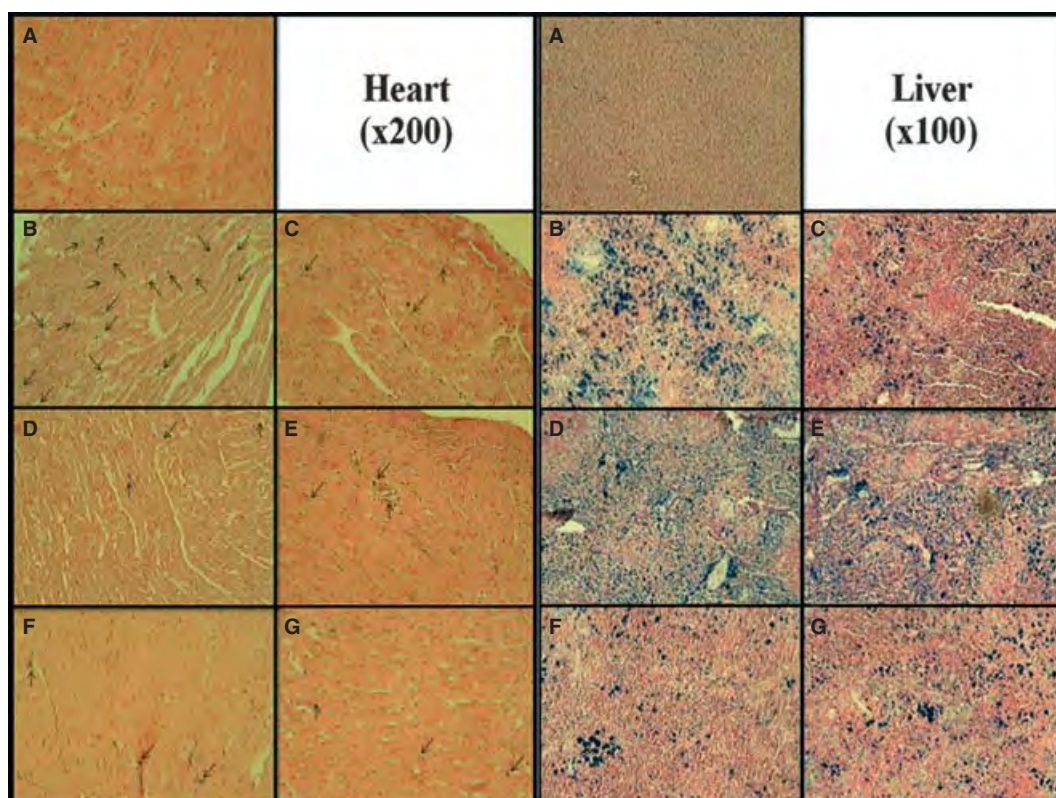


Figure 3 Prussian blue iron staining in the heart (x200) and liver (x100) of thalassemic mice. Control (A), Fe group (B), Fe/DFO (C), Fe/Verapamil (D), Fe/Nifedipine (E), Fe/Efonidipine (F), Fe/Ebselen (G). Arrow indicated iron staining in blue color.

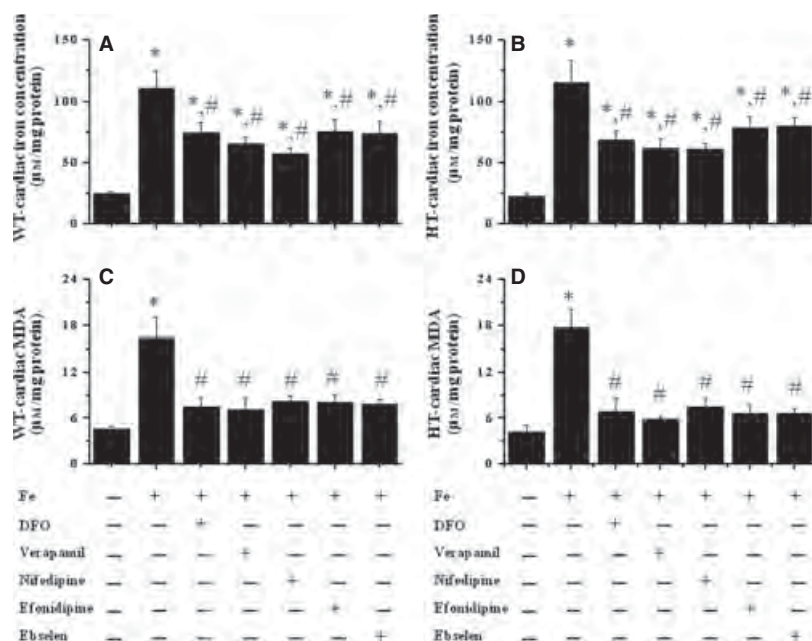


Figure 4 Effects of pharmacological interventions on cardiac iron concentration and cardiac malondialdehyde (MDA) content in wild-type (WT) and thalassemic mice (HT) ($n = 8-10/\text{group}$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. FE.

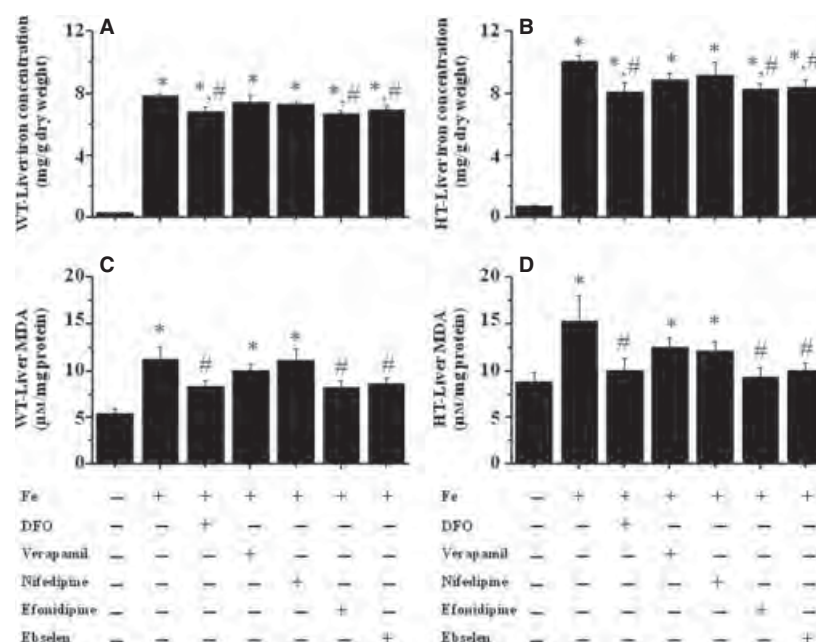


Figure 5 Effects of pharmacological interventions on liver iron concentration and liver malondialdehyde (MDA) content in wild-type (WT) and thalassemic mice (HT) ($n = 8-10$ /group). * $P < 0.05$ vs. control, # $P < 0.05$ vs. FE.

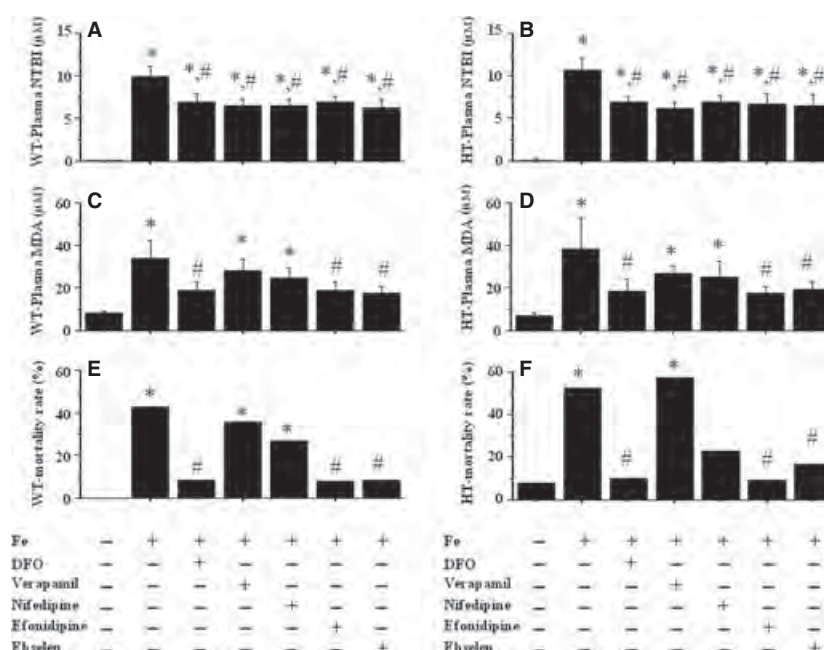


Figure 6 Effects of pharmacological interventions on plasma non-transferrin-bound iron (NTBI) level (A, B), plasma malondialdehyde (MDA) content (C, D) and mortality rate (E, F) in wild-type (WT) and thalassemic mice (HT) ($n = 8-10$ /group). * $P < 0.05$ vs. control, # $P < 0.05$ vs. FE.

Treatment with iron chelator (DFO), TTCC blocker (efonidipine) and DMT1 blocker (ebselen) decreased the mortality rate. However, the LTCC blockers verapamil and nifedipine did not decrease the mortality rate (Fig. 6E).

In iron-overloaded thalassemic mice, an increased mortality rate was found in the iron-loaded group, compared with the control group (Fig. 6F). Treatment with iron chelator (DFO), TTCC blocker (efonidipine) and DMT1 blocker (ebselen) decreased the mortality rate.

However, the LTCC blocker verapamil and nifedipine did not decrease the mortality rate (Fig. 6F). TTCC blocker (efonidipine) could reduce the mortality rate from 43% to 8% in WT and 53% to 9% in HT, whereas LTCC blocker did not provide this benefit (Fig. 6E,F).

Microarray study on LTCC and TTCC gene expressions in thalassemic heart

Microarray analysis showed that in the heart of iron-overload HT mice (HT/Fe group), calcium channel, voltage-dependent, T-type, alpha 1G subunit (*Cacna1g*) (NM_009783.1) was up-regulated (1.560263 fold), whereas calcium channel, voltage-dependent, L-type, alpha 1C subunit (*Cacna1c*) (NM_009781.1) was not altered, compared with the heart of HT mice without iron overload.

Real-time RT-PCR study on TTCC and LTCC mRNA expression

In iron-overloaded HT mice, an increased TTCC mRNA expression was found in the iron-loaded group, compared with the control group (Fig. 7A). Treatment with DFO, verapamil, nifedipine, efonidipine and ebselen did not alter TTCC mRNA expression. In contrast, LTCC mRNA expression was not changed in all groups of HT mice (Fig. 7B).

Discussion

The major findings in this study are that (i) iron-overload conditions are associated with increased Lf/Hf ratio, cardiac and liver iron, cardiac and liver MDA, plasma NTBI and plasma MDA, mortality rate and impaired LV function; (ii) treatment with LTCC, TTCC, DMT1 blocker and iron chelator (DFO) decreased the Lf/Hf ratio, cardiac iron and MDA, plasma NTBI and improved cardiac dysfunction in both types of mice (WT, HT), compared with the FE group; (iii) only TTCC and DMT1 blockers and iron chelator (DFO) decreased liver iron, liver MDA, plasma MDA and mortality rate in both types of mice (WT, HT), compared with the FE group, whereas LTCC blocker could not; (iv) in HT mice, only the TTCC blocker efonidipine increased EDP, $P_{\min}$ and maximum dP/dt ; (v) in HT/Fe group, TTCC was up-regulated compared with HT, and all of treatment did not changed TTCC expression.

In this study, the development of LV systolic dysfunction was observed in iron-overloaded mice as indicated by decreased ESP, $P_{\max}$, SV, CO and SW. LTCC blockers, verapamil and nifedipine, prevented this harmful effect of cardiac iron overload in both types of mice (WT, HT), which is consistent with a previous study in

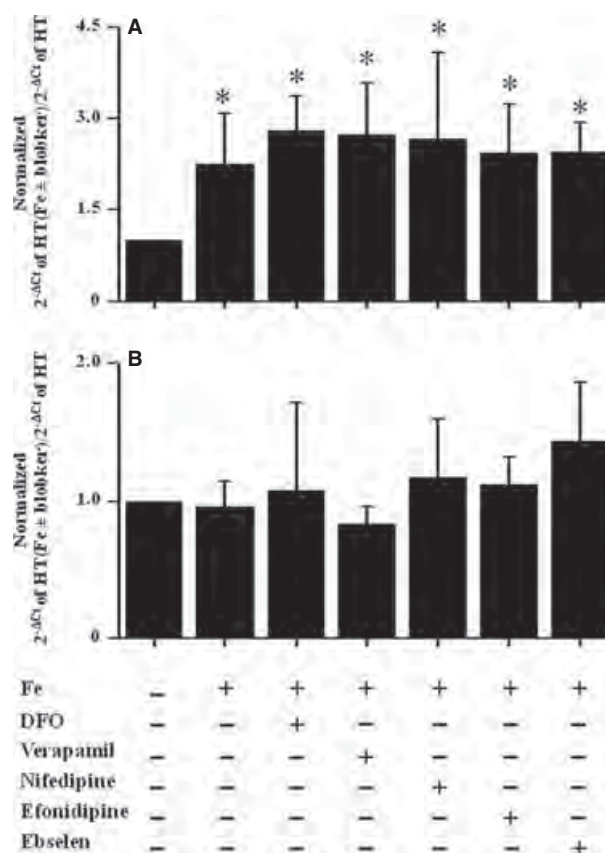


Figure 7 Real-time RT-PCR quantitation of TTCC and LTCC mRNA expression in the heart of thalassemic mice (HT). * $P < 0.05$ vs. control. LTCC, L-type calcium channels.

which the LTCC blockers, verapamil and amlodipine, improved cardiac dysfunction in iron-overloaded mice (10). Moreover, our results demonstrated that TTCC and DMT1 blockers as well as the iron chelator (DFO) could also attenuate cardiac dysfunction caused by iron overload in both WT and HT mice. In cultured thalassemic cardiomyocytes, a previous study demonstrated that treatment with the TTCC blocker, efonidipine, significantly reduced the cardiac iron uptake (11). In this study, our 'in vivo' iron-overload model demonstrated similar benefits of the TTCC blocker, emphasizing that TTCC could play an important role on cardiac iron uptake under iron-overload conditions. As the improvement of LV function was also observed in conjunction with the improved HRV following these pharmacological interventions, these findings suggested that LTCC, TTCC and DMT1 could play an important role in cardiac iron uptake.

It has been shown that chronic iron overload can lead to increased plasma NTBI, which can catalyze the production of highly toxic free hydroxyl radicals via Haber-Weiss and Fenton reactions (40, 41). The excess of free

radicals can damage cellular lipids, proteins, and DNA (40, 41), causing increased lipid peroxidation and leading to the increased cytotoxic aldehyde products such as 4-hydroxynonenal (HNE) and MDA (40, 41). The aldehyde products can form covalent links to proteins leading to the loss of cellular protein function (42). In this study, the increased cardiac iron concentrations in both types of mice under iron-overload conditions were associated with the increased cardiac MDA content, indicating the cardiac cellular damage caused by iron overload. This similar benefit of all pharmacological interventions in this study could be responsible for improved LV function and HRV found in these iron-overloaded mice.

Although all pharmacological interventions in this study could similarly reduce the cardiac iron concentration in both types of mice, the effects on MDA reduction in the plasma and liver were not the same. The TTCC blocker and DMT1 blocker as well as the DFO could reduce the liver iron concentration, liver MDA and plasma MDA to a greater extent than the LTCC blockers could in both WT and HT mice. In this study, LTCC blockers did not decrease liver iron, liver MDA and plasma MDA, suggesting that LTCC did not play an important role in iron uptake in the liver. These findings are consistent with previous studies which showed that efonidipine and ebselen could have an antioxidant effect (43, 44), whereas the LTCC blockers verapamil and amlodipine did not decrease liver iron in iron-overloaded mice as hepatocytes did not express LTCC (10). Our findings indicated that in addition to a beneficial cardiac effect, a TTCC blocker could also provide systemic protective effects, at least for plasma MDA and liver iron and MDA reduction, whereas an LTCC blocker did not. Considering all of these protective benefits, this could be the reason for the higher mortality rate observed in the LTCC blocker groups.

T-type Ca^{2+} channels are abundantly expressed in the embryonic cardiomyocytes, but their expression is suppressed in the adult cells (45). In addition, T-type Ca^{2+} channel current has been shown to reappear in ventricular myocytes under some pathological conditions such as ventricular hypertrophy (46, 47) and postmyocardial infarction (48). Our findings indicated that iron-overload conditions increased TTCC mRNA expression in HT mice. Although all of pharmacological treatment did not effect on gene expression, it might inhibit TTCC function, thus leading to improved LV function. These findings suggested that under pathologic conditions such as iron overload, TTCC expression was reexpressed in adult hearts and could play an important role in iron-overload cardiomyopathy.

In conclusion, as the TTCC blocker, efonidipine, could provide broader beneficial effects including the heart, liver, and plasma, and antioxidant in iron-overload

condition in both WT and HT mice, it is possible that efonidipine could be another drug of choice, in addition to an iron chelator and DMT1 blocker, for the treatment of the iron-overload condition. It is important to note here that although efonidipine is not a specific TTCC blocker as it could also block LTCC, its efficacy in blocking TTCC is much greater than that of LTCC (11, 49). Our findings conclude that efonidipine provided better protective effects than the LTCC blocker, indicating that the TTCC blocker effect of efonidipine could play an important role in the present study. Future clinical studies are also needed to validate the clinical significance for its use in iron-overload patients.

Study limitation

Although our findings indicated that TTCC expression was up-regulated, while LTCC was not altered in iron-overload hearts, the function of TTCC was not directly explored. Future studies are needed to investigate the TTCC current and the role of pharmacological interventions in iron-overload cardiomyocytes. Moreover, genetic manipulation of TTCC is needed to directly study its impact on iron uptake under iron-overload condition.

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Conflict of interests

The authors declare that there are no conflicts of interest.

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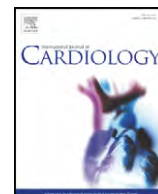
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Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia–reperfusion injury

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ABSTRACT

Background: Dipeptidyl peptidase-4 (DPP-4) inhibitor is a new anti-diabetic drug for type-2 diabetes mellitus patients. Despite its benefits on glycemic control, the effects of DPP-4 inhibitor on the heart during ischemia–reperfusion (I/R) periods are not known. We investigated the effect of DPP-4 inhibitor on cardiac electrophysiology and infarct size in a clinically relevant I/R model in swine and its underlying cardioprotective mechanism.

Methods: Fourteen pigs were randomized to receive either DPP-4 inhibitor (vildagliptin) 50 mg or normal saline intravenously prior to a 90-min left anterior descending artery occlusion, followed by a 120-min reperfusion period. The hemodynamic, cardiac electrophysiological and arrhythmic parameters, and the infarct size were determined before and during I/R. Rat cardiac mitochondria were used to study the protective effects of DPP-4 inhibitor on cardiac mitochondrial dysfunction caused by severe oxidative stress induced by H₂O₂ to mimic the I/R condition.

Results: Compared to the saline group, DPP-4 inhibitor attenuated the shortening of the effective refractory period (ERP), decreased the number of PVCs, increased the ventricular fibrillation threshold (VFT) during the ischemic period, and also decreased the infarct size. In cardiac mitochondria, DPP-4 inhibitor decreased the reactive oxygen species (ROS) production and prevented cardiac mitochondrial depolarization caused by severe oxidative stress.

Conclusions: During I/R, DPP-4 inhibitor stabilized the cardiac electrophysiology by preventing the ERP shortening, decreasing the number of PVCs, increasing the VFT, and decreasing the infarct size. This cardioprotective effect could be due to its prevention of cardiac mitochondrial dysfunction caused by severe oxidative stress during I/R.

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1. Introduction

Diabetes mellitus has been an important health problem in most nations with the number of patients dramatically soaring and expected to reach 366 million by the year 2030 [1]. Patients with type-2 diabetes mellitus (T2DM) have been shown to have a 2- to 4-fold higher risk in coronary heart disease and stroke mortality [2–6], and have a worse prognosis after cardiovascular events [7–9]. Although several new anti-diabetic drugs have been discovered in the past decades, the therapies have been limited by their adverse effects such as weight gain, hypoglycemia, fluid retention [10], and an unexpected cardiovascular risk

[11–13]. Therefore, new anti-diabetic drugs that could control hyperglycemia and reduce the risk of cardiovascular events are of potential benefits to T2DM patients.

In the past few years, a potent dipeptidyl peptidase-4 (DPP-4) inhibitor, which is a novel anti-diabetic drug, has been shown to be effective in treating T2DM patients. Its action is to inhibit the proteolytic enzyme DPP-4 activity, resulting in postponing the degradation of glucagon-like peptide-1 (GLP-1), thus improving glycemic control [14,15]. Although previous studies demonstrated the cardioprotective actions of GLP-1 in an ischemic heart model of ex vivo isolated rodent Langendorff heart [16,17], in vivo rats, rabbits, canine, and swine [18–21], as well as in acute myocardial infarction patients [22], reports on the cardioprotective effect of DPP-4 inhibitor are scant and controversial [23–26]. Furthermore, the effect of DPP-4 inhibitor on cardiac electrophysiology during ischemia–reperfusion (I/R) has never been investigated.

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The purpose of this study was to investigate the effect of vildagliptin, a DPP-4 inhibitor, on cardiac electrophysiology and infarct size in a clinically relevant I/R model in swine. We hypothesized that vildagliptin can attenuate the occurrence of cardiac arrhythmias, increase the ventricular fibrillation threshold (VFT), improve defibrillation efficacy by lowering the defibrillation threshold (DFT), and reduce the infarct size during I/R in the swine heart. To study the cardioprotective mechanism, we determined the effect of vildagliptin in isolated rat's cardiac mitochondria. We tested the hypothesis that the cardioprotective mechanism of vildagliptin is via its prevention of cardiac mitochondrial dysfunction caused by severe oxidative stress during I/R.

2. Materials and methods

2.1. Animal preparation

All experiments were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Pigs were anesthetized by intramuscular injection of a combination of atropine (0.04 mg/kg), zoletil® (4.4 mg/kg) and xylazine (2.2 mg/kg). After endotracheal intubation, anesthesia was maintained by 1.5–3.0% isoflurane delivered in 100% oxygen. Surface electrocardiogram (lead II), femoral arterial blood pressure (BP), heart rates (HR), core body temperature as well as blood gases and electrolytes were continuously monitored to maintain a normal physiological condition. Platinum coated titanium coil electrodes (34- and 68-mm) were advanced into the right ventricular apex (RV) and junction between right atrium and superior vena cava, respectively, to deliver electrical stimulus during VFT and DFT determinations [27]. After a median sternotomy, two pacing electrodes were attached to the epicardium at the right ventricular outflow tract (RVOT) and left ventricular apex (LV) to evaluate the effective refractory period (ERP) and diastolic pacing threshold (DPT) at each site. The electrode at the tip of the endocardial RV apex catheter was also used to determine the ERP and DPT at this site.

2.2. Experimental protocols

Fourteen domestic pigs (25 to 30 kg) were randomly divided into 2 groups ($n = 7$ /group). The first group was assigned to receive 30 ml of normal saline solution and the second group received vildagliptin (prepared by dissolving 50-mg vildagliptin in 30-ml saline solution). Both normal saline solution and vildagliptin (2 mg/Kg) were administered intravenously at a rate of 1.0 ml/min prior to the left anterior descending artery (LAD) occlusion. Hemodynamic and cardiac electrophysiological parameters including HR, systolic (SBP) and diastolic blood pressure (DBP), DPT, ERP, corrected QT interval (QT_c), VFT and DFT were determined at the beginning of the study as a baseline. Myocardial ischemia was induced by LAD occlusion at 5 cm above the distal end [28]. During the first 60 min of occlusion, if spontaneous ventricular fibrillation (VF) occurred, the defibrillation shock was delivered to determine the DFT. On the other hand, if VF did not occur, it was electrically induced with 50-Hz alternating current. Both VFT and DFT were determined using a three-reversal up/down protocol [28]. After 90 min of occlusion, LAD ligation was released to promote reperfusion for 120 min. All parameters were determined again at the end of the reperfusion. Ventricular arrhythmia, e.g. ventricular premature contractions (PVCs), ventricular tachycardia (VT) and spontaneous VF, was recorded throughout the experiment.

2.3. Diastolic pacing threshold (DPT) determination

A train of 10 S1 stimuli was delivered via the electrode at the tip of RV catheter. Current strength was begun with 0.1 mA and was increased in 0.1-mA steps until all stimuli in a train elicited a ventricular response (capture) [28]. The minimum current strength which captures ventricular response was defined as the DPT.

2.4. Effective refractory period (ERP) determination

An S2 stimulus ($2 \times$ DPT strength) was introduced in late diastole of the last S1 beat of a train of 10 S1 to elicit a capture. S1–S2 coupling interval was decreased in 10-ms steps until S2 failed to elicit a capture. ERP was defined as the longest S1–S2 interval which S2 stimulus failed to capture [28].

2.5. Ventricular fibrillation threshold (VFT) determination

The interval between the last S1 and the mid T-wave was determined for 3 times. An average was used as a coupling interval between the last S1 and S2 shock. VFT was performed by delivering S2 shocks starting at 100 V. If this shock induced VF, the decrement of 10-V step was used for each successive shock until VF was no longer induced. If the 100-V S2 shock did not induce VF, the increment of 10-V step was used for each successive shock until VF was induced. VFT was defined as the lowest shock strength that successfully induced VF [28].

2.6. Defibrillation threshold (DFT) determination

Defibrillation shock was delivered after 10 s of VF to determine the DFT using a three-reversal up/down protocol [28]. However, if the tested shock failed to defibrillate, a rescue shock (600–700 V) was delivered to successfully defibrillate the heart. The DFT was defined as the lowest shock strength required for successful defibrillation. A 4-minute interval was allowed between each VF induction episode to set the heart back to physiologic condition [28].

2.7. Infarct size determination

The infarct size was assessed with 0.5% Evans Blue and 1.0% Triphenyltetrazolium Chloride (TTC) staining as previously described [28]. In brief, at the end of the study, the LAD was re-occluded at the exact same location as during ischemia. Evans Blue was infused into the left and right coronary arteries to evaluate the area at risk (AAR). After being frozen overnight, the heart was cut into 5-mm thick slices perpendicular to the LAD from apex to the occlusion site. Each slice was incubated in TTC for 15 min to discriminate the infarct tissues from the viable myocardium. After overnight fixation with 4% paraformaldehyde, each slice was photographed with a digital camera. An area measurement was performed using Image Tool software version 3.0.

2.8. Histological analysis

Both infarct and normal myocardium were fixed with 4% neural buffered formaldehyde for 24 h at room temperature, followed by embedding in paraffin wax, and slicing into 5- μ m slices for subsequent Hematoxylin–Eosin staining [29,30]. The infarct tissues were evaluated for microscopic changes using the Lodge–Patch classification [31–33].

2.9. Isolated cardiac mitochondria study protocol

Male Wistar rats (300–350 g) were used for cardiac mitochondrial isolation as described previously [34]. H_2O_2 (2 mM, incubated for 5 min) was used to induce oxidative stress in cardiac mitochondria to mimic I/R condition [34]. Isolated cardiac mitochondria were divided into 6 groups ($n = 5$ /group): 1) Control, 2) Mitochondria treated with H_2O_2 , 3) Mitochondria treated with vildagliptin for 30 min, 4) Mitochondria pretreated with vildagliptin for 5 min followed by H_2O_2 treatment, 5) Mitochondria pretreated with vildagliptin for 15 min followed by H_2O_2 treatment, 6) Mitochondria pretreated with vildagliptin for 30 min followed by H_2O_2 treatment. Vildagliptin at doses of 0.33 mM, and 3.30 mM were used in this study.

The measurement of cardiac mitochondrial reactive oxygen species (ROS) production and mitochondrial membrane potential changes ($\Delta\Psi_m$) was determined in all groups as previously described [34]. In short, dichlorohydro-fluorescein diacetate dye was used to determine the level of ROS production in cardiac mitochondria. The ROS level was expressed as arbitrary units of fluorescence intensity determined at $\lambda_{excitation}$ 485 nm and $\lambda_{emission}$ 530 nm [34]. JC-1 was used to determine the change of cardiac mitochondrial membrane potential at $\lambda_{excitation}$ 485 nm and $\lambda_{emission}$ 530 nm for green and $\lambda_{emission}$ 590 nm for red [34]. Cardiac mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio.

2.10. Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical comparison of cardiac electrophysiological, hemodynamic, and arrhythmic parameters as well as cardiac mitochondria results was performed with the Student's *t* test. Chi-square test was performed to compare VT/VF incidence, and comparison of the infarct size was analyzed using Mann–Whitney's *U* test. All statistical analysis was performed with SPSS version 10.0. A *p*-value less than 0.05 was considered significant.

3. Results

The hemodynamic parameters including HR, SBP, DBP and cardiac electrophysiological parameters including QT_c , QRS complex, and DPT were not significantly different between the saline-treated group and the vildagliptin-treated group during baseline, ischemia, and reperfusion periods (Table 1). Pretreatment with vildagliptin significantly increased both VFT energy and VFT voltage at ischemic period compared with the saline-treated group (Fig. 1). However, there was no difference between the DFT of the vildagliptin-treated group and the saline-treated group at any time period (Fig. 2). For the ERP, no differences were found among ERPs recorded at three sites at baseline and reperfusion periods in both saline and vildagliptin treated groups (Fig. 3A and 3C). However, during ischemia the ERP at the LV epicardium (i.e. ischemic site) was significantly shortened, compared to the other two sites in the saline-treated group (Fig. 3B), thus creating the dispersion of the ERP in the heart during this period. In the

Table 1
Basic electrophysiological and hemodynamic parameters.

Parameters	Baseline		Ischemia 60 min		Reperfusion	
	NSS	Vil	NSS	Vil	NSS	Vil
HR (beat/min)	88.4 ± 5.2	98.0 ± 5.7	91.3 ± 3.6	95.3 ± 4.6	102.7 ± 8.5	107.6 ± 4.2
SBP (mm Hg)	101.1 ± 7.4	102.9 ± 5.1	87.1 ± 6.1	84.6 ± 6.1	86.1 ± 3.4	85.6 ± 4.7
DBP (mm Hg)	63.3 ± 5.4	64.0 ± 3.9	55.6 ± 4.1	52.0 ± 4.6	54.4 ± 2.3	53.9 ± 2.9
QT _c (ms)	466.7 ± 7.1	466.1 ± 14.0	451.2 ± 17.2	459.2 ± 18.5	441.7 ± 15.8	435.2 ± 20.3
QRS (ms)	64.4 ± 2.0	58.0 ± 5.0	59.6 ± 2.0	57.6 ± 4.5	60.8 ± 3.2	58.0 ± 2.2
DPT RV (mA)	0.20 ± 0.02	0.21 ± 0.01	0.29 ± 0.03	0.31 ± 0.05	0.33 ± 0.05	0.36 ± 0.04
DPT RVOT (mA)	0.19 ± 0.04	0.17 ± 0.02	0.14 ± 0.02	0.16 ± 0.03	0.40 ± 0.13	0.23 ± 0.07
DPT LV (mA)	0.11 ± 0.01	0.14 ± 0.02	0.22 ± 0.08	0.18 ± 0.04	0.42 ± 0.20	0.21 ± 0.04

NSS = Normal saline solution; Vil = Vildagliptin; HR = Heart rate; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; QT_c = Corrected QT interval; QRS = QRS complex; DPT = Diastolic pacing threshold; RV = Right ventricle; RVOT = Right ventricular outflow tract; LV = Left ventricle.

vildagliptin-treated group, the ERP from all 3 sites were not different during the ischemic period, indicating less dispersion of the ERP during ischemia in this group.

Regarding the occurrence of arrhythmias, the number of PVCs was markedly decreased in pigs treated with vildagliptin during 90 min of ischemia, compared to the saline-treated group (Fig. 4A). The number of PVCs during reperfusion was also smaller in the vildagliptin group, but it did not reach statistical significance (Fig. 4A). The VT/VF incidence (Fig. 4B) and the number of VT/VF episodes (Fig. 4C) were also smaller in the vildagliptin-treated group, but were not statistically significant. The time to the first VT/VF onset was not different between the vildagliptin and the saline treated groups (Fig. 4D). The area at risk (AAR) was not different between the saline (36.8 ± 2.7%) and the vildagliptin treated groups (33.4 ± 3.2%). However, the infarct size in the vildagliptin-treated group was significantly smaller than that in the saline-treated group (Fig. 5A), accounting for a

17% reduction in the infarct size. To determine stages of myocardial damage caused by I/R injury, myocardial tissues were obtained and evaluated based on microscopic changes. In the infarct area, thinning and waviness of myocardial fibers with distinct nuclei were observed (Fig. 5B).

In cardiac mitochondria, vildagliptin alone at doses 0.1 mg/ml (0.33 mM) and 1.0 mg/ml (3.30 mM) did not affect the mitochondrial ROS production and the mitochondrial membrane potential change (Fig. 6). H₂O₂ caused a markedly increased ROS level and decreased the red/green fluorescent intensity ratio (i.e. an indication of mitochondrial depolarization). Vildagliptin at 0.1 mg/ml neither attenuated cardiac mitochondrial ROS production nor prevented mitochondrial depolarization caused by H₂O₂ (Fig. 6A and 6B). However, treatment with 1.0 mg/ml of vildagliptin for 5, 15, and 30 min ameliorated cardiac mitochondrial dysfunction caused by H₂O₂, as indicated by lower ROS production (Fig. 6C) and less mitochondrial membrane potential changes (Fig. 6D), compared to those in H₂O₂-treated cardiac mitochondria.

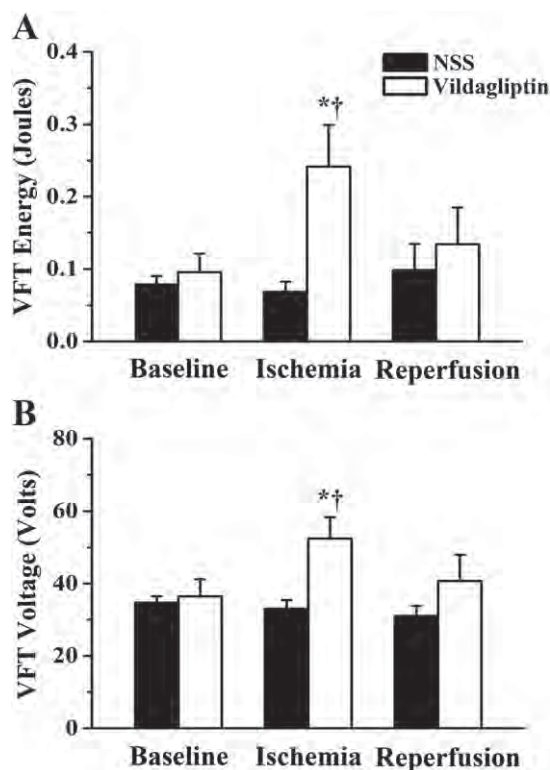


Fig. 1. Effect of vildagliptin on the VFT. During the ischemic period, vildagliptin significantly increased both VFT energy (panel A) and voltage (panel B) compared to normal saline group. * $p < 0.05$ vs. NSS at ischemic period; † $p < 0.05$ vs. vildagliptin at baseline.

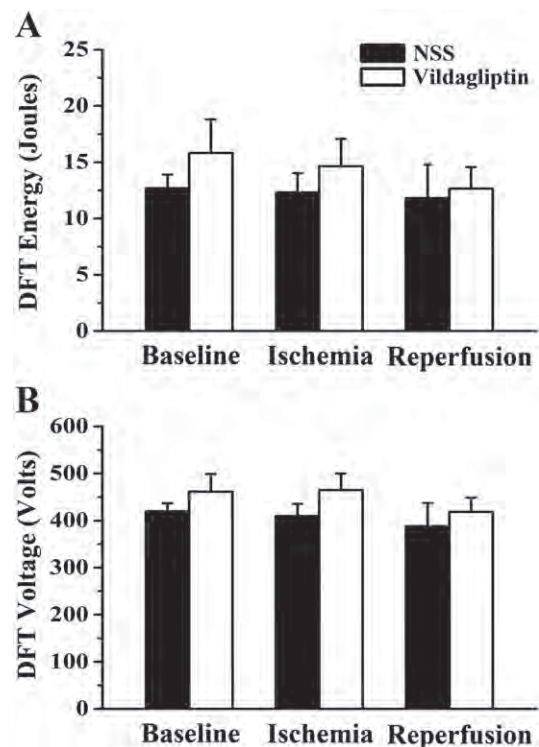


Fig. 2. Effect of vildagliptin on the DFT. Both vildagliptin and normal saline did not change the DFT energy (panel A) and DFT voltage (panel B).

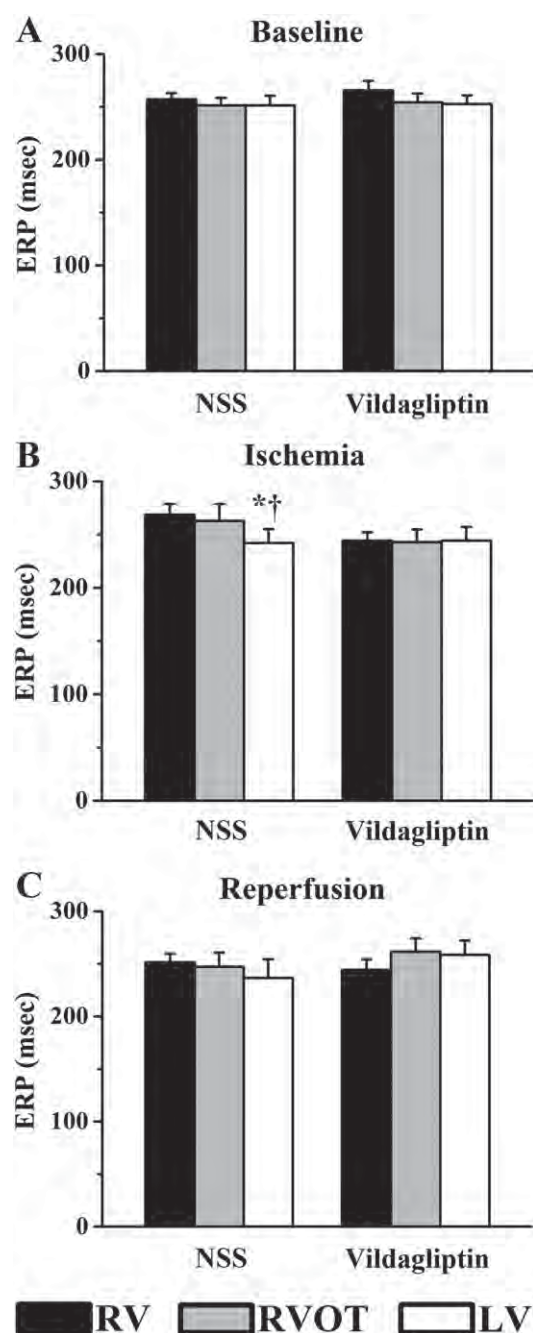


Fig. 3. Effect of vildagliptin on the ERP. ERP at baseline was not different among all 3 recording sites in both normal saline and vildagliptin groups (panel A). During ischemia, LV ERP was decreased compared to the other two recorded sites in the normal saline group (panel B), whereas the LV ERP was not different from the other two recorded sites in the vildagliptin treated group (panel B), indicating that vildagliptin prevented the ERP dispersion among all recording sites at ischemic period. At reperfusion, the ERPs were not different among all three recorded sites in both normal saline and vildagliptin treated groups (panel C). * $p < 0.05$ vs. RV; † $p < 0.05$ vs. RVOT. RV = Right ventricle; RVOT = Right ventricular outflow tract; LV = Left ventricle.

4. Discussion

In this study, we assessed the cardioprotective effects of vildagliptin in a clinically relevant swine I/R model. The major findings of this study were as follows. Vildagliptin demonstrated its cardioprotective effects by 1) attenuating ERP shortening caused by myocardial ischemia, thus decreasing the ERP dispersion in the heart during ischemia, 2) increasing the VFT, 3) reducing the number of PVCs, 4) decreasing

the infarct size, and 5) attenuating cardiac mitochondrial dysfunction due to oxidative stress caused by H_2O_2 .

In the present study, we demonstrated for the first time the cardiac electrophysiological effects of DPP-4 inhibitor, vildagliptin, in the I/R heart. Our study demonstrated that vildagliptin could attenuate the ERP shortening in the ischemic myocardium, thus decreasing the degree of the dispersion of refractoriness caused by cardiac ischemia. Since dispersion of refractoriness plays an important role in facilitating arrhythmias [35], prevention of the ERP dispersion in the heart during ischemia could attenuate the occurrence of arrhythmia in this study. Our study also demonstrated the decreased number of PVCs and the reduced VFT in the vildagliptin treated pigs, suggesting that vildagliptin could stabilize cardiac electrophysiology during ischemia, thus attenuating myocardial vulnerability to arrhythmia during ischemia.

In the present study, the AAR in both saline and vildagliptin treated groups was not different, indicating the similar extent of ischemic myocardium caused by an LAD occlusion in both groups. However, vildagliptin significantly decreased the infarct size, compared with that in the saline-treated group. The histological appearance of the infarct tissues represented an early myocardial infarction (stage 1) according to Lodge–Patch classification [31,33], which was consistent with the duration of I/R in this study. The beneficial effect on the infarct size reduction of vildagliptin in the swine I/R model as shown in our study was consistent with previous reports in I/R model in mice using DPP-4 inhibitor sitagliptin [24], and in pre-diabetic insulin resistance rat using vildagliptin [23]. Since a reduced infarct size has been shown to be associated with a decreased myocardial vulnerability to arrhythmias during I/R, the reduction of infarct size and decreased ERP dispersion caused by vildagliptin could help attenuate arrhythmia and reduce the VFT as found in this study. However, not all reports on the effects of DPP-4 inhibitors on the infarct size are consistent. In ischemic study of DPP-4 gene deleted mice and DPP-4 inhibitor treated mice, no infarct size reduction was observed in that study [26]. Furthermore, treatment with vildagliptin or valine pyrrolidide did not reduce the infarct size in rat I/R model [18,23]. This discrepancy in results could be due to the difference in animal models. Nevertheless, our study is the first study to demonstrate the infarct limiting effect of DPP-4 inhibitor in the swine I/R model. This finding suggests that the effect of DPP-4 inhibitor on the infarct size reduction could differ in different animal species. In the clinically relevant swine I/R model, the augmentation of GLP-1 level by GLP-1 analog could also reduce infarct size [21]. However, Kavianipour et al. [36] and Kristensen et al. [37] failed to show infarct size limiting profit. This inconsistent finding could be due to different type of drug, routes, and the timing of drug administration. In a swine study that showed infarct limiting benefit, exendin-4 (GLP-1 analog) was given at 10 μ g subcutaneously and intravenously prior to reperfusion and 10 μ g subcutaneously twice daily until day 3 of reperfusion [21]. However, in two swine studies that failed to show infarct limiting benefit, liraglutide (GLP-1 analog) was given at 10 μ g/kg/day subcutaneously for 3 days prior to the I/R conduction [37], and recombinant GLP-1 was given at 3 pmol/kg/min intravenously starting at 15 min before ischemia to the end of reperfusion [36]. All of these findings indicate the importance of species, type of drug, routes, and timing of drug administration in I/R models.

The present study further demonstrated the protective effect of vildagliptin on cardiac mitochondria. We demonstrated for the first time that DPP-4 inhibitor could effectively attenuate cardiac mitochondrial dysfunction caused by severe oxidative stress as found in the heart during I/R, suggesting that this mitochondrial protection could play a crucial role in the cardioprotective effect of vildagliptin. During I/R, it has been shown that the level of ROS could be markedly increased [38], resulting in the deterioration of the electron transport chain [39] and activation of the apoptotic pathway [40], and eventually myocardial death. In addition, the elevation of ROS level

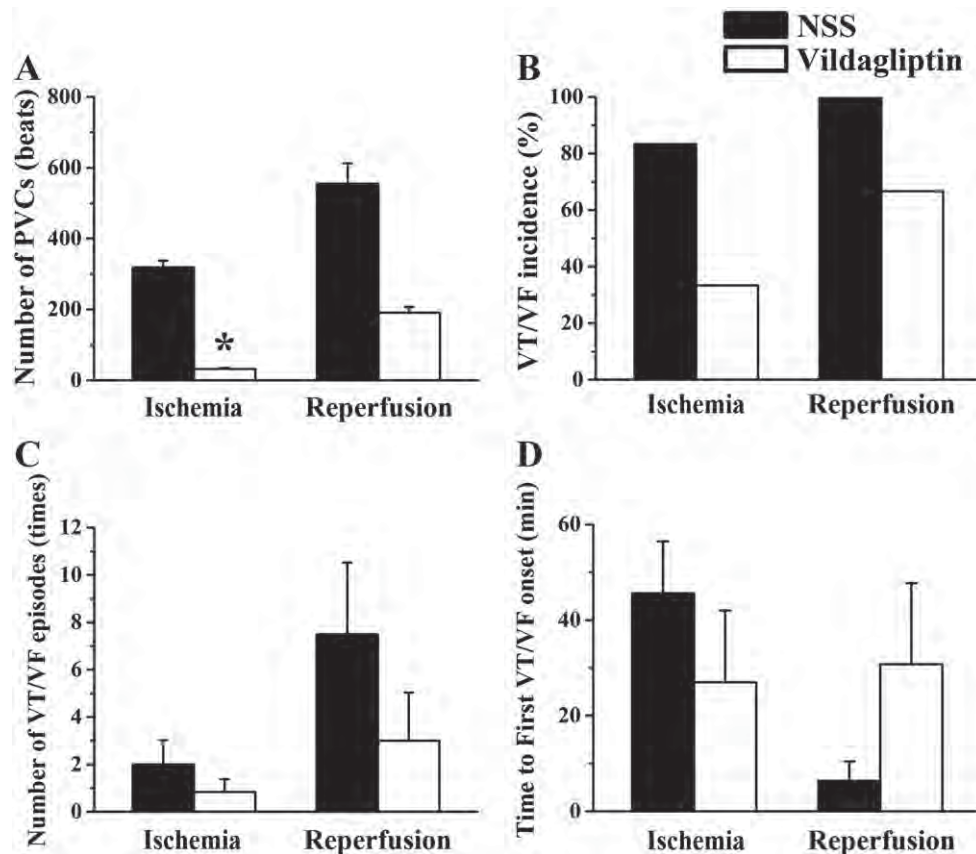


Fig. 4. Vildagliptin and the occurrence of cardiac arrhythmia. PVCs were markedly decreased in the vildagliptin treated group at the ischemic period and tended to decrease at reperfusion period, compared with normal saline group (panel A). Ventricular tachycardia (VT) and ventricular fibrillation (VF) incidence (panel B), VT/VF episode (panel C) and time to the first VT/VF onset (panel D) were not significantly different between vildagliptin and normal saline groups. * $p < 0.05$ vs. baseline.

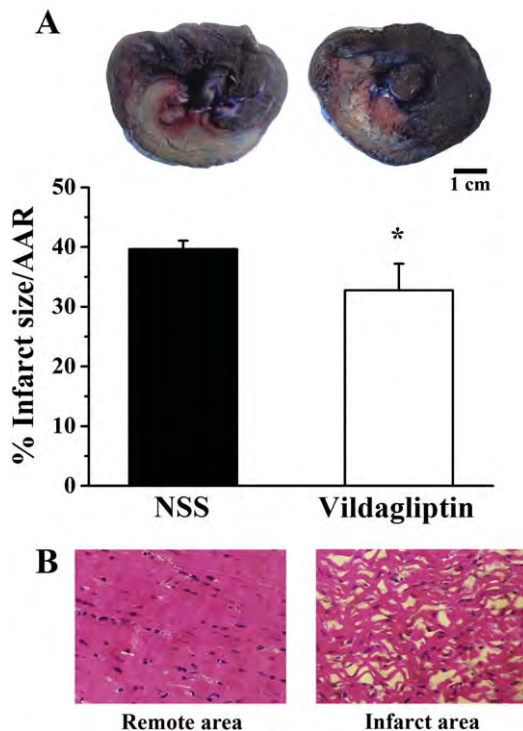


Fig. 5. Effect of vildagliptin on the infarct size. Panel A: The AAR was not different between the vildagliptin and normal saline treated groups. Vildagliptin could attenuate the infarct size by 17%, compared with normal saline treated group. Panel B: a microscopic appearance of the myocardium from the remote area (left) and infarct area (right). Thinning and waviness of myocardial fibers with distinct nuclei were found in the infarct area. * $p < 0.05$ vs. NSS. AAR = Area at risk.

could cause cardiac mitochondrial membrane depolarization, a condition that has been shown to be responsible for cardiac arrhythmias [41]. In our study, administration of vildagliptin at 3.30 mM to cardiac mitochondria could attenuate cardiac mitochondrial dysfunction caused by H_2O_2 , as indicated by the reduction of ROS production and attenuation of mitochondrial membrane potential changes. These effects could be a mechanism responsible for reduced myocardial vulnerability to arrhythmia and decreased infarct size found in the present study [42].

5. Conclusions

A DPP-4 inhibitor vildagliptin stabilized cardiac electrophysiology by attenuating ERP shortening and reducing the infarct size, resulting in decreasing myocardial vulnerability to cardiac arrhythmia during I/R. The cardioprotective effect of vildagliptin could be due to its ability to prevent cardiac mitochondrial dysfunction caused by severe oxidative stress during I/R.

Conflict of interest

None declared.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [43].

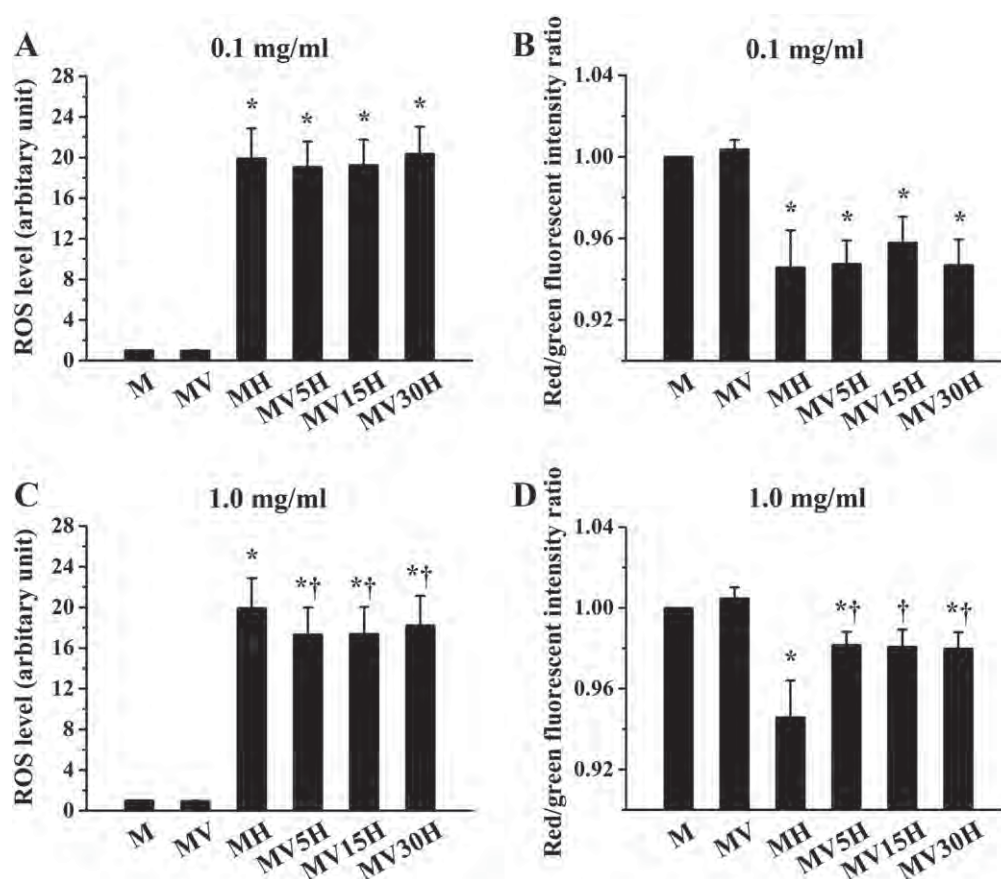


Fig. 6. Effect of vildagliptin on cardiac mitochondria. H₂O₂ caused markedly increased ROS level and decreased red/green fluorescent ratio. Vildagliptin at 0.1 mg/ml could not reduce ROS level (panel A) or prevent the red/green fluorescent ratio reduction (panel B) caused by H₂O₂. However, 1.0-mg/ml vildagliptin treated for 5, 15, and 30 min could significantly decrease ROS level (panel C) and attenuate mitochondrial depolarization (panel D) caused by H₂O₂. * $p < 0.05$ vs. M; † $p < 0.05$ vs. MH. M = Mitochondria; MV = Mitochondria + Vildagliptin; MH = Mitochondria + H₂O₂; MV5H = Mitochondria + Vildagliptin_{5 min} + H₂O₂; MV15H = Mitochondria + Vildagliptin_{15 min} + H₂O₂; MV30H = Mitochondria + Vildagliptin_{30 min} + H₂O₂.

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Role of p38 inhibition in cardiac ischemia/reperfusion injury

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Abstract The p38 mitogen-activated protein kinases (p38s) are Ser/Thr kinases that are activated as a result of cellular stresses and various pathological conditions, including myocardial ischemia/reperfusion. p38 activation has been shown to accentuate myocardial injury and impair cardiac function. Inhibition of p38 activation and its activity has been proposed to be cardioprotective by slowing the rate of myocardial damage and improving cardiac function. The growing body of evidence on the use of p38 inhibitors as therapeutic means for responding to heart problems is controversial, since both beneficial as well as a lack of protective effects on the heart have been reported. In this review, the outcomes from studies investigating the effect of p38 inhibitors on the heart in a wide range of study models, including *in vitro*, *ex vivo*, and *in vivo* models, are discussed. The correlations of experimental models with practical clinical usefulness, as

well as the need for future studies regarding the use of p38 inhibitors, are also addressed.

Keywords p38 Mitogen-activated protein kinase · Myocardial ischemia/reperfusion · p38 Inhibitors · Therapy

Introduction

Ischemic heart disease is considered to be the leading cause of death worldwide and is predicted to be the major cause of deaths in the future [1]. Myocardial ischemia exists when the reduction of the coronary flow is so severe that the supply of oxygen to the myocardium is inadequate for the oxygen demands of the tissue [2], resulting in the accumulation of metabolites in the ischemic region [2]. Severe and prolonged ischemia ultimately results in cellular necrosis. Currently, the most efficient method of reducing mortality in such patients experiencing ischemia is to achieve rapid reperfusion by thrombolysis or mechanical disruption of the occlusion. The mortality from acute myocardial infarction under these circumstances is inversely related to the amount of myocardial salvage achieved by reperfusion [3]. However, reperfusion itself can also be harmful, since it can damage the myocardium, a process known as “reperfusion injury” [4]. Different intracellular signaling pathways are considered to play a crucial role in the myocardial response to ischemia/reperfusion injury and consequent pathological remodeling. Many highly conserved serine/threonine mitogen-activated protein kinases (MAPK) are activated in response to myocardial ischemia/reperfusion [5]. In particular, the p38 MAPK has been widely studied.

A growing body of evidence from preclinical investigations indicates that the inhibition of p38 activation could reduce myocardial injury [6], suggesting the therapeutic

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potential of p38 inhibitors in ischemic heart disease. However, the findings of not all studies consistent, and these inconsistencies raise the question of whether p38 inhibition is truly cardioprotective. Only a few published reports on clinical trials with p38 inhibitor in cardiovascular disorders are currently available [7–10]. The aim of this article is, therefore, to comprehensively review the findings of relevant studies regarding the use of p38 inhibitors in the cardiac ischemia/reperfusion model, including *in vitro*, *ex vivo*, and *in vivo* models in both animal and clinical studies. Findings both consistent and inconsistent with the therapeutic potential of p38 inhibition are discussed, and the future direction of p38 inhibitor therapy in the cardiac ischemia/reperfusion model is addressed in the hope of elucidating the possible usefulness of p38 inhibitors in patients in the future.

Biological and biochemical properties of p38

The p38 MAPK is a family of serine/threonine protein kinases that plays an important role in cellular responses to external stress signaling and also functions in many cellular processes, including inflammation, cell differentiation, cell growth and death [11]. The human p38 was originally isolated as a 38-kDa protein that is rapidly tyrosine phosphorylated in response to lipopolysaccharide stimulation in human monocytes [11]. It was also identified as a target of a pyridinyl imidazole drug that blocked the production of tumor necrosis factor- α (TNF α), and was consequently called cytokine-suppressive anti-inflammatory drug-binding protein [11], and as a reactivating kinase for MAP kinase-activated protein (MAPKAP) kinase-2 [12]. Human p38 cDNA cloning revealed that the amino acid sequence of human p38 is 94% identical to mouse p38 [13, 14].

The activity of p38 is controlled by the dual phosphorylation of the Thr¹⁸⁰-Gly¹⁸¹-Tyr¹⁸² motif within the activation loop/lip [15]. The traditional view is that this dual phosphorylation event is achieved by upstream, dual specificity MAPK kinases (MAPKKs) or MKKs. The major activators of p38 *in vivo* are MKK3, MKK6 [16, 17], and MKK4 [18]. This serial phosphorylation relay from MKKK to MKK3/6 to p38, and finally to substrates is termed the “transphosphorylation” mechanism due to the transfer of the phosphate group from ATP to downstream signaling molecules. The pharmacological inhibitor, SB203580, inhibits p38 activity and attenuates the phosphorylation processes downstream of p38 [19, 20]. Although it is unlikely that SB203580 will inhibit dual phosphorylation of p38 itself, growing evidence demonstrates that the inhibitory effect of SB203580 and of structurally related compounds acts on p38 phosphorylation [21]. These findings could possibly be

explained by the finding that p38 can phosphorylate itself, a mechanism called “autophosphorylation” [22–24].

There are four isoforms of p38 that have been identified, including p38 α , β , γ , and δ . Sequence comparisons have revealed that each p38 isoform has more than 69% identity within this group, but only 40–45% to the other MAP kinase family members [25]. Among all isoforms, p38 α and β are highly homologous [26] and sensitive to pyridinyl imidazole molecules, such as SB203580 [27], but they have only 60% homology with p38 γ and δ , which are resistant to SB203580 [27]. p38 α is ubiquitously expressed in several tissues and is the best characterized and perhaps the most physiologically relevant kinase involved in inflammatory responses [26, 28]; it is also the isoform predominantly involved in myocardial ischemic injury [24].

p38 MAPK activation in myocardial ischemia/reperfusion

Myocardial ischemia is a potent stimulant of p38 activation, which is an important pro-apoptotic kinase in cardiomyocytes [29]. Evidence has been accumulating from preclinical investigations that the inhibition of p38 during prolonged ischemia slows the rate of infarction/death and inhibits the production of inflammatory cytokines, such as TNF α , interleukin-1 (IL-1), and IL-8, which are known to aggravate ischemic injury [6, 30]. In the clinical context, prompt reperfusion following coronary artery occlusion remains the most effective intervention to re-establish arterial patency and reduce ischemic myocardial injury [29]. However, reperfusion can re-activate p38, perhaps in response to stimuli such as reactive oxygen species (ROS) and osmotic stress [29]. Although this field of research is still evolving, compelling evidence supports a causative role of p38 in myocardial injury and dysfunction following ischemia/reperfusion [29, 31–33]. Many studies have elucidated the mechanisms, such as apoptosis and inflammation, through which p38 activation might contribute to ischemia/reperfusion injury [29]. Bogoyevitch et al. were the first to demonstrate that p38 α and β isoforms are activated in response to ischemia/reperfusion in the heart [34]. Later studies using ectopic gene expression found that the α isoform is implicated in cardiomyocyte apoptosis and that this isoform alone is sufficient to cause cell death following ischemia [24, 34–36].

Study models of p38 inhibitors on myocardial ischemia/reperfusion

The pro-apoptotic role of p38 in cardiomyocytes during ischemic injury has been highlighted in many studies using

a selective p38 inhibitor [34, 37]. These studies demonstrated that the inhibition of p38, using pharmacological inhibitors, could reduce the infarct size [38–43] and improve cardiac function [40, 42, 44–48] after myocardial infarction. However, there are some inconsistent findings indicating that treatment with p38 inhibitor neither reduced the infarct size nor improved cardiac function and that it abolished the beneficial effect of ischemic preconditioning [49–53]. These studies were performed in various model systems, including multiple *in vitro*, *ex vivo* (isolated whole heart), and *in vivo* animal models.

Reports of p38 inhibitor in an *in vitro* model of ischemia/reperfusion

Reports from *in vitro* experiments on the p38 inhibitor in the model of ischemia/reperfusion injury, either a cardiac cell line or isolated cardiomyocytes from many species, are summarized in Table 1. *In vitro* treatment with a p38 inhibitor, mainly SB203580, prior to ischemia at concentrations ranging from 1 to 15 μM was found to protect the cardiac cells from ischemia/reperfusion injury, suggesting an undesired effect of p38 activation in myocardial ischemia/reperfusion [36, 50, 54–63]. However, there have been some reports of beneficial effects following p38 activation, in which its activation could lead to the protection against injury rather than a harmful effect. Nagarkatti et al. [50] and Weinbrenner et al. [53] showed that the inhibition of p38 activation before ischemic preconditioning abolished the protective effect of preconditioning. Interestingly, in the same published work, applications with the same inhibitor and at a similar concentration before and during ischemia showed protection of the cardiac cell from ischemic injury [50]. These inconsistent findings could be due to the conditions of the heart at the time of p38 inhibition. The signal transduction cascade of ischemia can be divided into triggers and mediators. Triggers are important during the episode of preconditioning ischemia and reperfusion, while mediators are important during the prolonged index ischemia [64]. More importantly, the different signal transduction pathways and the consequences can possibly be due to differences in p38 downstream signalings or even end-effectors, which are specifically and differently activated according to prolonged ischemia or ischemic preconditioning (IPC). Identification of these specific targets of p38 is a challenge, as this information could prove helpful in understanding the complexity of p38 signaling, such as cross-talk between kinase pathways, desensitization to stimulation, and signal amplification, and ultimately lead to the discovery of powerful therapeutic agents with less harmful side effects.

Despite the fact that the *in vitro* model provides some valuable mechanistic information, which is also crucial to a better understanding of the mechanism of p38 activation

during ischemia/reperfusion, and in the hope that this will allow circumstance-specific inhibition and/or the identification of the harmful downstream pathways, it is important to note that the major limitation of *in vitro* studies is that they determine cell viability based on the release of metabolic enzymes as outcome measures and do not provide sufficient information on cardiac function. Therefore, studying the role of p38 in the whole heart would provide much insight into the function of the heart as an organ in the body.

Reports of p38 inhibitors in an *ex vivo* model of ischemia/reperfusion

A summary of *ex vivo* studies with cardiac ischemia/reperfusion is shown in Table 2. Studies of the inhibitory effect of p38 inhibitors in an *ex vivo* model were performed with concentrations of the inhibitor ranging from 1 to 10 μM , similar to most *in vitro* experiments. The pre-treatment of SB203580 and other p38 inhibitors prior to the ischemic period had a protective effect by reducing infarct size [38–43] and improving left ventricular (LV) function [40, 42, 44–48]. However, inconsistent findings were also reported in which inhibitor treatment in low-flow ischemia failed to reduce the infarct size [65] or abolished the protective effect of preconditioning [49, 66], which can also be seen in some *in vitro* data [50, 53]. There are many factors that possibly explain these inconsistent findings, such as dose of the inhibitor, timing of the treatment, study protocol of ischemia, and the specific animal model. For example, Gorog et al. [65] demonstrated that treatment with 1 μM of p38 inhibitor for 5 min before low-flow ischemia in a mouse model could not reduce the size of the infarct, whereas treatment with the same type and concentration of inhibitor in the same animal model for 10–15 min prior to the onset of ischemia did limit infarct size [43, 67]. However, treatment with 1 μM of p38 inhibitor for 5 min before ischemia was able to reduce infarct size in the rabbit model [38], suggesting different effects of the inhibitor in different species. The timing of the administration of p38 inhibitor in these experiments could be one of the major factors that should be considered. The concentration of p38 inhibitor used in most of these studies ranged from 1 to 10 μM , but the outcomes were apparently inconsistent. Some studies using either 1 or 10 μM p38 inhibitor showed that the therapy was protective [24, 38–41, 43, 45–48, 65, 67], whereas others reported no effect [42, 46, 49, 51]. These results suggest that timing of p38 inhibition is crucial for its cardioprotective effects during cardiac ischemia/reperfusion injury.

Similar to the findings in *in vitro* studies, inhibition of p38 activation prior to ischemic preconditioning also abolished the protective effect of preconditioning in an *ex vivo* model. In one study using the isolated rat heart model, 10 μM of p38 inhibitor given 5 min before the first precondition cycle

Table 1 Summary of reports on p38 inhibitor in an *in vitro* model

Species	Study model	Dosage of inhibitor	Treatment conditions	Readout	Outcomes	References
Rat	H9c2 rat cardiac myoblast cell line	15 μ M	1 hour before and during simulated ischemia	Cell viability by MTT assay	SB203580 increased cell viability	[50]
	HL-1 cardiac muscle cell line	1 μ M	30 min before and maintain during 7 hr sI	Cell viability by MTT assay	SB203580 increased cell viability	[36]
		10 μ M	SB before 45-min hypoxia SB before reoxygenation	Mitochondrial ROS, Apoptosis by propidium iodide (PI) staining	SB203580 decreased mitochondrial ROS and apoptosis	[63]
		10 μ M	2-h sI/5-h reperfusion SB203580 at and during 5-h reperfusion	Irreversible cellular injury by GFP-Bax or mCherry-Bax distribution	SB203580 decreased mitochondrial ROS	[54]
	Isolate neonatal rat cardiac myocytes	1 or 10 μ M	During 2-h sI	LDH release, and cell viability by MTT assay	SB203580 increased cell viability, reduced LDH release	[61]
Isolate neonatal rat cardiac myocytes		1 μ M	During 18-h hypoxia/1-h reoxygenation	Cell viability by trypan blue Annexin V-FITC, TUNEL, propidium iodide staining	SB203580 prevented hypoxia/reoxygenation induced cell death	[57]
		1 or 10 μ M	1 μ M SB30 min before 2 h of hypoxia 1-or 10 μ M 30 min before IPC	Cell viability by trypan blue exclusion assay	SB203580 increased cell viability did not block protective effect of IPC	[59]
		2–10 μ M	During 90-min ischemia	Cell viability by calcein acetoxymethyl ester and propidium iodide (PI) staining, LDH release	SB203580 reduced LDH release in a dose-dependent manner and significantly decreased cell death	[58]
		10 μ M	During 6-h sI/2-h reoxygenation	CK release, LDH release, and cell viability by MTT	SB203580 increased cell viability, reduced CK, LDH release	[35]
		10 μ M SB242719/ SB203580	15 min before and during ischemia/reperfusion	Apoptosis (TUNEL)	Both of SB242719 and SB203580 reduced apoptosis	[83]
Isolate neonatal rat cardiac myocytes (NRCMs)		10 mM	During 7-h ischemia	LDH release	Reduced LDH release	[84]
		10 μ M	15 min before and during sI and reoxygenation	Cytochrome C release, caspase 3 activation, DNA fragmentation	SB203580 prevented LDH release, cytochrome C release, caspase 3 activation, and DNA fragmentation	[60]
		500 nM–1 μ M RWJ	30 min before exposed to H ₂ O ₂ for 24 h	TUNEL	RWJ reduced apoptosis	[33]
Isolate adult rat cardiac myocytes (ARVMs)		1 and 10 μ M	30 min before and during 4-h sI	LDH release	SB203580 reduced LDH release	[36]
		10 μ M	20 min before exposed to H ₂ O ₂	apoptosis by Annexin V-FITC	SB203580 reduced apoptosis	[55]
		10 μ M	Throughout experiment of sI	Cell viability by MTS assay	SB203580 reduced apoptosis	[56]
Dog	Isolate cardiac cells	10 μ M	During 3-h hypoxia	TUNEL	SB203580 reduced apoptosis	[62]
Rat	H9c2 Rat cardiac myoblast cell line	15 μ M	1 hour before and during preconditioning and wash away after preconditioning	Cell viability by MTT	SB203580 blocked protective effect of IPC	[50]
	Isolate ventricular myocytes	10 μ M	5 min before IPC	Cell viability by trypan blue exclusion assay	SB203580 blocked protective effect of IPC	[53]

sI, simulated ischemia; IPC, ischemia preconditioning

The p38 inhibitor used in all of the *in vitro* models is SB203580, except where stated otherwise

Table 2 Summary of reports on p38 inhibitor in an *ex vivo* (isolated heart) model

Species	Study model	Dosage of inhibitor	Treatment conditions	Readout	Outcomes	References
Mouse	30 min global ischemia/120 min reperfusion	1 μ M SB203580	10 min before global ischemia	Infarct size	SB203580 reduced infarct size	[43]
	120 min of low-flow or moderate flow ischemia	1 μ M SB203580	5 min or 60 min after the onset of low-flow or moderate flow ischemia until the end of protocol	Infarct size and left ventricular function	Early SB203580 treatment in both low/moderate flow reduced infarct size, but failed to attenuate contractile dysfunction	[65]
	30 min low-flow ischemia/30 min of reperfusion or 60 min of low-flow ischemia/120 min of reperfusion	2 μ M SB203580	15 min before and 15 min after onset of ischemia/15 min before and after onset of reperfusion	Infarct size	SB203580 reduced infarct size	[39]
	30-min global ischemia/120-min reperfusion in p38 α drug-resistant mice	- 1 or 10 μ M SB203580 - 1 μ M BIRB-796	10 min before global ischemia	Infarct size, hemodynamic parameters	- SB203580 reduced infarct size and improved hemodynamic parameters in wildtype mice, but not in p38 α drug resistant (DR) mice - BIRB 796 reduced infarct size in both wildtype and p38 α DR mice	[24]
	Heart was perfused with 300 mM H ₂ O ₂ for 80 min	1 mM SB203580	Perfused 5 min before exposure to H ₂ O ₂	Developed pressure coronary flow, end-diastolic pressure, and creatine kinase in effluent	SB203580 reduced cell death and myocardial dysfunction	[47]
Rat	30-min global ischemia/120 min reperfusion	10 μ M SB203580	10 min before ischemia, throughout the period of reperfusion	DNA fragmentation, TUNEL assay, contractile function, CK activity	SB203580 before ischemia and during reperfusion reduced apoptosis and contractile function recovery	[46]
	20 min ischemia/25 min reperfusion -5 min I/5min R IPC before 20 min ischemia	10 μ M SB202190	- 5 min before index ischemia - 2 min before first cycle of IPC	Left ventricular developed pressure (LVDP), infarct size	- SB202190 reduced infarct size and enhanced function recovery - SB202190 had no effect on IPC	[41]
	30-min ischemia/180 min reperfusion	0.5-2 mg/kg FR 167653	1 hour via i.p. before ischemia and 1 mg/L in perfusion buffer 5 min before ischemia/reperfusion during reperfusion	Apoptosis by TUNEL, CK activity in effluent, Left ventricular function and caspase activity	FR 167653 reduced apoptosis, CK leakage, and improved cardiac contractile function during reperfusion	[44]
	25 min global ischemia/40 min reperfusion	20 μ M SB203580	5 min before ischemia/reperfusion	Left ventricular function and caspase activity	SB203580 reduced caspase-1,3,11 activation, and improved left ventricular function	[48]
	Rat heart subjected to hypothermic storage (8 h, 3°C) and rewarming reperfusion (30 min)	10 μ M SB202190	10 min before ischemia	Left ventricular function	SB202190 improved Left ventricular function recovery	[45]
	30-min regional ischemia/120-min reperfusion	10 μ M SB203580	10 min before ischemia	Infarct size, left ventricular function and CK and LDH activity in effluent, Apoptosis by TUNEL, caspase-3 assay	SB203580 reduced infarct size, improved Left ventricular function recovery, reduced cellular apoptosis and injury	[40]
	35-min regional ischemia/120-min reperfusion	1 μ M SB203580	10 min before ischemia/reperfusion	Infarct size, caspase-3 activation, and PARP cleavage	SB203580 before ischemia reduced apoptosis, SB203580 did not abolish protective effect of IPC	[67]
	30-min focal ischemia/120-min reperfusion	1 μ M SB203580	SB203580 5 min before I/R and 5 min after ischemia before reperfusion	Infarct size	SB203580 reduced infarct size	[38]
	30-min ischemia/120-min reperfusion in C57BL/6 or mkk3 ^{-/-}	1 μ M SB203580	15 min before ischemia or at perfuse Antimycin A	Infarct size and left ventricular function	SB203580 co-administered with Antimycin A abolished protective effect of Antimycin A preconditioning	[66]
	20-min ischemia/120 min reperfusion	10 μ M SB203580	After the onset of reperfusion for 30 min	Infarct size, left ventricular function and CK activity	SB203580 during reperfusion improved contractility but increased CK release and infarct size	[42]
Rat	4×4-min ischemia/6-min reperfusion followed by 30-min ischemia/120-min reperfusion in p38 α or p38 β DR mice	10 μ M SB203580	During IPC	Infarct size	SB203580 abolished protective effect of IPC in wildtype and p38 β DR but not p38 α DR	[85]
	30-min global ischemia/120-min reperfusion	10 μ M SB203580	10 min after the onset of reperfusion	DNA fragmentation, TUNEL assay, contractile function, CK activity and NBT staining	Perfusion of SB203580 at 10 min after reperfusion failed to protect the heart from ischemic injury	[46]
	35-min regional ischemia/120-min reperfusion	10 μ M SB203580	5 min before first preconditioning cycle and continued until 15 min into sustained ischemia SB 20 min before ischemia and during first 15 min of ischemia	Infarct size, left ventricular function	SB203580 blocked the protective effect of preconditioning	[49]
Rabbit	30-min focal ischemia/120-min reperfusion -5 min of global ischemia and 10 min reperfusion before the 30-min period of ischemia	2 μ M SB203580	20 min before ischemia	Infarct size	SB203580 alone had no effect on infarct size, treatment of SB203580 before ischemic precondition blocked the protective effect of preconditioning	[51]

prevented the cardioprotective effect of preconditioning [49]. However, treatment with a tenfold lower concentration of the same inhibitor for 10 min before ischemia, which is known to reduce infarct size in a similar animal model, could not abolish the subsequent protective effect of ischemic preconditioning [67]. These findings suggest that, in the ischemic preconditioning model, the dosage of the inhibitor has more influence on the cardioprotection effect than the timing of administration of the inhibitor. Similar to the finding in the heart of small rodents, infusion of p38 inhibitor in the isolated rabbit heart model also exerted a significant cardioprotective effect during sustained ischemia [38, 46], although this same inhibitor again blocked the cardioprotective effect of ischemic preconditioning [51]. Therefore, it may be concluded that p38 activation only during sustained ischemia appears to be proapoptotic, whereas its activation in ischemia preconditioning seemed to be more anti-apoptosis. Again, this inconsistency could possibly be explained by p38 activation playing different roles, namely, as a trigger or mediator, when subjected to different stimuli.

Reports of p38 inhibitors in an *in vivo* model of ischemia/reperfusion

The cardioprotective effect of p38 inhibitor that was demonstrated in *in vivo* studies was similar to the findings from both *in vitro* and *ex vivo* models. The *in vivo* study model provides valuable functional information which is closely related to the pathophysiology of myocardial ischemia/reperfusion. To date, *in vivo* studies on the use of p38 inhibitors have been reported with different doses, modes of treatment, duration of treatment, and animal species (Table 3).

Many studies have demonstrated the benefit of p38 inhibitors in the *in vivo* model of sustained ischemia, either in the small animal or large animal model, where p38 inhibitors were found to reduce the infarct size [21, 32, 68, 69] and improve LV function [33, 69–74]. Nevertheless, inconsistent reports do exist, mostly from studies performed in the large animal model. It is noticeable that, in large animal models such as pigs or dogs, p38 activation does not appear to be as clearly proapoptotic as found in rodents [32]. Kaiser et al. reported that SB239063 reduced the infarct size in the mouse model, but failed to protect the pig heart from ischemic injury [32]. The failure of the p38 inhibitor to protect the pig heart from ischemic injury was also reported in another study using different p38 inhibitors, such as BIX-645 and SB203580 [75]. The potential explanation for these inconsistent findings could be species, which can be explained by the concept that signal transduction varies among species. The findings in the large animal model also showed some inconsistencies.

The mode of drug administration and degree of coronary occlusion have been shown to play important roles in the cardioprotective effects of p38 inhibitor in a large animal model [75, 76]. Intracoronary infusion of SB203580 in low-flow ischemia failed to reduce the infarct size and limited the beneficial effect of IPC [75], whereas an intramyocardial injection of SB203580 in the ischemic area in complete coronary occlusion was able to reduce infarct size and did not abolish the IPC effect [76]. These findings emphasize the importance of the intensity of the ischemic stimuli that may cause variable degrees of signal transduction activation and responses. Nevertheless, in a dog model, the intracoronary infusion of SB203580 prior to ischemia/reperfusion or during IPC failed to reduce the infarct size and abrogated the protective effect of IPC, whereas the continuous treatment of SB203580 during sustained ischemia had a cardioprotective effect [52]. This report again emphasizes the importance of the timing and duration of p38 inhibitor administration in terms of its cardioprotective effect during ischemia/reperfusion.

It is widely accepted that an *in vivo* model is the best study model to determine the long-term effect of both drugs and physiological responses. Chronic studies investigating the long-term (1–14 weeks) effect of p38 inhibitor in ischemia/reperfusion have been reported. Most of these studies demonstrated that long-term treatment with p38 inhibitors following the induction of myocardial infarction had beneficial effects, such as improved cardiac function [33, 70–74, 77], inhibited infarct expansion [33], reduced scar size [77], and suppressed myocardial fibrosis [74].

p38 inhibitor: where do we go from here?

The important questions that still need to be clarified are whether p38 inhibitors really do have therapeutic potential in real clinical settings and if so, is the background information sufficient to ensure that the p38 inhibitor can be used effectively in real clinical treatment? It is noticeable that the majority of the experimental findings that initially indicated the efficiency of p38 inhibitors in reducing myocardial injury and impaired cardiac function were associated with pre-ischemic treatment. Prevention of p38 activation by the inhibitor prior to ischemia seems to be impractical in the actual clinical setting, as myocardial ischemia is an unpredictable episodic condition. Therefore, the timing of treatment and its therapeutic potential are critical issues that need to be addressed. It will be clinically more useful if the inhibition of p38 activation at the postischemic state, which includes the reperfusion period, can provide a cardioprotective effect. Nevertheless, the roles of p38 activation and the consequences of its inhibition in postischemic and reperfusion periods, especially in an *in vivo* model, have not been

Table 3 Summary of reports on p38 inhibitor in an *in vivo* model

Species	Study model	Dosage of inhibitor	Treatment conditions	Readout	Outcomes	References
Mouse	30-min regional ischemia/120-min reperfusion (LAD ligation)	2 mg/kg FR167653	Intraperitoneal injection 24 hours before ischemia	Infarct size	FR167653 reduced infarct size	[21]
	60-min regional ischemia/24-hr reperfusion (LAD ligation)	7 µg SB239063	Injected into tail vein 5 min before ischemia	Infarct size	SB239063 5 min before ischemia had 25% reduction infarct size compared to control	[32]
Rat	Regional ischemia/reperfusion (LAD ligation)	2 mg/kg SB203580-HCl	Intraperitoneal injection immediately after LAD ligation	Cardiomyocytes mitosis, Cardiac functions: % left ventricular fractional shortening (FS), end diastolic dimension, and end systolic dimension.	24 h after MI SB203580 treatment induced cardiomyocytes mitosis and significantly increased %FS.	[77]
	20-min regional ischemia/24-hr reperfusion (LAD ligation)	30 mg/kg SB239063 orally	Administered per oral 20 min before ischemia	Infarct size, myocardial myeloperoxidase	SB239063 reduced infarct size and polymorphonuclear cell accumulation	[68]
	Regional ischemia/reperfusion (LAD ligation)	1 mg/kg SB203580	Jugular vein injection 5 min before reperfusion	Rate-pressure product value, $\pm$ delta pressure/delta time max, apoptotic index, CK-MB activity, Area at risk (AAR)	SB203580 injection before reperfusion (postconditioning) improved cardiac function and reduce AAR.	[69]
Rabbit	1-hr regional Ischemia/3-hr reperfusion (LAD ligation)	1 mg/kg PD169316	Intravenous bolus injection 55 min after LAD ligation	Area at risk (AAR), plasma CK activity, myocardial myeloperoxidase	PD169316 reduced AAR, plasma CK activity and PMN accumulation	[86]
Pig	Regional ischemia/reperfusion (LAD ligation)	40 nM SB203580 or 5 mg/kg	Local intramyocardial infusion 1hr before index ischemia or 5 mg/animal 10 min before the onset of 1hr coronary occlusion	Infarct size	SB203580 reduced infarct size. Both local or systemic perfusion of SB203580 before and during IPC had no effect on IPC	[76]
Dog	Transplant dog heart	FR167653 (dose not mentioned)	Hearts from donor were left in situ for 30 min after cardiac arrest and subjected to coronary flushing and immersed in Celsior solution for 4 h with and without FR167653 during sustained ischemia	Cardiac output, LV pressure, End-systolic maximal elastance (EMax)	FR167653 improved heart-graft viability and cardiac function	[87]
Mouse	90-min occlusion/6-hr reperfusion	1.18 µg/kg/min SB203580	SB239068 in drinking water for a total of 2–14 weeks (for hemodynamic and biochemical studies) or 12 weeks (for survival observations).	Infarct size	Continuous treatment of SB203580 during sustained ischemia reduced the infarct size	[52]
	Long-term treatment: regional ischemia/reperfusion (LAD ligation) of MKK6bE transgenic mice	1200 ppm SB239068 in chow or food	Long-term treatment with SC-409 for 2- to 14-weeks post-MI.	Hemodynamic measurement by Pressure-volume loop analysis	SB239068 treatment reduced end-diastolic and end-systolic chamber stiffening; net chamber filling and output were restored toward baseline despite persistently depressed systolic function. SB prolonged life span.	[71]
	Long-term treatment: regional ischemia/reperfusion (LAD ligation)	30 mg/kg/day SC-409 in chow		Cardiac geometry and function by echocardiography	SC-409 reduced systolic blood pressure, increased LV ejection fraction and cardiac output, and decreased LV area at diastole	[72]

Table 3 (continued)

Species	Study model	Dosage of inhibitor	Treatment conditions	Readout	Outcomes	References
Rat	Long-term treatment: regional ischemia/reperfusion (LAD ligation)	50 mg/kg/day R WJ-67657	Daily treatment by gavage 21 days post-MI	Anterior and posterior wall thickness and LV fractional shortening, infarct size	Improved LV function and but had no effect on infarct size	[33]
	Long-term treatment: regional ischemia/reperfusion (LAD ligation)	2 mg/kg SB203580-HCl	Long-term treatment of SB203580 by intraperitoneal injection once every 3 days for 4 weeks	Cardiomyocytes mitosis, cardiac functions were assessed by percentage left ventricular fractional shortening (%FS), end diastolic dimension (EDD), and end systolic dimension (ESD). Cardiac function by echocardiography, myocardial fibrosis	-2 weeks after MI SB203580 treatment significantly increased %FS, prevented cardiac dilation as measured by EDD and ESD -at 3 months after injury, 4 weeks of SB203580 increased %FS by 30% and reduced scar size	[77]
	Long-term treatment: regional ischemia/reperfusion (LAD ligation)	2 mg/kg SB203580-HCl	Intraperitoneal injection once every 3 days for 1 and 4 weeks	Cardiac function by echocardiography, myocardial fibrosis	SB203580 suppressed myocardial fibrosis and LV remodeling	[74]
	Long-term treatment: regional ischemia/reperfusion (LAD ligation)	50 mg/kg/day RWJ-67657	Treatment by gavage for 7 days after MI compared with 12 weeks treatment	Cardiac function by echocardiography and hemodynamic measurement	Long-term RWJ treatment improved fractional shortening and attenuated the rise in LVEDP, and prevented the reduction in dP/dtmax	[70]
	Long-term treatment: regional ischemia/reperfusion (LAD ligation)	2 μ M SB239063	Intramyocardial injection with SB239063 encapsulated in microspheres formulated from the polymer (PCADK) and inject (0.5 mg/ml corresponding to 2 μ M SB239063) after LAD ligation	Cardiac function using MRI and Echocardiography 7 and 21 day post-MI	No significant improvement of cardiac function at 7 day post-MI but significantly improved cardiac function at 21 days post-MI	[73]
Pig	Regional 60-min ischemia/4-hr reperfusion (LAD ligation)	7 mg SB239063	SB directly injected into LV lumen and perfused for 5 min before LAD occlusion	Infarct size	SB239063 had no effect on infarct size	[32]

LAD, left anterior descending coronary artery; MI, myocardial infarction; LV, left ventricle; LVEDP, left ventricular end-diastolic pressure

intensively investigated. Studies in an *in vivo* model, either acute or long-term treatment, are essential and will provide significant and clinically useful information, which may be used to develop therapeutic strategies during the actual pathophysiological events that occur in humans.

In addition to the timing of drug administration, another important issue is the effect of p38 inhibition on potentially fatal cardiac arrhythmia during myocardial ischemia/reperfusion. Although a number of ischemia/reperfusion studies reported the incidence of fatal arrhythmias during ischemia/reperfusion [78, 79], no *in vivo* study has yet investigated the effect of p38 inhibitors on a lethal arrhythmia during ischemia/reperfusion. Similar to the postischemic mortality rate, no work has presented mortality data in animals treated with p38 inhibitors, which would support the long-term effect of using a p38 inhibitor. This crucial information needs to be obtained if the effect of p38 inhibitors in myocardial ischemia/reperfusion is to be of significant relevance.

The small molecule inhibitors of p38 have been studied for almost 20 years, predominantly in terms of the anti-inflammatory effect of the inhibitors [80]. However, most of the outcomes of using the p38 inhibitors in clinical trials have been disappointing as a result of adverse events stemming from drug toxicity [81]. Although many studies of the p38 inhibitor in myocardial ischemia seem to support the benefit of the p38 inhibitor in reducing myocardial injury and improving cardiac function, the majority of clinical trials with p38 inhibitors have been mainly aimed at studying its anti-inflammatory effect, not for myocardial infarctions. Therefore, at this point do we still have faith in the p38 inhibitor for attenuating cardiac damage in ischemic heart disease? Although there have been some clinical trials on a p38 inhibitor in cardiovascular disease [7–10], only one study has focused on the acute coronary syndrome [82], namely, the first clinical study of the p38 inhibitor GW856553 or Losmapimod (NCT00910962; GlaxoSmithKline, London, UK). In this trial, changes in high-sensitivity C-reactive protein and cardiac biomarkers are being measured as primary outcomes, as well as the infarct size and cardiac functions based on magnetic resonance imaging data in the sub-study [82]. This study is still ongoing, and the primary outcome data are expected to be available in April 2012 [82]. At the same time, it is necessary to look back to the pre-clinical data set derived from p38 inhibitors during myocardial ischemia/reperfusion in order to determine whether there are still some crucial gap(s) of information as these should be filled in an attempt to obtain useful information. This is an essential prerequisite to the exploitation of the wealth of pre-clinical data which suggests that the inhibition of p38 activation will benefit patients with ischemic heart disease.

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Na_v1.8, but not Na_v1.9, is upregulated in the inflamed dental pulp tissue of human primary teeth

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Abstract

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Aim To investigate alterations in Na_v1.8 and Na_v1.9 expression within inflamed dental pulp tissue of human primary teeth.

Methodology Dental pulp tissue obtained from both normal and inflamed pulps in primary teeth as well as pulps from normal and inflamed permanent teeth was used. The quantity of Na_v1.8 and Na_v1.9 expression in the dental pulp tissue was investigated using Western blot analysis. General neuron marker (PGP9.5) was used to quantify for neural density, and an increase in metalloproteinase-9 was used to indicate pulpal inflammation in inflamed teeth. Statistically significant differences for each determined

parameter between normal and inflamed teeth of both primary and permanent teeth were tested using the Mann–Whitney rank sum test.

Results There was no significant difference in neural density of normal and inflamed dental pulp tissue, although degrees of inflammation were increased in the inflamed dental pulp of both permanent and primary teeth ($P < 0.05$). Na_v1.8 and Na_v1.9 expression in inflamed pulps of permanent teeth increased significantly compared with normal permanent teeth ($P < 0.05$). However, only Na_v1.8 expression was increased significantly in the inflamed dental pulp of primary teeth ($P < 0.05$).

Conclusions Na_v1.8 alone may be the therapeutic target for treatment of painful pulpitis in primary teeth.

Keywords: dental pulp, inflammation, Na_v1.8, Na_v1.9, primary teeth.

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Introduction

Voltage-gated sodium channels (VGSCs) are transmembrane ion channels involved in the initiation and propagation of action potentials, and the spontaneous activity of VGSCs can lead to spontaneous pain. VGSCs are found in excitable cells, including neurons and muscle cells (Goodman 2008). VGSCs can be categorized into two groups according to their resistance to

the blocker, tetrodotoxin (TTX): TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) channels (Cummins *et al.* 2007). Na_v1.8 and Na_v1.9 are a subfamily of TTX-R VGSCs, which are dominantly found in unmyelinated C fibres, the largest group of sensory nerve fibres innervating dental pulp tissue (Amir *et al.* 2006). Na_v1.8, with its slow inactivation kinetics and high activation threshold, is involved in the electrogenesis of action potentials in C-type peripheral neurons (Renganathan *et al.* 2001), whereas Na_v1.9 is responsible for the generation of persistent action potentials (Dib-Hajj *et al.* 2002). Both Na_v1.8 and Na_v1.9 are believed to be involved in prolonged action potentials during painful stimuli (Cummins *et al.* 2007). Several previous studies have shown that Na_v1.8 and Na_v1.9 are upregulated

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in several chronic inflammatory pain models (Amaya *et al.* 2006, Joshi *et al.* 2006, Strickland *et al.* 2008).

Dental pulp innervation consists mostly of nociceptive nerve fibres that originate from the trigeminal ganglion, peripherally pass through the apical foramen, and terminate in the dental pulp as free nerve endings (Hildebrand *et al.* 1995). Dynamic changes in the density of pulpal innervation during pulpal inflammation have been found to be changed depending on the severity and timing of the inflammation (Byers *et al.* 1990). These changes can be either sprouting or degeneration of nerve fibres. Thus, the innervation density of dental pulp tissue may be either increased or decreased following inflammation. For example, a study by Rodd & Boissonade (2001) using immunohistochemistry revealed that neural density at the pulpal horn of inflamed dental pulp of human primary and permanent teeth increased with the depth of dental caries. In contrast, Western blot analysis indicated no significant difference in neural density of the dental pulp of inflamed human permanent teeth compared with normal teeth (Warren *et al.* 2008). In the dental pulp of permanent teeth with pulpitis, the upregulation of matrix metalloproteinase-9 (MMP-9), which is an enzyme responsible for extracellular matrix remodelling and degradation, was also found (Tsai *et al.* 2005). This finding indicates that MMP-9 is a marker for pulpal inflammation. The expression of Nav1.8 and Nav1.9 has been found at nerve endings in the dental pulp of permanent teeth (Renton *et al.* 2005, Wells *et al.* 2007), and the upregulation of those VGSC isoforms, Nav1.8 and Nav1.9, has been demonstrated during painful pulpitis of human permanent teeth (Renton *et al.* 2005, Wells *et al.* 2007, Warren *et al.* 2008). A recent study in rat dental pulp tissue found increased Nav1.8 mRNA expression in association with an increased degree of pulpal inflammation (Esmacili *et al.* 2011). In spite of the strong evidence for Nav1.8 and Nav1.9 expression in permanent human dental pulp tissue, no evidence of those sodium channel isoforms in the dental pulp of human primary teeth has been demonstrated. The innervation in primary teeth and in permanent teeth is different because the density of the dental nerve supply is lower in primary teeth (Rodd & Boissonade 2001, 2002). Therefore, the aim of this study was to investigate whether Nav1.8 and Nav1.9 expression is altered in human dental pulp of inflamed primary teeth compared with normal teeth. The hypothesis that the expression of Nav1.8 and Nav1.9 are altered following pulpal inflammation in primary teeth was tested.

Materials and methods

Subjects

The use of human subjects was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. Informed consent was obtained from all the subjects or accompanying guardians, in the case of subjects under the age of 20.

Teeth from subjects were divided into four groups: intact premolars extracted for orthodontic purposes ($n = 18$), permanent teeth diagnosed with irreversible pulpitis that required extraction ($n = 7$), intact primary teeth diagnosed with prolonged retention that required extraction ($n = 7$) and primary teeth diagnosed with reversible or irreversible pulpitis that required extraction ($n = 16$). Teeth with deep caries or mechanical pulp exposures with a history of pain on stimulation that rapidly disappeared or no history of pain and no radiographic periapical changes were diagnosed with reversible pulpitis. Teeth with irreversible pulpitis had deep caries and/or restorations with spontaneous pain. Normal or enlarged periodontal space was detected on the radiograph of teeth with irreversible pulpitis. All primary teeth had physiologic root resorption of not more than 2/3 root length. All teeth had no history of trauma, and all teeth with inflamed pulps were from deep caries. The subjects were interviewed for their history of pain before extraction. The information related to history of dental pain included sites of dental pain, duration, stimulating factors and severity of maximum pain was obtained using visual analogue scales (VAS).

Pulpal tissue collection

Immediately after extraction, a vertical groove almost to the depth of the pulp chamber was cut along the buccal surface of each tooth from the incisal edge to the apex in anterior teeth, and to the furcation in molar teeth. Then, the teeth were split open along the groove using an elevator. Pulpal tissues were removed and stored in 1.5 mL Eppendorf tubes in liquid nitrogen, before being transferred to a freezer and stored at -80°C for further investigation.

Western blot analysis

The method for Western blot analysis was modified from that of Warren *et al.* (2008). Frozen pulp tissue was weighed before being crushed with a plastic pestle

in a homogenization buffer at a ratio of 1 mg of pulpal tissue to 10 μL of buffer. The buffer consisted of RIPA (50 mmol L^{-1} of Tris-Cl pH7.5, 150 mmol L^{-1} of sodium chloride, 1 mmol L^{-1} of EDTA, 1% Triton X-100 and 0.1% SDS), two cocktails, of protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany), and 1% of sodium deoxycholate. The protein concentration of each sample was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were denatured by being boiled at 95 °C for 5 min and were detected via 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferring of proteins from gels to nitrocellulose membranes, and incubation of the membranes in primary antibody overnight. The primary antibodies used in this study were rabbit polyclonal antibody to PGP9.5, a general neuronal marker (1 : 400 in 0.1% TBST with 10% nonfat dry milk, ab10404; Biomed Diagnostics, Cambridge, MA, USA), rabbit polyclonal antibody to MMP-9, used as a marker of inflammation (1 : 200 in 0.1% TBST with 5% nonfat dry milk, ab38904; Biomed Diagnostics), rabbit polyclonal antibody to Nav1.8 (1 : 200 in 0.1% TBST with 5% bovine serum albumin, S2071; Sigma Aldrich Inc, St. Louis, MO, USA) and rabbit polyclonal antibody to Nav1.9 (1 : 200 in 0.1% TBST with 5% bovine serum albumin; S2196, Sigma Aldrich Inc). The membranes were then incubated in a secondary goat anti-rabbit antibody, conjugated with horseradish peroxidase (1 : 5000 in 0.1% TBST; Bio-Rad Laboratories). The proteins were visualized via a chemiluminescent detection system (Amersham ECL Western blotting detection reagents; GE Healthcare, Piscataway, NJ, USA). Band intensity was quantified by the Scion Image program (Scion Corporation, Frederick, MD, USA). The data were normalized with β -actin to control for the amount of protein loading and transfer.

Data analysis

Data were shown as mean $\pm$ SE. Statistically significant differences for each determined parameter between normal and inflamed teeth of both primary and permanent teeth were tested using the Mann-Whitney rank sum test. Significance levels were set at $P < 0.05$.

Results

Mean ages of subjects with permanent teeth with normal pulps, permanent teeth with inflamed pulps, primary teeth with normal pulps and primary teeth

with inflamed pulps were 17.3 ± 1.1 , 35.4 ± 6.3 , 9.4 ± 0.9 and 6.1 ± 0.7 (mean $\pm$ SE) years, respectively. The average duration of pain was 56 ± 51 days in the subjects with permanent teeth with inflamed pulps and 15 ± 6 days in subjects with primary teeth with inflamed pulps. No pain was reported in subjects with normal primary and normal permanent teeth. All permanent teeth with inflamed pulps were diagnosed with irreversible pulpitis. Of 16 inflamed primary teeth, eight were clinically diagnosed with irreversible pulpitis, whereas another eight were clinically diagnosed with reversible pulpitis.

To quantify the amount of nerve fibres in the dental pulp, the levels of PGP9.5 compared with β -actin in each dental pulp were measured. There was no significant difference in the relative amount of PGP9.5 between normal and inflamed dental pulp of either primary or permanent teeth (Fig. 1). The average levels of inflammation were represented by the ratio of MMP-9 to β -actin. The relative amounts of MMP-9 were significantly higher in inflamed primary teeth ($P < 0.05$) and inflamed permanent teeth ($P < 0.05$) than in normal teeth (Fig. 2). The expression

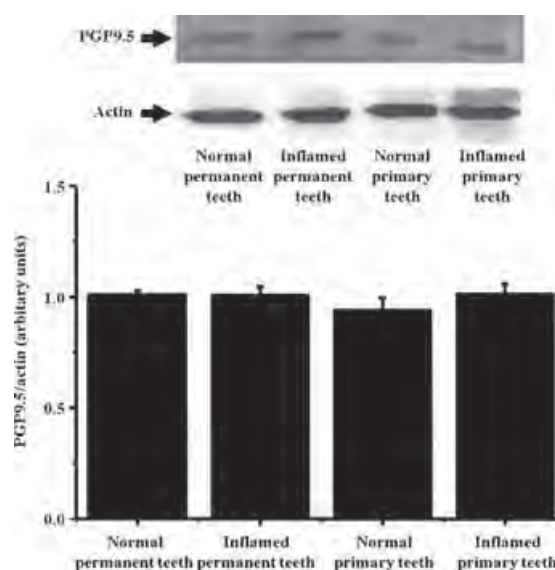


Figure 1 Relative amounts of PGP9.5 expression. PGP9.5, the neural marker, was detected in the dental pulp of permanent and primary teeth as indicated by the arrow. The band intensity of each sample was normalized with β -actin and shown as mean $\pm$ SE. The data show that the relative amount of PGP9.5 in dental pulp of inflamed permanent ($n = 7$) and primary teeth ($n = 16$) was not significantly different from that in normal permanent ($n = 18$) and primary ($n = 7$) teeth.

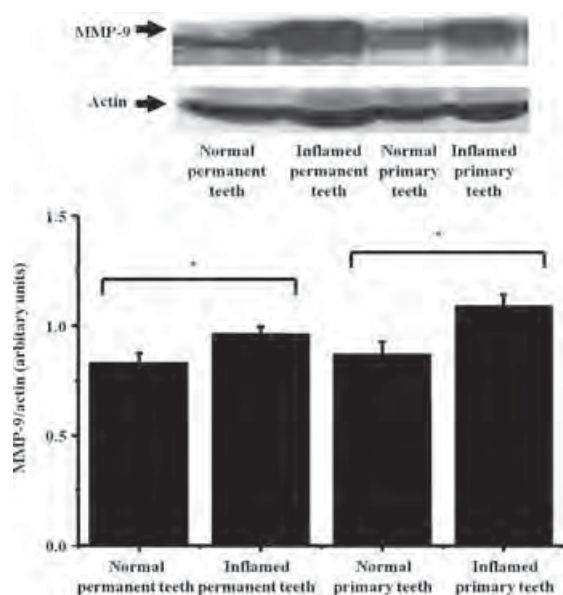


Figure 2 Relative amounts of metalloproteinase-9 (MMP-9) expression. The expected molecular weight of MMP-9 is indicated by the arrow. The band intensity of each sample was normalized with β -actin and shown as mean $\pm$ SE. There was a significant increase in MMP-9 in inflamed pulp compared with normal pulp of both permanent ($n = 7$ and 18 , for inflamed and normal pulp, respectively) and primary teeth ($n = 16$ and 7 , for inflamed and normal pulp, respectively) ($*P < 0.05$).

of $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in the dental pulp of permanent teeth with inflamed pulps was significantly greater than in normal permanent teeth ($P < 0.05$, as shown in Figs 3 and 4). In primary teeth, only $\text{Na}_v1.8$ expression was significantly higher in the inflamed dental pulp than in normal dental pulp ($P < 0.05$, Fig. 3). However, no significant difference between the amount of $\text{Na}_v1.9$ expression in inflamed and normal pulp tissues in primary teeth was observed (Fig. 4).

Only $\text{Na}_v1.8$ was correlated with maximum pain intensity in the group with permanent teeth ($P < 0.05$, $r = 0.674$). However, no correlation between all protein expression and pain intensity in groups with primary teeth was found ($P > 0.05$).

Discussion

The present study is the first to demonstrate the expression of $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in dental pulp tissue of human primary teeth. $\text{Na}_v1.8$ was upregulated in the inflamed dental pulp of both permanent and primary teeth, whereas $\text{Na}_v1.9$ was upregulated only

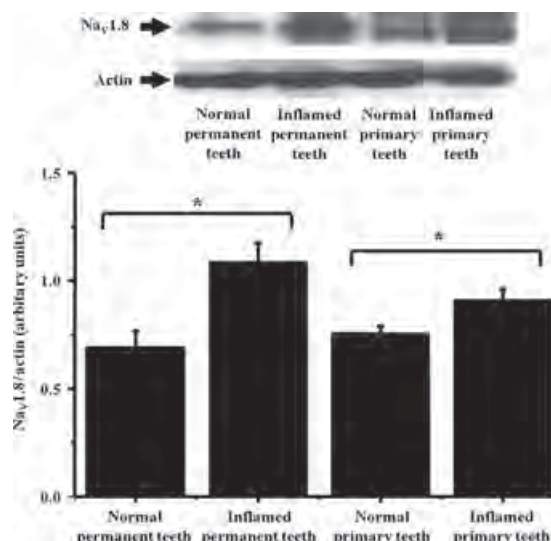


Figure 3 Relative amounts of $\text{Na}_v1.8$ expression. The expected molecular weight of $\text{Na}_v1.8$ is indicated by the arrow. The band intensity of each sample was normalized with β -actin and shown as mean $\pm$ SE. The data indicate a significant increase of $\text{Na}_v1.8$ in the inflamed dental pulp of permanent ($n = 5$) and primary teeth ($n = 9$) compared with normal dental pulp ($n = 6$ and 6 , for normal permanent and primary teeth, respectively) ($*P < 0.05$).

in the inflamed dental pulp of permanent teeth. The results also showed that there was no change in the overall amount of nerve fibres in inflamed pulps compared with normal pulps in both permanent and primary teeth.

Changes in pulpal innervation during inflammation by sprouting of nerve fibres have been reported (Byers et al. 1990). However, the depth of carious lesions and degrees of pulpal inflammation resulting in the degenerative changes of pulpal tissues may explain the findings with no change in the overall amount of innervation in teeth with and without pulpitis. The comparability of overall innervation between inflamed and normal dental pulp tissue may also suggest a similarity in the number of potential sites of sodium channel expression. Therefore, the increased expression of $\text{Na}_v1.8$ in primary and permanent teeth with pulpitis and of $\text{Na}_v1.9$ in permanent teeth with pulpitis results not only from an increase in the number of potential sites of their expression, but may also result directly from the upregulation of the $\text{Na}_v1.8$ and $\text{Na}_v1.9$ expression itself. The increased expression of $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in human permanent dental pulp with painful pulpitis, as in the present study, has previously

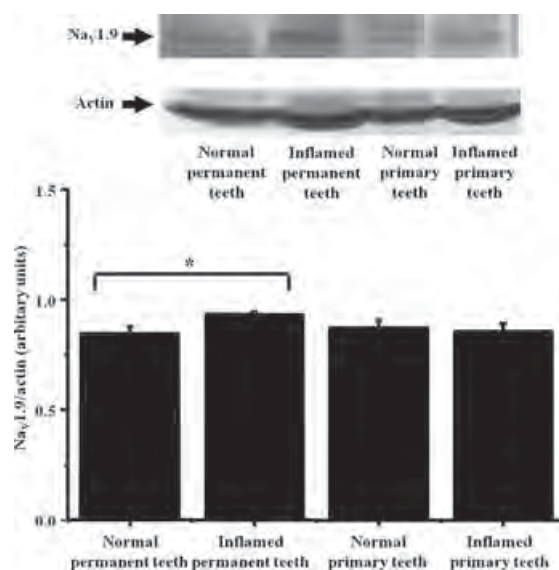


Figure 4 Relative amounts of Nav1.9 expression. The expected molecular weight of Nav1.9 is indicated by the arrow. The band intensity of each sample was normalized with β -actin and shown as mean $\pm$ SE. There was a significant increase of Nav1.9 in the inflamed dental pulp of permanent teeth ($n = 5$) compared with normal permanent teeth ($n = 8$) ($*P < 0.05$). However, there was no significant difference in Nav1.9 expression between normal ($n = 5$) and inflamed ($n = 9$) dental pulp of primary teeth.

been reported (Renton *et al.* 2005, Wells *et al.* 2007, Warren *et al.* 2008), but the altered expression of Nav1.8 and Nav1.9 in primary teeth with pulpitis has never been investigated. Despite the dominant expression of Nav1.8 and Nav1.9 in nociceptive neurons, the localized expression of both sodium channel subtypes may not be similar. Nav1.8 is remarkably localized in unmyelinated and small myelinated sensory neurons, whereas Nav1.9 is located only in unmyelinated neurons (Amaya *et al.* 2000). The possible explanation for no upregulation of the Nav1.9 expression in inflamed primary teeth could be due to the insufficient degree of pulpal inflammation in inflamed primary teeth to activate the upregulation of Nav1.9 expression. Previous studies demonstrated that the expression of Nav1.8 may be more sensitive to inflammatory pain stimuli, suggesting that the threshold for upregulation of Nav1.8 may be lower than that of Nav1.9 (Benn *et al.* 2001, Yu *et al.* 2011). As the inflammatory mediators secreted following injuries, such as prostaglandin E₂ (PGE₂), serotonin and adenosine, are capable to modulate TTX-R currents, contributing to hyperalgesia (Gold 1999), and that inflammatory

mediators have been found to be increased with age (Brünnsgaard & Pedersen 2003), it is possible that the levels of inflammatory mediators could be higher in the group with permanent teeth than the group with primary teeth.

Axonal demyelination is a common event in the inflamed dental pulp that causes a dynamic change in the proportion of myelinated and demyelinated fibres (Henry *et al.* 2009). The study of Henry *et al.* (2009) in human permanent inflammatory dental pulp showed that there was an increase in the atypical nodal sites, representing axonal demyelination. That study also found the accumulation of sodium channels at the atypical nodal sites but there was no overall upregulation of sodium channel expression (Henry *et al.* 2009). That finding suggested that pulpal inflammation can upregulate sodium channels at only some specific sites without changes in total sodium channel expression. The upregulation of Nav1.8, but not Nav1.9, expression in the inflamed primary teeth found in this study suggests that Nav1.8, rather than Nav1.9, should have a significant role in primary tooth pulpal inflammatory pain. However, the finding that Nav1.9 did not increase in primary teeth with pulpitis does not mean that there was no change in Nav1.9 expression, but there may be changes in specific sites of Nav1.9 accumulation without overall quantitative changes. Further investigations to localize the sites of Nav1.8 and Nav1.9 expression and studies in the field of stimulating pathways of Nav1.8 and Nav1.9 are still required. It was also demonstrated that only Nav1.8 expression in permanent teeth was correlated with pain score; therefore, the findings may emphasize that only Nav1.8, but not Nav1.9, plays a role in chronic pulpal inflammatory pain. However, the reasons for the absence of correlation between Nav1.8 and pain scores in the group with primary teeth may be a result of the difference in their ability to recall previous pain between adults and children.

Conclusions

Evidence exists to support the concept that Nav1.8 plays an important role in chronic inflammatory pain (Amaya *et al.* 2000, Joshi *et al.* 2006, Strickland *et al.* 2008). In contrast, the evidence for the involvement of Nav1.9 in inflammatory pain is controversial (Porreca *et al.* 1999, Coggeshall *et al.* 2004, Amaya *et al.* 2006, Strickland *et al.* 2008). In the present study, there was an increase in both Nav1.8 and Nav1.9 expression in

permanent teeth with inflamed pulps compared with permanent teeth with normal pulps, and there was an increase of only Nav1.8, but not Nav1.9, expression in primary teeth with inflamed pulps compared with normal primary teeth. This suggests that, in permanent teeth, both Nav1.8 and Nav1.9 may be the targets for the development of novel analgesic drugs and novel anaesthetic agents for the treatment of pulpal inflammatory pain, whereas, in primary teeth, only Nav1.8 may be the target for the treatment of pulpal inflammatory pain.

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Cardioprotective effects of incretin during ischaemia-reperfusion

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Abstract

Incretin is a gut derived peptide hormone secreted in the intestine after food ingestion, and is degraded rapidly after secretion by dipeptidyl peptidase (DPP)-4. Incretin-based therapy, such as glucagon-like peptide (GLP)-1 and the DPP-4 inhibitor, has been proposed as a new therapeutic approach for the treatment of type 2 diabetic patients. In the past few years, growing evidence also demonstrated the cardioprotective effects of incretin-based therapy, especially during ischaemia-reperfusion (I/R) injury in both the animal models and in clinical studies. However, inconsistent reports exist regarding the use of these pharmacological interventions. In this article, a comprehensive review regarding both basic and clinical studies reporting the effects of GLP-1 and DPP-4 inhibitors on I/R hearts is presented and discussed. The consistent findings as well as controversial results are summarised, focusing on the effects of incretin on the infarct size, left ventricular function and haemodynamic improvement during an I/R injury.

Keywords

Incretin, GLP-1, DPP-4 inhibitor, ischaemia-reperfusion injury, heart

Introduction

Diabetes mellitus (DM) has become a significant health problem in most nations with the number of patients dramatically soaring and expected to reach 366 million by the year 2030.¹ It has been shown that patients with type 2 diabetes mellitus (T2DM) have a two- to four-fold higher risk of coronary disease and stroke mortality.^{2–6} Although several new anti-diabetic drugs have been discovered in the past decades, the therapies have been limited by their adverse effects such as weight gain, hypoglycaemia, fluid retention and an unexpected cardiovascular risk.^{7–10} Therefore, new anti-diabetic drugs that could control hyperglycaemia and reduce the risk of cardiovascular events are of potential benefit to T2DM patients.

Incretin is a gut derived peptide hormone which enhances endogenous insulin secretion and reduces glucagon secretion, resulting in reduced blood glucose after food consumption.^{11–14} Its secretion is greatly influenced by ingestion.¹⁵ The incretin hormone has been classified into glucose-dependent insulintropic peptide (GIP) and glucagon-like peptide (GLP)-1. GIP is secreted by the enteroendocrine K cell of the proximal intestine, while GLP-1 is released from the enteroendocrine L cell of the distal intestine. GLP-1 is responsible for the majority of the incretin effect on pancreatic β -cell function. The secretion of GLP-1 is lower in patients with T2DM than normal, suggesting that this hormone contributes to the pathogenesis of the disease.¹⁶

The circulating GLP-1 has two isotypes: GLP-1 (7-36) and GLP-1 (7-37), in which GLP-1 (7-36) is responsible for 80% of active GLP-1.¹⁷ GLP-1 has insulintropic, insulino-mimetic and glucagonostatic effects.¹⁸ GLP-1 binds to GLP-1 receptors (GLP-1Rs) leading to regulatory actions which are an enhancement of β -cell function and proliferation, enhancement of glucose-dependent insulin secretion from β -cell, activation of insulin biosynthesis, suppression of elevated glucagon secretion, suppression of food intake and slowing of gastric emptying.^{11,19} However, the therapeutic drawback is that the circulating GLP-1 level decreases rapidly, i.e. less than two minutes, after secretion due to being degraded by dipeptidyl peptidase (DPP)-4 enzymes and renal clearance.^{20–23} DPP-4 enzyme degrades GLP-1 (7-36) by removing an N-terminal

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dipeptide, resulting in its metabolite, GLP-1 (9-36), which has 1000-fold lower affinity to GLP-1Rs.²¹ It has been shown that the inhibition of DPP-4 could enhance the level of intact GLP-1 and prolong its action time.²⁴ Thus, two classes of drugs have been recently used for incretin enhancement in T2DM, including GLP-1 analogues and DPP-4 inhibitors. While GLP-1 analogues (i.e. exenatide, liraglutide and albiglutide) increase the GLP-1 level to a supraphysiological level, DPP-4 inhibitors (i.e. vildagliptin, sitagliptin and saxagliptin) conserve and prolong intact GLP-1 availability within a physiological level.

Although the primary physiological function of GLP-1 is related to the control of plasma glucose, GLP-1Rs have been ubiquitously found in a variety of extra-pancreatic tissues including the central and peripheral nervous system, kidney, lung, gastrointestinal tract, blood vessel and heart both in rodents and humans.^{13,25} Since GLP-1Rs in the heart are similar to in the pancreas,²⁵ it has been suggested that drugs targeting incretin enhancement may potentially affect the heart.

DM has been shown to increase cardiovascular risk including increased incidence of myocardial infarction.² With the undesirable effects of several anti-diabetic drugs such as those in the thiazolidinedione group, i.e. rosiglitazone, that increased the cardiovascular morbidity and mortality,^{8,9} it is essential that any new anti-diabetic drug be investigated for both beneficial and harmful effects on the cardiovascular system. In this review, reports from basic and clinical studies regarding the effects of GLP-1 and DPP-4 inhibitor on cardiac function and infarct size in an ischaemic-reperfused heart are comprehensively summarised. Key results are critically discussed with emphasis placed on consistent findings. Inconsistent results are also highlighted to more fully explore what is known and what remains to be discovered.

Effects of GLP-1 on the infarct size

GLP-1 has been shown to have an infarct limiting effect in both in vitro and in vivo models of ischaemia-reperfusion (I/R) injury (Table 1). GLP-1 co-administered with DPP-4 inhibitor, valine pyrrolide (VP), could reduce the infarct size ranging from 39% to 58% in isolated Langendorff rat hearts whether given prior to ischaemia or during the reperfusion period.^{26–29} However, the administration of GLP-1²⁸ or DPP-4 inhibitor^{26–28} alone failed to reduce the infarct size. These studies suggested the synergistic effect of DPP-4 inhibition and exogenous GLP-1 on the infarct limiting effect in which DPP-4 inhibitor enhanced endogenous GLP-1 level and reduced exogenous GLP-1 degradation.

Another line of drug that has been used to enhance an incretin effect is a DPP-4 resistant GLP-1R agonist. Exendin-4, a hormone found in the saliva of the Gila monster, at 0.03 and 0.3 nM has been shown to reduce the infarct size, while 3.0 nM exendin-4 could not show this benefit,

suggesting a biphasic infarct limiting effect.³⁰ Human transferrin (Tf) was also demonstrated to prolong the GLP-1 action. Administration of GLP-1-Tf could effectively reduce the infarct size in rabbits, given either before or after coronary occlusion.³¹ Recently, albiglutide, another GLP-1 analogue, has been shown to increase cAMP in an ischaemic myocardium and improve cardiac metabolic efficiency by increasing glucose metabolism and reducing fat oxidation, which results in 26% reduction of infarct size.³²

In a pig model, exenatide has been shown to reduce the infarct size,³³ whereas the other two studies with a shorter period of I/R using recombinant GLP-1 (rGLP-1)³⁴ or liraglutide³⁵ could not demonstrate any improvement in infarct size (Table 2). This discrepancy could be due to a different duration and site of occlusion, therapeutic drugs and drug concentrations. This hypothesis is supported by a report by Noyan-Ashraf and colleagues, demonstrating that the optimal time and dose of liraglutide, GLP-1 analogue, played an important role in infarct size reduction, improved survival rate and contractile function in both normoglycaemic and diabetic mice.³⁶

Several mechanisms underlying the infarct limiting effect of GLP-1 have been proposed to be independent of weight loss.³⁶ First, GLP-1 has been shown to activate the cAMP-PKA pathway,^{26,32,36} the pro-survival kinase associated with reperfusion injury signalling kinase (RISK) pathway,³⁷ including the following: PI3K, Akt, MAPK, PPAR β/δ , Nrf-2, HO-1 and Akt-p70s6K-BAD pathways.^{18,26,27,33,36,38} Second, GLP-1 has been shown to reduce oxidative stress and increase antioxidants, leading to decreased apoptosis.^{26,31,33,36} Third, since proinflammatory cells such as neutrophils (PMNs) play an important role during the time of blood return to the heart,^{39,40} GLP-1 has been shown to attenuate the PMN activation and accumulation in the myocardium, thus reducing injuries caused by reperfusion.⁴¹ Recent studies demonstrated that the infarct limiting effects were diminished with the administration of either GLP-1 metabolite, i.e. GLP-1 (9-36),^{29,30} or when giving GLP-1 with the GLP-1R antagonist, namely GLP-1 (9-39).^{26,29,30,36} Moreover, the administration of liraglutide failed to activate cardioprotective kinase in the GLP-1R depleted mice. Taken together, the effect of GLP-1 on the infarct size reduction is proposed to be a GLP-1R dependent mechanism.

Effects of GLP-1 on left ventricular function

Besides its infarct limiting effect, GLP-1 has been shown to improve left ventricular (LV) recovery from ischaemic myocardial stunning after reperfusion (Table 3). The administration of GLP-1 as a post-conditioning agent, which demonstrated a higher plasma GLP-1 level, could improve LV performance by decreasing the wall motion abnormality, and increasing the ejection fraction and LV

Table 1. Summary of the beneficial effects of GLP-1 on the infarct size.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Rabbit	I/R LCX ligation 30-min ischaemia 180-min reperfusion	GLP-1-Tf 10 mg/kg Pretreatment 12 h, SC At reperfusion, IV	1. GLP-1-Tf decreased infarct size in both pretreatment and post-ischaemia by 23% and 25%, respectively. 2. GLP-1-Tf decreased apoptotic index.	GLP-1-Tf decreased infarct size by reducing myocardial apoptosis whether given pretreatment or post-ischaemia.	Matsubara et al. ³¹
Normal mice	MI LAD ligation 28-day ischaemia	Liraglutide Pretreatment 75 or 200 µg/kg, IP, twice daily for 7 days 75 µg/kg, IP, twice daily for 7 days	1. Liraglutide at dose 75 and 200 µg/kg increased survival rate in both normal and diabetic mice. 2. Liraglutide at dose 200 µg/kg reduced infarct size by 27% and reduced HW/BW ratio. 3. Liraglutide activated cardioprotective signalling pathway, e.g. pAkt, pGSK3β, PPARβ/δ, nNrf-2, and HO-1 before MI through GLP-1R. 4. Liraglutide increased pAkt, pGSK3β, and decreased caspase 3, ANP and MMP-9 after MI. 5. Liraglutide increased cAMP level and decreased caspase 3 in cardiomyocytes. 6. Concomitant administration of GLP-1R antagonist abolished effects of liraglutide on cardiomyocytes.	Liraglutide decreased infarct size. The mechanism could be due to activation of cardioprotective signalling pathway, e.g. pAkt, pGSK3β, and reduce caspase-3 activation through GLP-1R dependent pathway.	Noyan-Ashraf et al. ³⁶
High fat diet with streptozotocin-induced diabetic mice					
Pig	I/R LCX ligation 75-min ischaemia 3-day reperfusion	Exenatide 10 µg, SC and IV 5 min before reperfusion 10 µg, SC, twice daily during reperfusion	1. Exenatide reduced infarct size by 39%. 2. Exenatide increased Akt and Bcl-2 phosphorylation. 3. Exenatide decreased caspase 3. 4. Exenatide decreased nuclear oxidative stress. 5. Exenatide increased antioxidant enzyme.	Exenatide decreased infarct size by activating pro-survival kinase, Akt and Bcl-2 phosphorylation as well as decreasing pro-apoptotic caspase 3. Exenatide also increased anti-oxidant, resulting in decreased nuclear oxidative stress.	Timmers et al. ³³
Rat	I/R LAD ligation 30-min ischaemia 120-min reperfusion	Pretreated with VP 20 mg/kg, SC, 30 min before experiment + infusion with GLP-1 4.8 pmol/ kg per min throughout experiment	VP + GLP-1 reduced infarct size by 58%.	DPP-4 inhibitor combined with GLP-1 could reduce infarct size.	Bose et al. ²⁶
Rat	I/R LAD ligation 30-min ischaemia 120-min reperfusion	rGLP-1 30 pM/kg per min, infusion beginning at 28 min after occlusion until the end of reperfusion	1. rGLP-1 reduced infarct size by 79%. 2. rGLP-1 attenuated PMN activation. 3. rGLP-1 decreased PMN accumulation in ischaemic myocardium. 4. In vitro, rGLP-1 attenuated PMN activation; this effect was abolished by GLP-1R antagonist.	GLP-1 mitigated I/R injury by reduced infarct size and inhibited PMN-mediated reperfusion injury through GLP-1R dependent pathway.	Dokken et al. ⁴¹
Rat	I/R LAD ligation 30-min ischaemia 24-h reperfusion	Albiglutide 1, 3, 10 mg/kg per day, SC, once daily, for 3 days	1. Albiglutide at doses 3 and 10 mg/kg reduced infarct size by 26%. 2. Albiglutide increased cAMP level in ischaemic myocardium. 3. Albiglutide increased myocardial glucose uptake. 4. Albiglutide increased carbohydrate oxidation and reduced fat oxidation.	Infarct limiting effect of albiglutide was associated with improvement of cardiac metabolic efficiency.	Bao et al. ³²

(Continued)

Table 1. (continued)

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Isolated rat heart	I/R Left main coronary artery ligation (regional ischaemia) 35-min ischaemia 120-min reperfusion	VP 20 mg/L + GLP-1 0.3 nM in perfusion buffer, throughout experiment	1. VP + GLP-1 reduced infarct size by 55%. 2. Using GLP-1 inhibitor, cAMP inhibitor, PI3K inhibitor and MAPK inhibitor abolished infarct limiting effect. 3. VP + GLP-1 treated group increased phosphorylated BAD.	Infarct limiting effect of GLP-1 could be due to activation of pro-survival kinase including cAMP, PI3K, MAPK and promote BAD phosphorylation.	Bose et al. ²⁶
Isolated rat heart	I/R Left main coronary artery ligation (regional ischaemia) 35-min ischaemia 120-min reperfusion	VP 20 mg/L + GLP-1 0.3 nM in perfusion buffer, throughout experiment	1. GLP-1 + VP reduced infarct size by 50%. 2. Rapamycin, p70s6K inhibitor, abolished infarct limiting effect. 3. GLP-1 + VP did not increase phosphorylated p70s6K.	GLP-1 with DPP-4 inhibitor could reduce infarct size by activation of Akt-p70s6K-BAD pathway.	Bose et al. ²⁸
Isolated rat heart	I/R Left main coronary artery ligation (regional ischaemia) 35-min ischaemia 120-min reperfusion	Pretreatment VP 20 mg/L + GLP-1 0.3 nM, 15 min before I/R VP 20 mg/L + GLP-1 0.3 nM, during 15 min of reperfusion	Given VP + GLP-1 before ischaemia or at reperfusion reduced infarct size by 49% and 48%, respectively.	GLP-1 with DPP-4 inhibitor could use as preconditioning or post-conditioning agent to reduce infarct size.	Bose et al. ²⁷
Isolated rat heart	I/R 35-min global no flow ischaemia 120-min reperfusion	VP 40 µM + GLP-1 0.3 nM, during 15 min of reperfusion	1. VP + GLP-1 reduced infarct size by 39%. 2. GLP-1 with GLP-1R antagonist could not reduce infarct size.	Infarct limiting effect of GLP-1 was mediated by GLP-1R.	Ossum et al. ²⁹
Isolated rat heart	I/R 45-min global no flow ischaemia 120-min reperfusion	Exendin-4 0.03, 0.3, 3.0 nM, during 15 min at reperfusion	1. Exendin-4 at 0.03 and 0.3 nM reduced infarct size by 53% and 56%, respectively. 2. Exendin-4 with GLP-1R antagonist cannot reduce infarct size.	Infarct limiting effect of exendin-4 was mediated by GLP-1R.	Sonne et al. ³⁰

I/R: ischaemic-reperfusion, LCX: left circumflex coronary artery; GLP-1-Tf: glucagon-like peptide-1-transferrin, SC: subcutaneous route, IV: intravenous route, MI: myocardial infarction, LAD: left anterior descending coronary artery, IP: intraperitoneal route, HW/BW: heart weight/body weight; GLP-1R: GLP-1 receptor; pAkt: phosphorylated Akt; pGSK: phosphorylated glycogen synthase kinase; PPAR: peroxisome proliferated-activated receptors; HO-1: heme-oxygenase-1; ANP: atrial natriuretic peptide; MMP: matrix metalloproteinase; VP: valine pyrrolide; rGLP-1: recombinant GLP-1; PMN: polymorphonuclear neutrophils; DPP-4: dipeptidyl peptidase-4; PI3K: phosphoinositide 3-kinase; MAPK: mitogen-activated protein kinase; BAD: Bcl-2 associated death promoter.

Table 2. Summary of the neutral effects of GLP-1 on the infarct size.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Pig	I/R LAD ligation 60-min ischaemia 120-min reperfusion	rGLP-1, 3 pmol/kg per min, 120 min before experiment + rGLP-1, 3 pmol/kg per min, IV perfusion during I/R rGLP-1, 3 pmol/kg per min, IV perfusion during I/R	1. rGLP-1 did not reduce infarct size. 2. rGLP-1 increased plasma insulin level. 3. rGLP-1 lowered blood glucose level. 4. rGLP-1 decreased interstitial levels of pyruvate and lactate.	rGLP-1 did not reduce infarct size, but altered myocardial glucose utilisation, which was associated with insulin level.	Kavianipour et al. ³⁴
Pig	I/R Balloon LAD occlusion 40-min ischaemia 150-min reperfusion	Liraglutide Pretreatment 10 µg/kg, SC, once daily for 3 days	1. Liraglutide did not reduce infarct size. 2. VF incidence and time to VF was not different between liraglutide and vehicle.	Liraglutide did not reduce infarct size.	Kristensen et al. ³⁵
Isolated rat heart	I/R Left main coronary artery ligation (regional ischaemia) 35-min ischaemia 120-min reperfusion	GLP-1 0.3 nM in perfusion buffer, throughout experiment	GLP-1 alone did not reduce infarct size.	GLP-1 alone could not reduce infarct size. DPP-4 inhibitor was important for exogenous GLP-1 function to reduce infarct size.	Bose et al. ²⁸
Isolated rat heart	I/R 35-min global no flow ischaemia 120-min reperfusion	VP 40 µM + GLP-1 (9-36) 0.3 nM, during 15 min of reperfusion	GLP-1 (9-36) + VP did not reduce infarct size.	GLP-1 metabolite with DPP-4 inhibitor did not reduce infarct size.	Ossum et al. ²⁹
Isolated rat heart	I/R 45-min global no flow ischaemia 120-min reperfusion	GLP-1 (9-36) 0.03, 0.3, 3.0 nM, during 15 min of reperfusion	GLP-1 (9-36) did not reduce infarct size.	GLP-1 metabolite did not reduce infarct size.	Sonne et al. ³⁰

I/R: ischaemia-reperfusion; LAD: left anterior descending coronary artery; rGLP-1: recombinant GLP-1; IV: intravenous route; SC: subcutaneous route; VP: valine pyrrolide; VF: ventricular fibrillation; DPP-4: dipeptidyl peptidase-4.

developed pressure,^{29,31} compared with giving GLP-1 as a pre-conditioning agent, which has a lower plasma GLP-1 level.³¹ Nevertheless, it is possible that the time for which GLP-1 was given as a preconditioning treatment in those studies was too short, thus allowing insufficient time for its action. This is supported by a report by Noyan-Ashraf and colleagues which demonstrated that the GLP-1 analogue required the optimal pre-treatment time and dose for the activation of the cardiac gene and protein to promote LV enhancement.³⁶ Other possible factors including routes and times of drug administration during I/R could also play an important role in this finding. Interestingly, like the infarct limiting effect, exendin-4 also improved the mechanical function in a biphasic manner.³⁰ In a clinically relevant swine model, exenatide improved regional and global systolic function.³³ In coronary artery disease patients with good LV function, a continuous infusion of rGLP-1 for 30 minutes before dobutamine stress echocardiography demonstrated an improved ejection fraction and LV regional wall function as well as mitigated post-ischaemic stunning,

predominantly in the ischaemic wall segment.⁴² The beneficial effect of a 72-hour GLP-1 infusion on the post-infarction recovery, including an increased ejection fraction and improvement of both global and regional wall motion was also observed in acute myocardial infarction patients with LV ejection fraction of less than 40% after successful primary percutaneous coronary intervention.⁴³ Long-term GLP-1 infusion also improved myocardial stunning, which was characterised by enhanced LV wall motion and relaxation in I/R dogs.⁴⁴

Surprisingly, the benefit of exenatide and GLP-1 on LV function was also observed in the presence of GLP-1R antagonist and in GLP-1R deleted mice, suggesting that the improved LV function of GLP-1 may be mediated independent of GLP-1R.^{30,38} However, a recent study demonstrated that GLP-1 co-administered with sitagliptin, DPP-4 inhibitor, could not improve contractile functional recovery in GLP-1R deleted mice, whereas this treatment provided the benefit in wild-type mice.³⁸ Taken together, unlike the infarct limiting effect, the LV function amelioration of

Table 3. Summary of the effects of GLP-1 on left ventricular function.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Rabbit	I/R LCX ligation 30-min ischaemia 180-min reperfusion	GLP-1-Tf 10 mg/kg Pretreatment 12 h, SC At reperfusion, IV	1. GLP-1 level in post-ischaemic treatment significantly higher than pre-ischaemic treatment. 2. Given GLP-1-Tf at reperfusion decreased wall motion abnormality. 3. Given GLP-1-Tf at reperfusion improved ejection fraction. 4. Pretreatment with GLP-1-Tf failed to improve ejection fraction and wall motion abnormality.	GLP-1-Tf at higher concentration, post-ischaemic treatment, improved LV function by limiting myocardial stunning.	Matsubara et al. ³¹
Dog	I/R Ballooned LCX occlusion 10-min ischaemia 1-day reperfusion	GLP-1 1.5 pmol/kg per min, IV perfusion, during reperfusion	1. GLP-1 caused an earlier and completely recovered regional wall motion. 2. GLP-1 restored diastolic relaxation to normal. 3. GLP-1 did not improve fractional shortening. 4. GLP-1 did not improve LV contractility.	GLP-1 enhanced functional recovery from ischaemic myocardial stunning independently of changing systemic haemodynamic or global systolic function.	Nikolaïdis et al. ⁴⁴
Pig	I/R LCX ligation 75-min ischaemia 3-day reperfusion	Exenatide 10 µg, SC and IV, 5 min before reperfusion + 10 µg, SC, twice daily during reperfusion	1. Exenatide increased systolic wall thickening. 2. Exenatide increased fractional area shortening. 3. Exenatide increased ejection fraction. 4. Exenatide reduced myocardial stiffness.	Exenatide improved systolic function	Timmers et al. ³³
Rat	I/R LAD ligation 30-min ischaemia 24-h reperfusion	Albiglutide 1, 3, 10 mg/kg per day, SC, once daily, for 3 days	1. Albiglutide increased dP/dt_{max} 2. Albiglutide increased myocardial glucose uptake. 3. Albiglutide increased carbohydrate oxidation and reduced fat oxidation. 4. Albiglutide increased ejection fraction and end systolic volume.	Albiglutide increased cardiac function by improving cardiac metabolic efficiency.	Bao et al. ³²
Isolated mice heart	I/R 30-min global ischaemia 40-min reperfusion	Liraglutide 0.3, 3, 30 nM, in perfusion buffer, 20 min before ischaemia 30 nM, in perfusion buffer, at reperfusion for 20 min Pretreatment with liraglutide 200 µg/kg, IP, b.i.d., 1- or 7-day VP 40 µM + GLP-1 0.3 nM, during 15 min of reperfusion	1. Acute treatment with liraglutide before or after ischaemia failed to improve LVDP. 2. Pretreatment with liraglutide for 1- or 7-day improved LVDP.	LV improvement of liraglutide required optimal dose and pretreatment time.	Noyan-Ashraf et al. ³⁶
Isolated rat heart	I/R 35-min global no flow ischaemia 120-min reperfusion	GLP-1 slightly increased LVDP, rate pressure product and maximal rate of LV systolic pressure development.		GLP-1 + VP tended to improve LV function.	Ossum et al. ²⁹
Isolated rat heart	I/R 35 min global no flow ischaemia 120-min reperfusion	VP 40 µM + GLP-1 (9-36) 0.3 nM, during 15 min of reperfusion	GLP-1 (9-36) decreased LVDP, rate pressure product and maximal rate of LV systolic pressure development.	GLP-1 metabolite acted as a strong negative inotrope in post-ischaemic hearts.	Ossum et al. ²⁹

Table 3. (continued)

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Isolated rat heart	I/R 45-min global no flow ischaemia 120-min reperfusion	Exendin-4 0.03, 0.3, 3.0 nM, for 15 min at reperfusion GLP-1 (9-36) 0.03, 0.3, 3.0 nM, for 15 min at reperfusion	1. Exendin-4 at 0.03 and 0.3 nM improved LVDP and rate pressure product during the last 60 min of reperfusion. 2. GLP-1 (9-36) at 0.03 and 0.3 nM improved LVDP and rate pressure product during the last 60 min of reperfusion. 3. GLP-1 (9-39) partly affected on beneficial effect of LV performance of exendin-4 and GLP-1 (9-36). 1. GLP-1 enhanced LV end diastolic pressure. 2. GLP-1 increased LVDP. 3. GLP-1 increased myocardial glucose uptake. 4. GLP-1 increased GLUT-1 and GLUT-4 translocation.	GLP-1 and GLP-1 (9-36) increased ventricular performance. Action of GLP-1 and GLP-1 (9-36) was mediated independently with GLP-IR. GLP-1 improved post-ischaemic contractile dysfunction by enhancing myocardial glucose uptake.	Sonne et al. ³⁰
Isolated rat heart	I/R 30-min global low flow ischaemia 30-min reperfusion	GLP-1 500 pmol/L in perfusion buffer, pretreatment 1 min before ischaemia and throughout reperfusion	1. GLP-1 enhanced LV end diastolic pressure. 2. GLP-1 increased LVDP. 3. GLP-1 increased myocardial glucose uptake. 4. GLP-1 increased GLUT-1 and GLUT-4 translocation.	GLP-1 improved post-ischaemic contractile dysfunction by enhancing myocardial glucose uptake.	Zhao et al. ¹⁸
Isolated rat heart and isolated mitochondria	I/R 20-min global low flow ischaemia 30-min reperfusion	Exendin-4 1 nM/kg, SC, 6 day, once daily, after birth	Juvenile age (4–6 weeks) 1. HW, HW/BW, and HW/tibia length were similar between groups. 2. Exendin enhanced per cent recovery of LVDP. 3. Rates of state 3 and 4 respiration were comparable in control and exendin treated group. 4. Exendin did not reduce oxidative stress. 5. Exendin did not increase antioxidant, MnSOD. Adult age (9 months) 1. HW, HW/BW, and HW/tibia length were similar between groups. 2. Exendin improved recovery of LVDP. 3. Rates of state 3 respiration were lower in exendin-treated animal, while state 4 respiration was comparable. 4. Mitochondria from exendin-treated group took up less calcium.	Short course treatment with exendin-4 improved LV function in adult after I/R insult by alteration of mitochondrial phenotype which reduced rates of oxidative phosphorylation and mitochondrial calcium uptake.	Brown et al. ⁴⁷
Isolated mice heart of wild type and GLP-IR ^{-/-}	I/R 30-min global no flow ischaemia 40-min reperfusion	GLP-1 Exendin-4 0.3, 3, 5 nM in perfusion buffer Pretreatment for 20 min before ischaemia Post-treatment for 20 min at reperfusion GLP-1 (9-36) 0.3, 3, 6 nM in perfusion buffer Pretreatment for 20 min before ischaemia Post-treatment for 20 min at reperfusion	1. Pretreatment with GLP-1 (0.3nM) and Exendin-4 (5 nM) increased LVDP in wild-type mice. 2. Pretreatment with GLP-1 (0.3 nM) increased LVDP in GLP-IR ^{-/-} mice. 3. Pretreatment with exendin-4 failed to increase LVDP in GLP-IR ^{-/-} mice. 4. Pretreatment with GLP-1 (9-36) worsened LV function in wild type mice. 5. Post treatment with GLP-1 (9-36) improved LVDP in both wild-type and GLP-IR ^{-/-} mice. 6. With sitagliptin, effect of GLP-1 on LVDP was diminished in GLP-IR ^{-/-} but improved in wild-type mice.	Effects of GLP-1 on LV performance were mediated via both GLP-IR dependent and independent pathways.	Ban et al. ³⁸

(Continued)

Table 3. (continued)

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Acute MI patients with EF<40%	After successful primary angioplasty	rGLP-1 1.5 pmol/kg per min 72 h after angioplasty	1. rGLP-1 improved LV ejection fraction. 2. rGLP-1 decreased global and regional wall motion score index.	Infusion of GLP-1 improved regional and global LV function in patient after successful primary angioplasty.	Nikolaïdis et al. ⁴³
Patients	Coronary artery disease with normal resting LV function	GLP-1 1.2 pmol/kg per min, intravenous infusion starting at 30 min before dobutamine stress echocardiography	1. GLP-1 level was increased after GLP-1 administration. 2. GLP-1 lowered plasma glucose level. 3. Insulin level was increased in GLP-1 treated group during dobutamine stress. 4. Free fatty acid level was lowered in GLP-1 treated group. 5. GLP-1 improved ejection fraction and regional wall function during peak dobutamine stress and 30 min of recovery. 6. GLP-1 had a greater beneficial effect on ischaemic than on non-ischaemic segments.	GLP-1 improved both global and regional LV performance in response to stress and also reduced post-ischaemic stunning.	Read et al. ⁴²

I/R: ischaemia-reperfusion; LCX: left circumflex coronary artery; GLP-1-Tf: glucagon-like peptide-1-transferrin; SC: subcutaneous route; IV: intravenous route; LV: left ventricle; LAD: left anterior descending coronary artery; IP: intraperitoneal route; LVDP: left ventricular developed pressure; VP: valine pyrolysate; GLP-1R: GLP-1 receptor; GLUT: glucose transporter; MnSOD: manganese superoxide dismutase; HW: heart weight; BW: body weight; EF: ejection fraction; rGLP-1: recombinant GLP-1.

GLP-1 was mediated by both GLP-1R dependent and independent pathways. Although the administration of GLP-1 (9-36) could not reduce the infarct size,^{29,30} it exerts a functional recovery benefit which might mediate through the GLP-1 (9-36)-cGMP-NO pathway, independent of GLP-1R activation.^{30,38} However, the inotropic effect of GLP-1 (9-36) is still questionable.²⁹

Enhanced myocardial glucose uptake by up-regulation of glucose transporter (GLUT)-1 and GLUT-4 may be one of the underlying mechanisms to explain the beneficial effect of GLP-1 on LV functional improvement.^{18,32} In a normal physiological condition, the heart mainly uses fatty acid as a fuel in maintaining its function. Reduced availability of oxygen during low-flow ischaemia reduces the heart's ability to produce energy from fatty acid oxidation and carbohydrates. The enhancement of glycolysis through diverse mechanisms or pharmacologic interventions can delay and prevent ischaemic damage and improve the recovery of the contractile function.^{45,46} GLP-1 has been shown to increase myocardial glucose uptake, which ameliorates the post-ischaemia myocardial dysfunction.^{18,32} This action of GLP-1 might be used as a therapeutic target for high risk cardiovascular disease patients, especially in insulin resistant T2DM. The long-term effect of GLP-1 analogue has been demonstrated in neonatal rats receiving exenatide once daily for six days.⁴⁷ This persistent beneficial effect is mediated by an altered mitochondrial phenotype which decreased the cardiac mitochondria calcium uptake and reduced oxidative phosphorylation, resulting in improved functional recovery after ischaemia reperfusion injury. Surprisingly, the cardioprotective effect of GLP-1 and GLP-1 analogue was not associated with any improvement in haemodynamic parameters (Table 4).^{26,31-35,44,47}

Unlike GLP-1, information regarding the effects of DPP-4 inhibitor on the I/R heart is scarce and controversial.⁴⁸⁻⁵⁰ DPP-4 inhibitors inhibit the enzyme activity of DPP-4, resulting in decreasing degradation rate, thereby maintaining higher GLP-1 levels.^{24,51,52} Although growing evidence demonstrated the infarct limiting effect of DPP-4 inhibitors (Table 5), many studies suggested that the physiological level of GLP-1 may not be sufficient to reduce the infarct size (Table 6). Besides the infarct limiting effect, the beneficial effect of DPP-4 inhibitors on LV function after I/R injury has also been documented (Table 7).

Effects of DPP-4 inhibitor on the infarct size

As DM increases cardiovascular risk including myocardial infarction, pretreatment with vildagliptin, DPP-4 inhibitor, has been shown to attenuate the infarct size in insulin resistant rats.⁵³ This cardioprotective effect was also observed in normoglycaemic rats with pretreatment of sitagliptin.⁴⁸ A longer period of pre-treatment with sitagliptin resulted in smaller infarct size, suggesting that the plasma drug level

Table 4. Summary of the effects of GLP-1 on haemodynamic parameters.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Rabbit	I/R LCX ligation 30-min ischaemia 180-min reperfusion	GLP-1-Tf 10 mg/kg Pretreatment 12 h, SC At reperfusion, IV	Treatment with GLP-1-Tf did not affect heart rate and mean arterial blood pressure between groups.	GLP-1-Tf did not alter haemodynamic parameters.	Matsubara et al. ³¹
Pig	I/R LAD ligation 60-min ischaemia 120-min reperfusion	rGLP-1, 3 pmol/kg per min, 120 min before experiment + rGLP-1, 3 pmol/kg per min, IV perfusion during I/R rGLP-1, 3 pmol/kg per min, IV perfusion during I/R	rGLP-1 did not affect heart rate, cardiac output, mean arterial blood pressure, pulmonary artery wedge pressure and central venous pressure.	rGLP-1 did not alter haemodynamic parameters.	Kavianipour et al. ³⁴
Pig	I/R Balloon LAD occlusion 40-min ischaemia 150-min reperfusion	Liraglutide Pretreatment 10 µg/kg, SC, once daily for 3 days	Liraglutide increased heart rate but had no effect on blood pressure and cardiac output.	Liraglutide slightly increased heart rate but had no effect on other haemodynamic parameters.	Kristensen et al. ³⁵
Pig	I/R LCX ligation 75-min ischaemia 3-day reperfusion	Exenatide 10 µg, SC and IV 5 min before reperfusion 10 µg, SC, twice daily during reperfusion	Exenatide did not affect heart rate, mean arterial blood pressure and cardiac output.	Exenatide did not alter haemodynamic parameters.	Timmers et al. ³³
Rat	I/R LAD ligation 30-min ischaemia 120-min reperfusion	Pretreated with VP 20 mg/kg, SC, 30 min before experiment + infusion with GLP-1 4.8 pmol/kg per min throughout experiment	VP + GLP-1 did not affect heart rate and mean arterial blood pressure.	GLP-1 with DPP-4 did not alter haemodynamic parameters.	Bose et al. ²⁶
Dog	I/R Balloon LCX occlusion 10-min ischaemia 1-day reperfusion	GLP-1 1.5 pmol/kg per min, IV perfusion, during reperfusion	GLP-1 did not affect heart rate, coronary blood flow, mean arterial blood pressure and LV end diastolic pressure.	Long term GLP-1 infusion did not alter haemodynamic parameters.	Nikolaidis et al. ⁴⁴
Isolated mice heart of wild type and GLP-IR ^{-/-}	I/R 30-min global no flow ischaemia 40-min reperfusion	GLP-1 Exendin-4 0.3, 3, 5 nM in perfusion buffer Pretreatment for 20 min before ischaemia Post-treatment for 20 min at reperfusion GLP-1 (9-36) 0.3, 3, 6 nM in perfusion buffer Pretreatment for 20 min before ischaemia Post-treatment for 20 min at reperfusion	1. Pretreatment with GLP-1 improved pre- and post-ischaemic coronary flow. 2. Pretreatment with GLP-1 (9-36) improved pre-ischaemic coronary flow. 3. Post treatment with GLP-1 (9-36) improved post-ischaemic coronary flow. 4. GLP-1 and GLP-1 (9-36) increased cGMP level in wild-type and GLP-IR ^{-/-} mice. 5. Exendin-4 did not produce cGMP release. 6. Functional recovery after I/R was reduced in GLP-1 treated with L-NAME, NOS inhibitor.	Vascular effects of GLP-1 were mediated via GLP-1 (9-36) and GLP-1R independent pathway, through NOS-dependent cGMP formation.	Ban et al. ³⁸
Patients	Coronary artery disease with normal resting LV function	GLP-1 1.2 pmol/kg per min, IV infusion starting at 30 min before dobutamine stress echocardiography	GLP-1 did not affect heart rate, systolic and diastolic blood pressure, and rate-pressure product.	GLP-1 did not alter haemodynamic parameters.	Read et al. ⁴²

I/R: ischaemia-reperfusion; LCX: left circumflex coronary artery; GLP-1-Tf: glucagon-like peptide-1-transferrin; SC: subcutaneous route; LAD: left anterior descending coronary artery; IV: intravenous route; rGLP-1: recombinant GLP-1; VP: valine pyrrrolide; DPP-4: dipeptidyl peptidase-4; LV: left ventricle; GLP-1R: GLP-1 receptor; L-NAME: NG-monomethyl-L-arginine; NOS: nitric oxide synthase.

Table 5. Summary of the beneficial effects of DPP-4 inhibitor on infarct size.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Diet-induced obesity, insulin resistant rat	I/R LAD ligation 35-min ischaemia 30-min reperfusion	Vildagliptin Pretreatment 10 mg/kg per day, PO, for 4 weeks	1. Vildagliptin reduced infarct size in diet induced obesity rat by 38%. 2. Vildagliptin increased plasma GLP-I level to normal in diet-induced obesity rat. 3. Vildagliptin increased beta cell/alpha cell ratio to normal in diet-induced obesity rat. 4. Vildagliptin increased phosphorylation of Akt and ERK at serine 42.	Vildagliptin reduced infarct size in insulin resistance rat by increased phosphorylation of Akt and ERK at serine 42.	Huisamen et al. ⁵³
Rat	I/R LAD ligation 30-min ischaemia 4-hour reperfusion	Sitagliptin Pretreatment 300 mg/kg per day, PO, for 3 or 14 days	1. Pretreatment with sitagliptin for 3 and 14 days reduced infarct size by 47% and 64%, respectively. 2. Using PKA inhibitor completely abolished infarct limiting effect of sitagliptin. 3. Sitagliptin increased cAMP level.	Protective effect of sitagliptin was via cAMP-dependent PKA activation.	Ye et al. ⁴⁸

I/R: ischaemia-reperfusion; LAD: left anterior descending coronary artery; PO: per oral; GLP-I: glucagon-like peptide-I; ERK: extracellular regulated kinase; PKA: protein kinase A.

might influence its cardioprotective effect. The infarct limiting mechanism of DPP-4 inhibitor in these studies was proposed to be due to the activation of Akt and cAMP-PKA pro-survival kinase in RISK pathway as observed in GLP-1 administration.^{48,53} However, some studies demonstrated that prolonged availability of intact GLP-1 caused by a DPP-4 inhibitor failed to demonstrate the infarct limiting effect.^{26–28,54} Moreover, inhibition or reduction of DPP-4 activity using DPP-4 deleted mice or pretreatment with sitagliptin before ischaemia in high fat diet-induced diabetic mice could not reduce the infarct size, but improved the survival rate by activation of cardioprotection kinase such as GSK3 β , ANP and Akt.⁴⁹ These discrepant results suggest that the different animal models, the duration of ischaemia in the heart models and the minimal dose for a specific time may play a pivotal role in the reduction of the infarct size. Tables 5 and 6 summarise significant effects of DPP-4 inhibitors on the infarct size.

Effects of DPP-4 inhibitor on left ventricular function

DPP-4 inhibitors also exert a cardioprotective effect by the improvement of post-ischaemia myocardial stunning. Table 7 summarises the effects of DPP-4 inhibitors on LV function. In DPP-4 deleted mice and mice pretreated with sitagliptin for 12 hours prior to aortic occlusion, improved LV developed pressure after I/R injury was observed.⁴⁹ However, acute treatment with sitagliptin for 20 minutes prior to I/R insult failed to improve ventricular performance.⁴⁹ The discrepancy in these findings could be due to the differences in the time of drug administration as well as study models (in vivo versus ex vivo), and might

suggest that the benefit of DPP-4 inhibitor required an optimal time and an intact cardiovascular system process. However, a recent report in rats with ischaemic heart failure demonstrated that early or late treatment with vildagliptin had no beneficial effect on ventricular performance.⁵⁴ In a clinical study, single dose treatment with sitagliptin improved regional and global wall function, which improved post-ischaemic stunning in patients with coronary artery disease awaiting revascularisation.⁵⁰ These improvements are independent of insulin⁵⁰ or haemodynamic parameter alterations.^{49,50,53} Table 8 summarises the effect of DPP-4 inhibitors on haemodynamic parameters.

Conclusion

Growing evidence suggests that incretin exerts a cardioprotective effect during I/R injury. The infarct limiting effect is mediated through GLP-1R, while LV function improvement is mediated through both GLP-1R dependent and independent (via GLP-1 (9-39)) pathways. The underlying mechanisms were due to the activation of pro-survival kinase, increased antioxidant and myocardium glucose utilisation, reduction of oxidative stress and pro-apoptotic kinase, and attenuation proinflammatory cell activation and accumulation in the myocardium. Although many mechanisms have been proposed, the exact mechanisms have never been fully elucidated. Therefore, further investigations are required to determine the cardioprotective mechanisms of incretin. Ultimately, large clinical trials are the essential step to validate those effects reported in animal studies and to warrant their clinical use in the I/R condition.

Table 6. Summary of the neutral effects of DPP-4 inhibitor on infarct size.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Normoglycaemic rat	I/R LAD ligation 35-min ischaemia 30-min reperfusion	Vildagliptin Pretreatment 10 mg/kg per day, PO, for 4 weeks	Vildagliptin did not reduce infarct size in normal rat.	Vildagliptin did not reduce infarct size in normal rat.	Huisamen et al. ⁵³
DPP-4 ^{-/-} mice	MI LAD ligation for 4 weeks	DPP-4 ^{-/-} mice	1. DPP-4 ^{-/-} mice had improved survival rate. 2. DPP-4 ^{-/-} mice did not have reduced infarct size. 3. pGSK3 β , pANP, pAkt were increased in DPP-4 ^{-/-} mice.	DPP-4 ^{-/-} did not reduce infarct size, but improved survival rate after MI in normoglycaemia. The mechanism could be due to activation of pGSK3 β , pANP and pAkt.	Sauve et al. ⁴⁹
High fat diet with streptozotocin-induced diabetic mice	MI LAD ligation for 4 weeks	Sitagliptin Pretreatment 250 mg/kg per day, PO, 12 weeks	1. Sitagliptin improved survival rate. 2. Sitagliptin did not reduce infarct size. 3. Sitagliptin increased GLP-I (7-36) amide level. 4. Sitagliptin lowered HbA1c level. 5. HO-1, ANP, pAkt increased in sitagliptin group. 6. pGSK3 β tended to increase in sitagliptin-treated group.	Sitagliptin did not reduce infarct size, but improved survival rate after MI in diabetic mice. The mechanism could be due to activation of HO-1, ANP, pAkt and improved blood glucose.	Sauve et al. ⁴⁹
Rat	I/R LAD ligation 30-min ischaemia 120-min reperfusion	VP 20 mg/kg, SC, 30 min before ischaemia	VP did not reduce infarct size.	Intact GLP-I alone did not reduce infarct size.	Bose et al. ²⁶
Rat	Ischaemic heart failure 12-week LAD occlusion	Vildagliptin 15 mg/kg per day, PO, 2 days prior to LAD occlusion 15 mg/kg per day, PO, beginning after LAD occlusion for 3 weeks	1. Vildagliptin increased active plasma GLP-I level and decreased DPP-4 activity. 2. Vildagliptin did not mitigate the MI-induced decrease in capillary density. 3. Vildagliptin could not reduce cardiomyocyte size.	Early or late vildagliptin treatments had no infarct limiting effect on ischaemic cardiac remodelling.	Yin et al. ⁵⁴
Isolated rat heart	I/R Left main coronary artery ligation (regional ischaemia) 35-min ischaemia 120-min reperfusion	VP Pretreatment 20 mg/L VP 20 mg/L at reperfusion	VP did not reduce infarct size whether given as preconditioning or at reperfusion.	Intact GLP-I alone did not reduce infarct size.	Bose et al. ²⁷
Isolated rat heart	I/R Left main coronary artery ligation (regional ischaemia) 35-min ischaemia 120-min reperfusion	VP 20 mg/L, in perfusion buffer, throughout experiment	VP alone did not reduce infarct size.	Intact GLP-I alone did not reduce infarct size.	Bose et al. ²⁸

I/R: ischaemia-reperfusion; LAD: left anterior descending coronary artery; PO: per oral; MI: myocardial infarction; pGSK: phosphorylated glycogen synthase kinase; pANP: phosphorylated atrial natriuretic peptide; pAkt: phosphorylated Akt; GLP-I: glucagon-like peptide-I; HbA1c: haemoglobin A1c; HO-1: heme oxygenase-I; VP: valine pyrrolide; SC: subcutaneous route; DPP-4: dipeptidyl peptidase-4.

Table 7. Summary of the effects of DPP-4 inhibitor on left ventricular function.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Rat	Ischaemic heart failure 12-week LAD occlusion	Vildagliptin 15 mg/kg per day, PO for 2 days prior to LAD occlusion 15 mg/kg per day, PO for 3 weeks after LAD occlusion	1. Vildagliptin did not improve ejection fraction and fractional shortening. 2. Progressive LV dilatation and wall thinning after MI was attenuated in vildagliptin-treated groups. 3. Vildagliptin did not improve dP/dt_{max} and dP/dt_{min} .	Early and late treatment with vildagliptin had no beneficial effect on LV function.	Yin et al. ⁵⁴
Isolated mice heart	I/R 30-min global no flow ischaemia 40-min reperfusion	Sitagliptin 20 mg/kg, IP, 12 h and 1 h prior to heart excision DPP-4 ^{-/-} mice Sitagliptin 5 μ mol/L, in perfusion buffer, for 20 min before I/R insult	1. Sitagliptin improved LVDP in normoglycaemic mice. 2. LVDP was increased in DPP-4 ^{-/-} mice compared with DPP-4 ^{+/+} mice. 3. Administration of sitagliptin immediately before I/R injury failed to improve LVDP in normoglycaemic mice.	Genetic deletion and pharmacological inhibition of DPP-4 activity improved LV function. Acute reduction of cardiac DPP-4 activity was not sufficient to improve LV function.	Sauve et al. ⁴⁹
Patients	Coronary artery disease and preserved LV function	Sitagliptin 100 mg, single PO	1. Sitagliptin increased plasma GLP-I level. 2. Sitagliptin lowered plasma glucose level. 3. Insulin level was comparable between groups. 4. Free fatty acid level was comparable between groups. 5. Sitagliptin improved ejection fraction and regional wall function during peak dobutamine stress and 30 min of recovery. 6. Sitagliptin had a greater beneficial effect on ischaemic than on non-ischaemic segments.	Sitagliptin improved both global and regional LV performance in response to stress and also reduced post-ischaemic stunning.	Read et al. ⁵⁰

LAD: left anterior descending coronary artery; PO: per oral; LV: left ventricle; MI: myocardial infarction; I/R: ischaemia-reperfusion; IP: intraperitoneal route; LVDP: left ventricle developed pressure; DPP-4: dipeptidyl peptidase-4; GLP-I: glucagon-like peptide-1.

Table 8. Summary of the effects of DPP-4 inhibitor on haemodynamic parameters.

Animal species	Study model	Drug/dose/route	Major findings	Interpretation	References
Mice	MI LAD ligation for 4 weeks	DPP-4 ^{-/-} mice	Aortic flow and mitral flow were comparable between DPP-4 ^{-/-} mice and DPP-4 ^{+/+} mice.	Genetic inhibition of DPP-4 activity did not alter haemodynamic parameters.	Sauve et al. ⁴⁹
Mice	MI LAD ligation for 4 days	Sitagliptin 250 mg/kg per day, PO, for 11 days	Aortic flow and mitral flow were comparable between sitagliptin and control group.	Pharmacological inhibition of DPP-4 activity did not alter haemodynamic parameters.	Sauve et al. ⁴⁹
Rat	Ischaemic heart failure 12-week LAD occlusion	Vildagliptin 15 mg/kg per day, PO for 2 days prior to LAD occlusion 15 mg/kg per day, PO for 3 weeks after LAD occlusion	Vildagliptin did not alter heart rate, systolic and diastolic blood pressure, LV systolic and end diastolic pressure.	Vildagliptin had no beneficial effect on haemodynamic parameters.	Yin et al. ⁵⁴
Isolated rat heart	I/R 45-min global low flow ischaemia 30-min reperfusion	Vildagliptin Pretreatment 10 mg/kg per day, PO, for 4 weeks	Vildagliptin did not improve basal aortic output and basal coronary flow in both normal and insulin resistant rat.	Vildagliptin did not alter haemodynamic parameters.	Huisamen et al. ⁵³
Patients	Coronary artery disease and preserved LV function	Sitagliptin 100 mg, single oral dose	Sitagliptin did not affect heart rate, systolic and diastolic blood pressure, and rate-pressure product.	Sitagliptin did not alter haemodynamic parameters.	Read et al. ⁵⁰

MI: myocardial infarction; LAD: left anterior descending coronary artery; DPP-4: dipeptidyl peptidase-4; PO: per oral; LV: left ventricle; I/R: ischaemia-reperfusion.

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Conflicts of interest

No conflicts of interest have been declared.

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Effects of *Kaempferia parviflora* Wall. Ex. Baker and Sildenafil Citrate on cGMP Level, Cardiac Function, and Intracellular Ca^{2+} Regulation in Rat Hearts

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AU2

Abstract: Although *Kaempferia parviflora* extract (KPE) and its flavonoids have positive effects on the nitric oxide (NO) signaling pathway, its mechanisms on the heart are still unclear. Because our previous studies demonstrated that KPE decreased defibrillation efficacy in swine similar to that of sildenafil citrate, the phosphodiesterase-5 inhibitor, it is possible that KPE may affect the cardiac NO signaling pathway. In the present study, the effects of KPE and sildenafil citrate on cGMP level, modulation of cardiac function, and Ca^{2+} transients in ventricular myocytes were investigated. In a rat model, cardiac cGMP level, cardiac function, and Ca^{2+} transients were measured before and after treatment with KPE and sildenafil citrate. KPE significantly increased the cGMP level and decreased cardiac function and Ca^{2+} transient. These effects were similar to those found in the sildenafil citrate-treated group. Furthermore, the nonspecific NOS inhibitor could abolish the effects of KPE and sildenafil citrate on Ca^{2+} transient. KPE has positive effect on NO signaling in the heart, resulting in an increased cGMP level, similar to that of sildenafil citrate. This effect was found to influence the physiology of normal heart via the attenuation of cardiac function and the reduction of Ca^{2+} transient in ventricular myocytes.

Key Words: *Kaempferia parviflora*, sildenafil citrate, heart, cGMP, cardiac function, Ca^{2+} transient

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INTRODUCTION

Kaempferia parviflora Wall. Ex. Baker or Krachai-dam belongs to the plant in Zingiberaceae family.¹ The rhizome has been used in Thai traditional medicine for several

purposes such as the treatment of allergy, gastrointestinal disorders and rectifying male impotence.¹ Various studies demonstrated that the crude extract of *K. parviflora* rhizome and its flavonoids exert a positive effect on the nitric oxide (NO) signaling pathway both in the in vivo and in vitro studies.^{2–6} *K. parviflora* extract (KPE) was shown to increase the expression of endothelial nitric oxide synthase (eNOS) mRNA and the protein level in primary cell culture of human umbilical vein endothelial cells.² Moreover, it has been shown recently that some flavonoids extracted from *K. parviflora* rhizome can inhibit the activity of phosphodiesterase type 5 (PDE-5),³ the enzyme that specifically cleaves the NO mediator cGMP to 5'GMP.⁷ These effects of *K. parviflora* results in an increased intracellular cGMP level.

Previous studies demonstrated that exposure to KPE produced vasodilation in both in vivo and ex vivo studies.^{4–6,8,9} similar to the effects of NO on vasculature. It has been shown that L-N^G-nitroarginine methyl ester (L-NAME), a nonspecific nitric oxide synthase (NOS) inhibitor, can significantly decrease KPE-induced vasorelaxation of the aortic ring.⁴ Moreover, the vasorelaxant effect of flavonoids extracted from *K. parviflora* rhizome was found to be related to NO signaling pathway.^{5,6} In addition, KPE was shown to improve the endothelial dysfunction in streptozotocin-induced diabetic rats.¹⁰ This effect was found to be due to the increase of NO bioavailability by the effect of *K. parviflora*.¹⁰

Our previous study in swine demonstrated that KPE decreased the defibrillation efficacy and increased the vulnerability to arrhythmia,¹¹ similar to that of the supratherapeutic concentration of sildenafil citrate, the PDE-5 inhibitor.^{12,13} This finding suggested that KPE may affect the heart via NO/cGMP-dependent mechanism. However, the effect of KPE on NO signaling pathway in the heart is still unknown. In the present study, we hypothesized that KPE has the positive effect on NO signaling pathway in the heart via increased cGMP level. Because NO signaling pathway plays a crucial role in attenuating intracellular Ca^{2+} concentration $[(\text{Ca}^{2+})_i]$ via the modulation of L-type Ca^{2+} channel and Ca^{2+} handling proteins of the heart,^{14–17} the effects of KPE and sildenafil citrate on Ca^{2+} transient and cardiac function were also investigated in the present study. We hypothesized that KPE attenuates cardiac function via the reduction of Ca^{2+} transient amplitude similar to that of sildenafil citrate.

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MATERIALS AND METHODS

Preparation of KPE

K. parviflora rhizomes were obtained in the form of coarsely ground powder from Thanyaporn Co, Ltd., Samutprakarn province, Thailand. The preparation of KPE was performed using the protocol as described previously.¹¹ Briefly, the coarse powder of *K. parviflora* rhizomes was weighed for 3000 mg, and extracted with 90°C saline 70 mL for 15 minutes. Then, the solution was filtered with Whatman filtered paper No.1 to collect the extract. The final volume of the extract was ~50 mL (the concentration of the extract stock solution was at 60 mg/mL).

In the present study, 2 different concentrations of KPE were used base on their effects on cardiac electrophysiological parameters as previously described.¹¹ The 100-mg/kg KPE, the highest concentration from our preclinical study was found to alter major cardiac electrophysiological parameters,¹¹ whereas the 12.5 mg/kg did not change any cardiac electrophysiological parameters.¹¹

In an in vivo model, the extract was collected and diluted with saline for the final concentration of KPE at 12.5 or 100 mg/kg. In an in vitro model, the extract was added into the bath solution for the final concentrations at 75, 150, or 300 µg/mL.

The Experimental Protocol in an In Vivo Model

Animal Preparation and Cannulation for Intravenous Injection

The study was approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. Rats were received standard pelted rat diet and water ad libitum before the study. Male Wistar rats (~300 g) were anesthetized with thiopental (100 mg/kg, intraperitoneally). Rats were operated to expose the left internal jugular vein and cannulated with polyethylene tubing (PE-60, Intramedic, Clay Adams, NJ) containing heparinized saline (40 U/mL) for subsequent infusion of KPE, drugs or saline vehicle.

The Measurement of cGMP Level in the Heart

The cardiac cGMP level was measured according to the modified method of Favory et al.¹⁸ Briefly, frozen minced ventricular samples were weighed and homogenized with 6% trichloroacetic acid in deionized water at a ratio of 1 g wet weight: 3 mL trichloroacetic acid solution. The tissue suspension was vortexed briefly before centrifuging at 2000g at 4°C for 15 minutes. The supernatant was washed 3 times with water-saturated diethyl ether and the upper ether layer was discarded after each wash. The aqueous extract was heated with 70°C in water bath for 5 minutes to discard the remaining diethyl ether. The final solution was lyophilized and analyzed for cGMP by a commercial ELISA kit (Parameter, R&D Systems, Minneapolis, MN).

Western Blot Analysis for NOS Expression

Frozen minced samples were homogenized with ice-cold potassium phosphate buffer (pH 8.3) with protease

inhibitor cocktail, and subsequently centrifuged at 800g for 10 minutes at 4°C. The supernatant was centrifuged at 15,000g for 20 minutes at 4°C, and the protein concentration in the resultant supernatant was measured. Equal amount of proteins were subjected to sodium dodecyl sulfate—polyacrylamide gel electrophoresis. Blots were probed with primary antibodies against eNOS, nNOS, and iNOS (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected with the ECL detection system (GE Healthcare Bio-sciences Corp, Piscataway, NJ). Subsequent detection of the specific proteins was normalized to β-actin using Scion Image for Windows.

Experimental Protocol for Studying the Effect of KPE on cGMP Level and NOS Expression in the Heart

The rats were divided into 4 groups (n = 8 in each group). Group 1: saline; group 2: sildenafil citrate (Pfizer; 4 mg/kg, comparable to the supratherapeutic concentration); groups 3 and 4: KPE (12.5 and 100 mg/kg, respectively).

After cannulation, the solutions including KPE, sildenafil citrate or saline (1 mL each) were administered (0.5 mL/min, intravenously (i.v.)). Thirty minutes after the end of the treatments, the ventricles were rapidly removed, cleaned in ice-cold saline, and then minced and divided into 2 halves for cGMP level measurement and Western blot analysis for NOS expression. The period of the study (30 min) was chosen because our previous study indicated that the effect of KPE on cardiac electrophysiology reached a peak 30 minutes after KPE infusion.¹¹

Pharmacological Protocol for Studying the Effect of KPE on the Downstream Cascade From NOS in the Heart

The rats were divided into 5 groups (n = 8 in each group). Group 1: saline-treated in combination with NO-donor, nitroglycerin (NTG, Schwarz, Germany), and nonspecific NOS inhibitor, L-NAME (Sigma Chemical, St. Louis, MO); group 2: 12.5-mg/kg KPE with NTG and L-NAME; group 3: 12.5-mg/kg KPE combined with NTG only; group 4: 100-mg/kg KPE with NTG and L-NAME; and group 5: 100-mg/kg KPE combined with NTG only.

After internal jugular vein cannulation, rats in groups 1, 2, and 4 received a bolus of L-NAME (10 mg/kg,¹⁹ 0.25 mL, intravenously). In other groups, saline was administered instead of L-NAME. After L-NAME or saline injection, the KPE or saline (1 mL each) were infused (0.5 mL/min). Subsequently, a bolus of NTG (150 µg/kg,²⁰ 0.25 mL, intravenously) was administered and maintained (50 µg/kg/min, intravenously) throughout the experiment in all groups. Thirty minutes after KPE or saline infusion, the ventricles were rapidly removed, cleaned in ice-cold saline, and processed to assess cGMP levels.

Experimental Protocol for Studying the Effect of KPE on Cardiac Function

The rats were divided into 6 groups (n = 8 in each group). Group 1: saline; groups 2 and 3: KPE (12.5 and

100 mg/kg, respectively); groups 4 and 5, sildenafil citrate (2 and 4 mg/kg, the latter concentration is comparable to the therapeutic concentration); groups 6: L-NAME (10 mg/kg).

After the surgery for the insertion of the cardiac pressure-conductance catheter (Scisense Inc, London, Ontario, Canada), left ventricular (LV) function was assessed. LV hemodynamic data was digitized and recorded using a data acquisition system. After each experiment, end-systolic pressure (ESP), end diastolic pressure, maximum $+dP/dt$ and $-dP/dt$, end-systolic volume (ESV), end-diastolic volume (EDV), ejection fraction (EF), and heart rate (HR) were extracted during baseline and after administration of saline, KPE (12.5 and 100 mg/kg), sildenafil citrate (2 and 4 mg/kg), or L-NAME (1 mL each, 0.5 mL/min, intravenously) in groups 1–6, respectively. The LV hemodynamics were measured again after the end of saline, KPE, or sildenafil citrate infusion, and the data were compared against baseline.

The Experimental Protocol in an In Vitro Model

Preparation of Isolated Ventricular Myocytes

Rat ventricular myocytes were isolated using an enzymatic technique.²¹ The rats were injected with 0.2 mL heparin intraperitoneally. After deep anesthesia, the hearts were excised, and the aortas were cannulated rapidly. The cannulated hearts were perfused on a Langendorff perfusion apparatus with oxygenated normal Tyrode solution containing (in mM): 130 NaCl, 5.4 KCl, 0.75 $CaCl_2$, 1.4 $MgCl_2$, 0.4 NaH_2PO_4 , 10 glucose, 4.2 HEPES, 20 taurine, 10 creatine) at 37°C in the first column at constant flow (~2 mL/min) for 10 minutes. Then, the hearts were switched to the second column and perfused with oxygenated Ca^{2+} -free Tyrode solution. After 5 minutes, the hearts were switched to the third column (enzymatic buffer), where they were digested with the 30-mL normal Tyrode solution containing 1 mg/mL of collagenase type II (Gibco, Invitrogen Corporation, CA), for another 7 minutes. After that, the hearts were cut off the cannula, and the aortas and atria were removed from the hearts. The remaining ventricular tissues were cut into several pieces with scissors in the same enzymatic buffer from the third column. Then the cardiomyocytes in the solution were dispersed gently by a wide tipped pipette and filtered through nylon mesh into a plastic tube. After the cardiomyocytes were pelleted by gravity for ~6 minutes, the supernatant was aspirated and the cell pellet was resuspended and incubated in the warm normal Tyrode solution before the use.

The Measurement of Ca^{2+} Transient

The Ca^{2+} transient in rat ventricular myocytes was measured by a fluorimetric ratio technique.²² The fluorescent Ca^{2+} indicator Fura-2 was loaded by incubating the cardiomyocytes at room temperature for 20–30 minutes with 25 μM of Fura-2/AM (Sigma Chemical, St. Louis, MO). The background and cell autofluorescence were cancelled out by zeroing the output using cells without Fura-2/AM loading. Ultraviolet light at the wavelengths of 340 and 387 nm were used for the excitation of the Fura-2 from a xenon arc lamp

controlled by a microfluorometry system (Cell, Olympus, Tokyo, Japan), and the excitation light beam was directed into an inverted microscope (IX-81; Olympus). The ratio of emitted fluorescence signals from the Fura-2/AM loaded cardiomyocytes at 510 nm were recorded. The Ca^{2+} transient parameters including the Ca^{2+} transient amplitude, the Ca^{2+} transient rising and decay rate, and the diastolic Ca^{2+} level were measured during 1-Hz field-stimulation with 10-ms supramaximal threshold strength square-wave pulses. The fluorescence ratio data was processed and stored in a computer using Xcellence imaging software (Olympus).

Experimental Protocol for Studying the Effects of KPE and Sildenafil Citrate on Ca^{2+} Transient in Isolated Ventricular Myocytes

The ventricular myocytes were divided into 6 groups ($n = 8–12$ in each group). Before the saline, KPE, or sildenafil citrate superfusion, the Ca^{2+} transient in each group was measured as the baseline. After that, in group 1, saline was superfused to serve as a control group. In groups 2, 3, and 4, KPE at several concentrations (75, 150, and 300 $\mu g/mL$, respectively) was superfused. The 300 $\mu g/mL$ KPE (approximately consistent with the 20-mg/kg KPE in our in vivo studies) was chosen because it decreased the Ca^{2+} transient in isolated ventricular myocytes within 5 minutes of superfusion. The concentration of KPE higher than 300 $\mu g/mL$ was shown to have the cytotoxic effects; cells displayed obvious shrinkage within 5 minutes after superfusion of the extract.

In groups 5 and 6, sildenafil citrate (30 and 60 $\mu g/mL$, respectively, approximately comparable with the concentration of 2 and 4 mg/kg in our in vivo study), were superfused. The Ca^{2+} transient was measured again at 5 minutes after sildenafil citrate superfusion and at 5 and 10 minutes after KPE or saline superfusion.

Experimental Protocol for Studying the Effects of KPE and Sildenafil Citrate in Combination With L-Name On Ca^{2+} Transient in Isolated Ventricular Myocytes

The ventricular myocytes were treated with L-NAME (1 mM) for 5 minutes, after which the Ca^{2+} transient was measured as the baseline value. These L-NAME-treated cardiomyocytes were divided into 6 treatment groups ($n = 8–12$ in each group): saline, KPE (75, 150, and 300 $\mu g/mL$), or sildenafil citrate (30 and 60 $\mu g/mL$). In the sildenafil-treated groups, Ca^{2+} transients were measured at 5 minutes after sildenafil superfusion. In saline and KPE-treated groups, Ca^{2+} transients were measured at 10 minutes (the effective time obtained from the in vitro protocol) after saline or KPE superfusion.

Experimental Protocol for Studying the Effects of KPE and Sildenafil Citrate in Combination With L-NAME and NTG on Ca^{2+} Transient in Isolated Ventricular Myocytes

The ventricular myocytes were treated with L-NAME (1 mM) in combination with NTG (10 μM) for 5 minutes, after which the Ca^{2+} transient was measured as the baseline. These cardiomyocytes were divided into 3 groups ($n = 8–12$

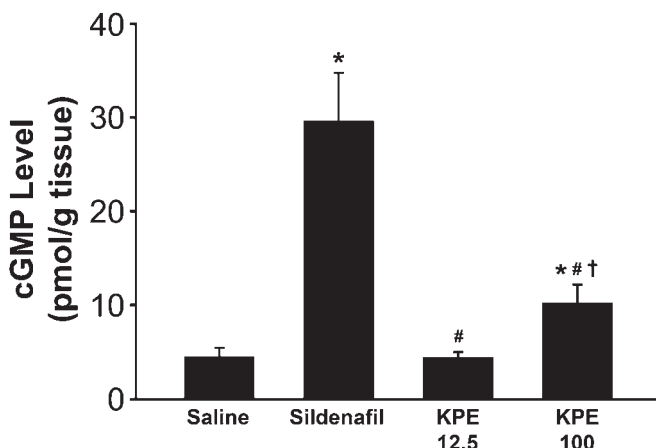


FIGURE 1. Effects of KPE (12.5 and 100 mg/kg), sildenafil citrate (4 mg/kg), and saline ($n = 8$ in each group) on the cGMP level in rat hearts. * $P < 0.05$ vs. saline; # $P < 0.05$ vs. sildenafil citrate; † $P < 0.05$ vs. KPE12.5.

in each group) as follows: saline, KPE (300 $\mu\text{g/mL}$), or sildenafil citrate (60 $\mu\text{g/mL}$). Five minutes after sildenafil citrate superfusion, Ca^{2+} transient was measured again to compare against baseline. In saline-treated and KPE-treated groups, Ca^{2+} transients were measured again at 10 minutes after superfusion of saline or the extract to compare against the baseline.

Statistical Analysis

Values are expressed as mean $\pm$ SD in all studies. Comparisons of variables at baseline and after saline, KPE, sildenafil citrate or L-NAME treatment in each group were performed using the Wilcoxon Signed Ranks test. Comparisons of variables between experimental groups were

performed using the Mann–Whitney U test. $P < 0.05$ was considered statistically significant.

RESULTS

The In Vivo Studies

Effect of KPE on cGMP Level in the Hearts

At 30 minutes after treatment, the intravenous administration of both 100-mg/kg KPE and 4-mg/kg sildenafil citrate significantly increased cGMP level in the ventricles (Fig. 1). The level of cGMP in the sildenafil citrate-treated group (the positive control) was also higher than that of the 100-mg/kg KPE-treated group. However, the cGMP level in the 12.5-mg/kg KPE group was not changed compared with the saline-treated group (Fig. 1).

Effect of KPE and Sildenafil Citrate on NOS Expression in the Hearts

Figure 2 represents the expression of NOS in the ventricles after KPE, sildenafil citrate, or saline treatment. KPE at any concentration, sildenafil citrate (4 mg/kg), or saline did not alter the expression of eNOS and the other 2 types of NOS, nNOS and iNOS, in the ventricles at 30 minutes after intravenous administration of the treatment.

Effect of KPE on Downstream Cascade From NOS in the Hearts

Because we found that the 100-mg/kg KPE increased cGMP level but did not change the expression of any type of NOS in rat ventricles, we further hypothesized that KPE increases cGMP by inhibiting PDE-5, similar to that of sildenafil citrate. To test this hypothesis, L-NAME, the nonspecific NOS inhibitor, was used to inhibit all types of NOS, and NTG, the NO donor, was used as the source of NO to control the level of exogenous NO in all groups. This

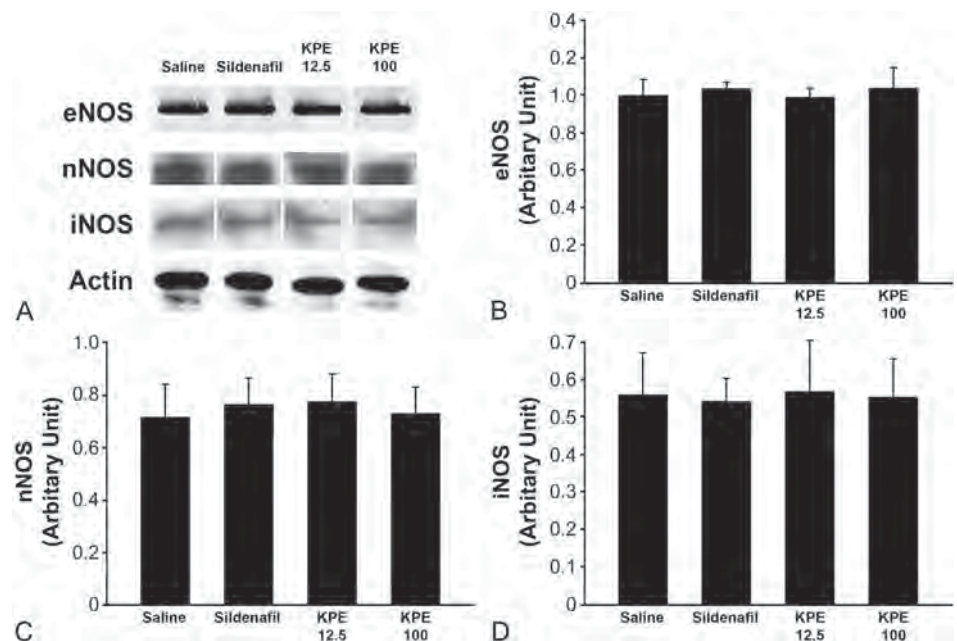


FIGURE 2. Effects of KPE (12.5 and 100 mg/kg), sildenafil citrate (4 mg/kg), and saline ($n = 8$ in each group) on NOS expression in rat hearts. A, Proteins by Western blot analysis in rat hearts; B, eNOS; C, nNOS; D, iNOS.

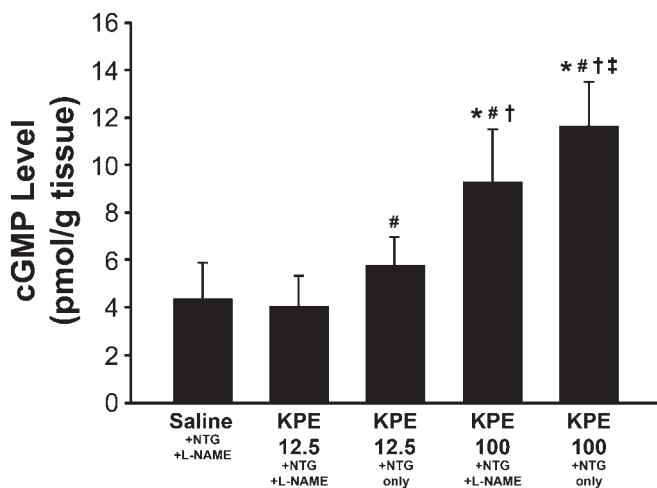


FIGURE 3. Effects of KPE (12.5 and 100 mg/kg) and saline combination with NO donor, NTG, and NOS inhibitor, L-NAME, or with NO donor only ($n = 8$ in each group) on the cGMP level in rat hearts. * $P < 0.05$ vs. saline; # $P < 0.05$ vs. KPE12.5 + NTG + L-NAME; † $P < 0.05$ vs. KPE12.5 + NTG only; ‡ $P < 0.05$ vs. KPE100 + NTG + L-NAME.

would allow us to elucidate whether increased cGMP was caused by the effect of KPE on NOS or its downstream cascade. Our results demonstrated that in the group treated with 100-mg/kg KPE combined with L-NAME and NTG, the cGMP level in the ventricles was significantly higher than the control (ie, saline plus L-NAME and NTG) group (Fig. 3).

Moreover, in the group treated with 100-mg/kg KPE combined with NTG only, the cGMP level was significantly higher than that in the KPE-treated group with L-NAME and NTG (Fig. 3). The cGMP level of both 12.5-mg/kg KPE-treated groups (in combination with NTG) with or without L-NAME was not different from that in the control group. However, the cGMP level in the group without L-NAME was significantly higher than that in the group with L-NAME (Fig. 3).

Effects of KPE and Sildenafil Citrate on Cardiac Function

Table 1 shows a tabulation of LV hemodynamic parameters including ESP, end-diastolic pressure, maximum +dP/dt and -dP/dt, ESV, EDV, EF, and HR after the treatments of KPE, sildenafil citrate, L-NAME, or saline compared with baseline. We found that after 30 minutes, KPE (100 mg/kg) degraded cardiac function as shown by the significantly decrease of ESP, maximum +dP/dt and -dP/dt, and ESV compared with baseline of the same group. Additionally, both concentrations of sildenafil citrate significantly degraded cardiac function by the same parameters as 100-mg/kg KPE-treated group within 5 minutes after the end of drug administration. In L-NAME-treated group, ESP, maximum +dP/dt and -dP/dt, ESV, and EDV were found to increase within 5 minutes after the end of L-NAME administration. However, EF and HR were not changed in these groups. KPE at 12.5 mg/kg or saline did not alter any LV hemodynamic parameters.

TABLE 1. Effects of KPE (12.5 and 100 mg/kg), Sildenafil Citrate (2 and 4 mg/kg), L-NAME (10 mg/kg), and Saline on the Cardiac Function ($n = 8$ in Each Group)

Parameters	Group 1		Group 2		Group 3	
	Baseline	Saline	Baseline	KPE (12.5 mg/kg)	Baseline	KPE (100 mg/kg)
ESP (mm Hg)	176 ± 16	178 ± 16	188 ± 21	191 ± 21	185 ± 12	171 ± 8*
EDP (mm Hg)	47 ± 4	48 ± 4	50 ± 3	51 ± 2	44 ± 3	43 ± 2
Maximum +dP/dt (mm Hg/s)	8821 ± 767	8839 ± 675	8954 ± 649	8827 ± 845	8424 ± 327	7840 ± 403*
Maximum -dP/dt (mmHg/s)	4554 ± 933	4407 ± 980	4698 ± 802	4794 ± 842	4145 ± 538	2963 ± 243*
ESV (% of baseline)	100	103 ± 14	100	104 ± 18	100	98 ± 16
EDV (% of baseline)	100	100 ± 4	100	99 ± 2	100	89 ± 6*
EF (% of baseline)	100	98 ± 7	100	98 ± 10	100	96 ± 10
HR (bpm)	442 ± 31	439 ± 35	425 ± 43	418 ± 50	420 ± 22	417 ± 14

Parameters	Group 4		Group 5		Group 6	
	Baseline	Sildenafil Citrate (2 mg/kg)	Baseline	Sildenafil Citrate (4 mg/kg)	Baseline	L-NAME
ESP (mm Hg)	187 ± 9	176 ± 14*	181 ± 6	165 ± 8*	186 ± 30	224 ± 43*
EDP (mm Hg)	52 ± 3	51 ± 2	43 ± 5	43 ± 4	45 ± 3	49 ± 6*
Maximum +dP/dt (mm Hg/s)	8791 ± 471	8173 ± 337*	8807 ± 499	7586 ± 603*	8433 ± 474	9476 ± 378*
Maximum -dP/dt (mmHg/s)	4459 ± 848	3058 ± 309*	4018 ± 430	2739 ± 697*	4298 ± 940	5053 ± 891*
ESV (% of baseline)	100	94 ± 17	100	97 ± 31	100	183 ± 54*
EDV (% of baseline)	100	92 ± 8*	100	89 ± 11*	100	138 ± 24*
EF (% of baseline)	100	99 ± 6	100	96 ± 21	100	86 ± 21
HR (bpm)	436 ± 16	444 ± 16	434 ± 37	439 ± 40	434 ± 22	416 ± 15

Values are expressed as mean ± SD.

* $P < 0.05$ versus baseline of the same group.

EDP, end diastolic pressure.

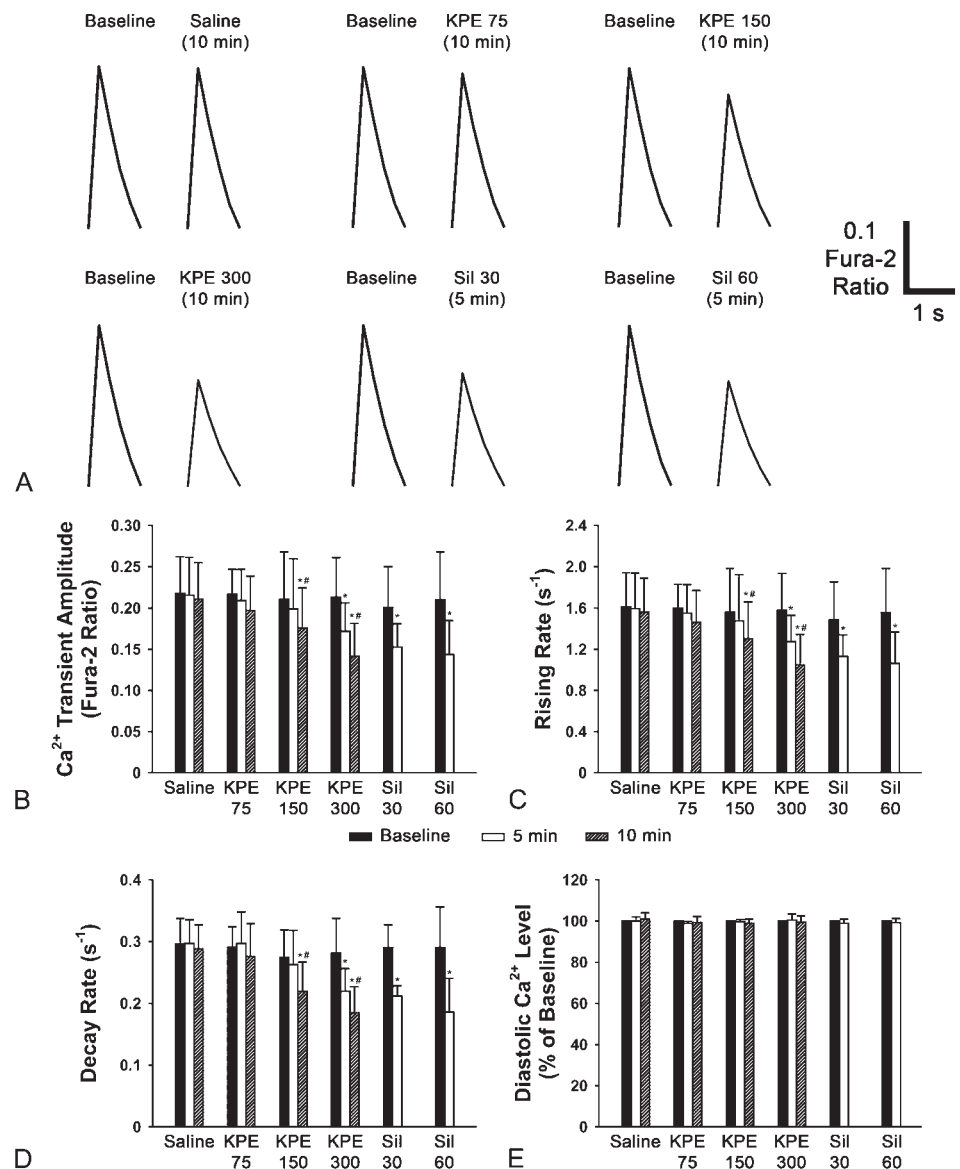


FIGURE 4. Effects of KPE at 75 ($n = 9$), 150 ($n = 10$), and 300 $\mu\text{g/mL}$ ($n = 8$); sildenafil citrate (Sil) at 30 ($n = 11$) and 60 mg/mL ($n = 12$); and saline ($n = 12$) on the Ca^{2+} transient in ventricular myocytes. A, the representative Ca^{2+} transient tracings; B, Ca^{2+} transient amplitude; C, Ca^{2+} transient rising rate; D, Ca^{2+} transient decay rate; E, diastolic Ca^{2+} level. $*P < 0.05$ vs. baseline; $\#P < 0.05$ vs. 5 minutes of the same group.

The In Vitro Studies

Effects of KPE and Sildenafil Citrate on Ca^{2+} Transient in Isolated Ventricular Myocytes

[F4]

Figure 4A represents the Ca^{2+} transient tracings of isolated ventricular myocytes treated with KPE, sildenafil citrate, and saline. KPE at the concentration of 300 $\mu\text{g/mL}$ significantly decreased the Ca^{2+} transient amplitude (Fig. 4B), rising (Fig. 4C) and decay rate (Fig. 4D) within 5 minutes (19%, 19%, and 22% respectively) and showed the progressive decrease of these parameters at 10 minutes of KPE superfusion (33%, 33%, and 34%, respectively). The superfusion of KPE at 150 $\mu\text{g/mL}$ decreased Ca^{2+} transient amplitude, rising and decay rate at 10 minutes of KPE superfusion (17%, 17%, and 20%, respectively). KPE at 75 $\mu\text{g/mL}$ did not change the Ca^{2+} transient amplitude, rising and decay rate.

After the superfusion of sildenafil citrate at 60 and 30 $\mu\text{g/mL}$ for 5 minutes, the Ca^{2+} transient amplitudes were decreased by 32% and 25%, respectively (Fig. 4B), the Ca^{2+} transient rising rates were decreased by 32% and 25%, respectively (Fig. 4C), and the Ca^{2+} transient decay rates were decreased by 36% and 27%, respectively (Fig. 4D). Saline did not alter Ca^{2+} transient amplitude, rising and decay rate. Additionally, the diastolic Ca^{2+} level was not altered from its baseline after being treated with any of the tested concentrations of KPE, sildenafil citrate, and saline (Fig. 4E).

Effects of KPE and Sildenafil Citrate on Ca^{2+} Transient in Combination With L-Name in Isolated Ventricular Myocytes

This study was performed to determine whether KPE and sildenafil citrate modulate Ca^{2+} transient through the

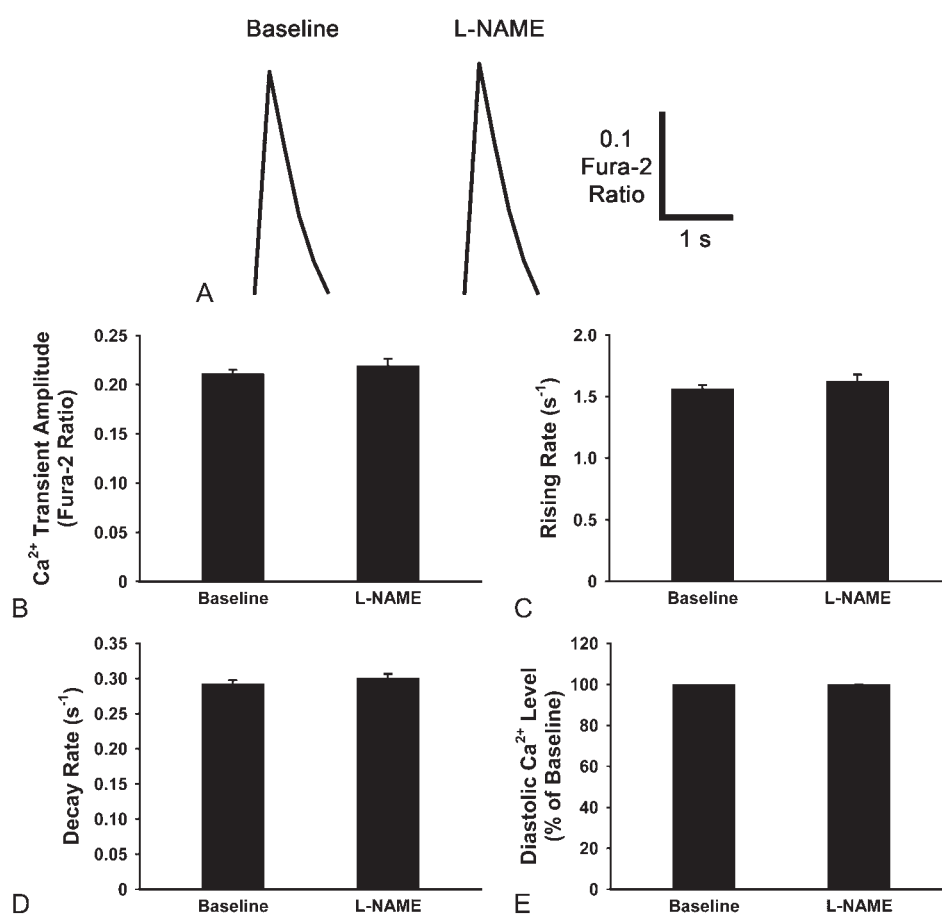


FIGURE 5. Effects of L-NAME (1 mM) on the Ca^{2+} transient in ventricular myocytes. A, the representative Ca^{2+} transient tracings; B, Ca^{2+} transient amplitude; C, Ca^{2+} transient rising rate; D, Ca^{2+} transient decay rate; E, diastolic Ca^{2+} level.

NO/cGMP signaling pathway. It is known that this pathway attenuates intracellular Ca^{2+} level via the regulation of L-type Ca^{2+} channel and Ca^{2+} handling proteins.^{14–17} To prevent intracellular cGMP production via NO before KPE or sildenafil citrate was applied to the ventricular myocytes, all types of NOS were inhibited with nonspecific NOS inhibitor L-NAME in this series of experiment. We hypothesized that the disturbance of cGMP production by L-NAME could abolish the effects of KPE and sildenafil citrate on Ca^{2+} transient.

F5 Figure 5 represents the effect of L-NAME on the Ca^{2+} transient of isolated ventricular myocytes. It was found that after 5 minutes of L-NAME treatment, the Ca^{2+} transient amplitude, rising and decay rate or diastolic Ca^{2+} level did not change compared with the baseline. These effects of L-NAME–treated cardiomyocytes served as baseline for further studying the effects of KPE and sildenafil citrate treatments.

F6 In L-NAME–treated cardiomyocytes, neither KPE of any concentrations nor sildenafil citrate altered the Ca^{2+} transient amplitude, rising or decay rate, compared with the ventricular myocytes treated with L-NAME alone (baseline) (Fig. 6). Moreover, the diastolic Ca^{2+} level was not altered in any of these groups. Saline did not alter Ca^{2+} transient after L-NAME superfusion in isolated ventricular myocytes.

Experimental Protocol for Studying the Effects of KPE and Sildenafil Citrate in Combination With L-NAME and NTG on Ca^{2+} Transient in Isolated Ventricular Myocytes

In L-NAME–treated cardiomyocytes in combination with NTG, KPE (300 $\mu\text{g}/\text{mL}$) and sildenafil citrate (6 $\mu\text{g}/\text{mL}$) decreased the Ca^{2+} transient amplitude, rising and decay rate, compared with the pretreatment of the extract or sildenafil citrate (Fig. 7). However, the diastolic Ca^{2+} level was not altered in these groups. Saline did not alter Ca^{2+} transient after L-NAME combined with NTG superfusion in isolated ventricular myocytes.

F7

DISCUSSION

The major findings of the present study are as follows: (1) The 100-mg/kg KPE increased cardiac cGMP level via downstream cascade from NOS; (2) The 100-mg/kg KPE and both supratherapeutic and therapeutic concentrations of sildenafil citrate decreased cardiac function in the normal rat heart; (3) The 100-mg/kg KPE and both supratherapeutic and therapeutic concentrations of sildenafil citrate decreased Ca^{2+} transient via the NO signaling pathway from its downstream cascade from NOS; (4) L-NAME increased cardiac function without the changing of Ca^{2+} transient.

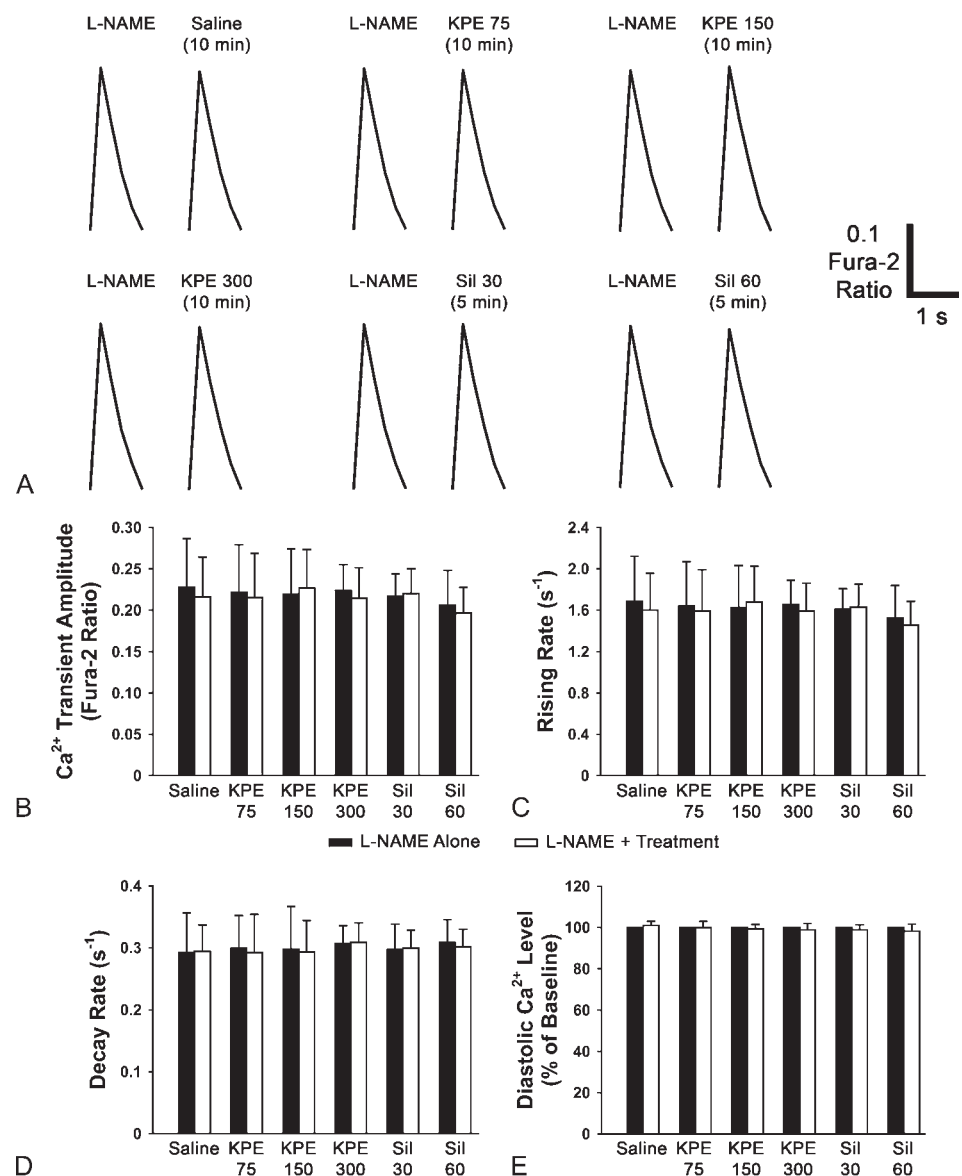


FIGURE 6. Effects of KPE at 75 (n = 9), 150 (n = 10) and 300 $\mu\text{g/mL}$ (n = 8); sildenafil citrate (Sil) at 30 (n = 10) and 60 $\mu\text{g/mL}$ (n = 11); and saline (n = 8) on the Ca^{2+} transient in ventricular myocytes in combination with the NOS inhibitor, L-NAME. A, the representative Ca^{2+} transient tracings; B, Ca^{2+} transient amplitude; C, Ca^{2+} transient rising rate; D, Ca^{2+} transient decay rate; E, diastolic Ca^{2+} level.

Although various studies demonstrated that KPE and its bioactive flavonoids have the positive effects on the NO signaling pathway in the vessels and endothelial cell culture,²⁻⁶ its effect on the heart had not been investigated. Our study is the first to demonstrate that a high concentration of KPE (100 mg/kg) also affected the NO signaling pathway in rat hearts, and that its effect was similar to that of sildenafil citrate (a PDE-5 inhibitor), the positive control. In the present study, a high concentration of KPE (100 mg/kg) significantly degraded cardiac function in vivo at 30 minutes after the end of KPE infusion. This effect of KPE is possibly due to the increased cGMP level in the heart. This is supported by our findings that sildenafil citrate, the PDE-5 inhibitor that increased cGMP intracellularly and represented the positive effect of NO signaling pathway on cardiac function in our study, could decrease the cardiac function in the same manner of the high concentration of KPE. These results indicate that

NO-cGMP signaling pathway could possibly explain the mechanism by which KPE modulates cardiac function. In L-NAME-treated group, the cardiac function was found to increase compared with baseline. This result suggested that the inhibition of NO signaling pathway could cause the augmentation of cardiac contractility, whereas the enhancement of this pathway causes the contrary result.

In the present study, we found that at 30 minutes after the end of KPE infusion, cGMP level in rat hearts increased, even though NOS expression in the heart was not changed. Although the study of Wattanapitayakul et al² in 2007 found that KPE increased the expression of eNOS mRNA and protein in human umbilical vein endothelial cells, we did not observe the change of cardiac eNOS and the other 2 types of NOS protein expressions in our study. This could be due to the different organs used, period of the treatment, animal species, and study protocol. Furthermore, we also found that

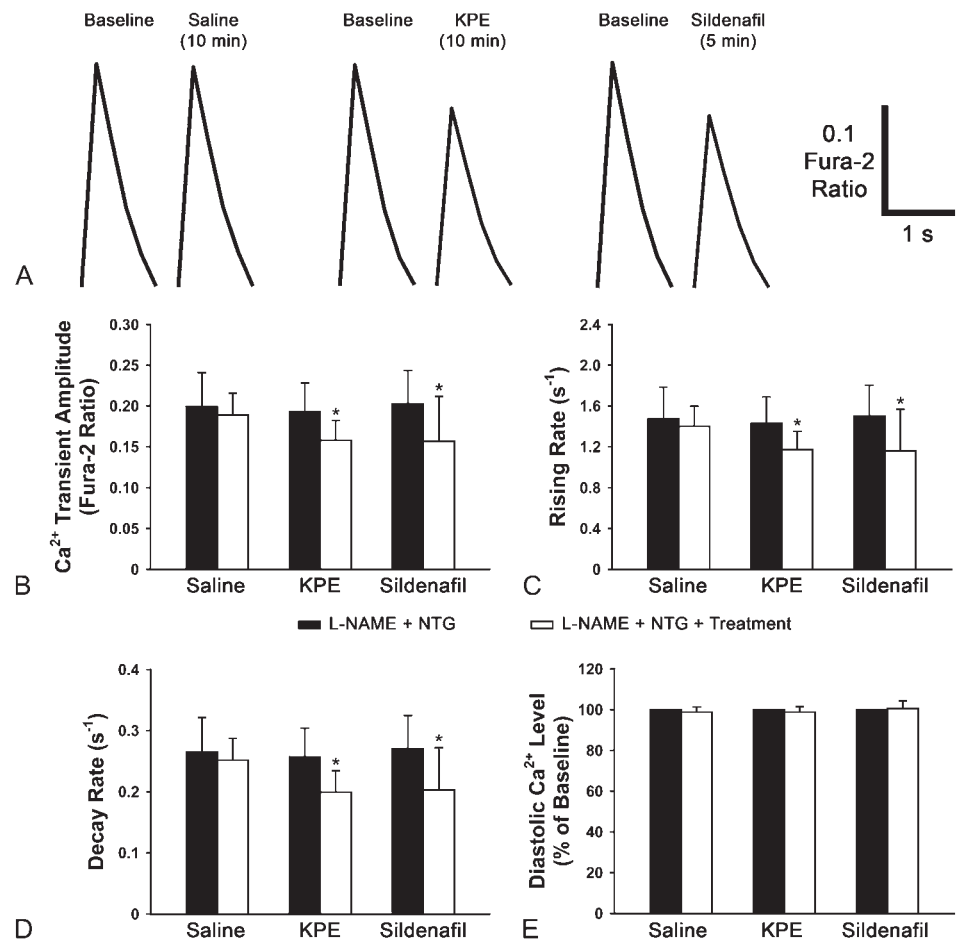


FIGURE 7. Effects of KPE (300 $\mu\text{g/mL}$, $n = 10$), sildenafil citrate (60 $\mu\text{g/mL}$, $n = 8$); and saline ($n = 8$) on the Ca^{2+} transient in ventricular myocytes in combination with the NOS inhibitor, L-NAME, and NO donor, NTG. A, the representative Ca^{2+} transient tracings; B, Ca^{2+} transient amplitude; C, Ca^{2+} transient rising rate; D, Ca^{2+} transient decay rate; E, diastolic Ca^{2+} level. * $P < 0.05$ vs. baseline.

when all types of NOS were inhibited by L-NAME, cGMP level was still increased after the administration of the high concentration of KPE (ie, saline vs. KPE100 + NTG + L-NAME, Fig. 3). These results suggest that the increased cGMP level by KPE in the heart depend on neither the increasing NOS expression nor NOS activity. Because *K. parviflora* has been shown to have many kinds of flavonoids,^{23–26} and previous studies demonstrated that some flavonoids and polyphenolic compounds from *K. parviflora* and other herbal plants exhibit PDE-5 inhibitory effect.^{3,27–29} Therefore, we hypothesized that KPE used in the present study could have the PDE-5 inhibitory effect in the heart. Although we found that the 100-mg/kg KPE can increase cGMP level in the ventricle, it was found to be much lower than the effect of 4-mg/kg sildenafil citrate. This finding suggests that KPE could have mild PDE-5 inhibitory effects compared with that of sildenafil citrate as observed in the present study. Additionally, we found that cardiac cGMP level in the KPE-treated groups that NOS was not inhibited (ie, groups 3 and 5, Fig. 3) were higher than that of the same KPE-treated groups, and NOS was inhibited by L-NAME (ie, groups 2 and 4, Fig. 3). These findings suggest that the endogenous NO from NOS also plays a role in the difference of cGMP production in these groups.

Because KPE was found to cause vasorelaxation through the NO-cGMP signaling mechanism,⁴ it is possible that KPE decreased the cardiac function through the direct effect on the heart via the modulation of excitation–contraction coupling or the indirect effect via its effect on vasculature. We investigated this hypothesis by studying the effect of KPE in isolated ventricular myocytes which could provide evidence for cardiac contractility via the Ca^{2+} induced Ca^{2+} release and excitation–contraction coupling without the interference of vascular effect of KPE. It is well recognized that the NO-cGMP signaling pathway regulates many types of ions via the modulation of ion channels and ion-handling proteins in cardiomyocytes.^{14–17,30–32} Among various types of ions, Ca^{2+} is the important ion that plays a role in the regulation of electrical and mechanical function of the heart, that is the generation of action potential and excitation–contraction coupling in each cardiac cycle.³³ The NO-cGMP signaling pathway is one of the crucial pathways that attenuate the $(\text{Ca}^{2+})_i$ via the modulation of L-type Ca^{2+} current ($I_{\text{Ca-L}}$) and Ca^{2+} recycling by the sarcoplasmic reticulum.^{14–17} Our results demonstrated that KPE and sildenafil citrate decreased Ca^{2+} transient amplitude, rising and decay rate, in isolated ventricular myocytes. When treated with the nonspecific NOS inhibitor L-NAME to completely inhibit the

generation of cGMP intracellularly, the effects of both KPE and sildenafil citrate were completely eliminated. These effects reappeared again when the NO donor NTG was added to activate the downstream cascade from NOS. These findings suggest that cGMP is the important mediator of KPE and sildenafil citrate in the modulation of Ca^{2+} transient in ventricular myocytes. Decreased Ca^{2+} transient amplitude and rising rate of ventricular myocytes by KPE and sildenafil citrate may result in the reduction in the cardiac contractility as shown by the decreased maximum $+\text{dP}/\text{dt}$, whereas the decreased Ca^{2+} transient decay rate may result in diastolic dysfunction as shown by the decreased maximum $-\text{dP}/\text{dt}$ in our in vivo study. Although KPE and sildenafil citrate decreased Ca^{2+} transient decay rate in isolated cardiomyocytes, the baseline diastolic Ca^{2+} levels were not altered. These findings suggest that KPE not only decreased the Ca^{2+} influx but also slowed down the process of getting rid of Ca^{2+} from the cytoplasm during each cardiac cycle.

Our results also demonstrate that L-NAME alone did not change Ca^{2+} transient amplitude, rising or decay rate, and diastolic Ca^{2+} level in isolated ventricular myocytes. This result suggests that the basal level of cGMP does not alter Ca^{2+} transient of isolated ventricular myocytes. This finding is consistent with the previous study that L-NAME did not change the Ca^{2+} transient in LV-free wall of guinea pig hearts.³⁴ Although our cardiac function study showed that L-NAME increased cardiac contractility, this finding does not depend on the direct effect of L-NAME on intracellular Ca^{2+} regulation. In addition, the other study in isolated-perfused rabbit hearts also found that the non-specific NOS inhibitor, L- N^{G} -nitroarginine, did not change cardiac contractility.³⁵ Because L-NAME is the drug that was used to increase the blood pressure in hypertensive animal model,³⁶ the increase of cardiac function after L-NAME treatment may be due to the compensatory mechanism against hypertensive effect of L-NAME (ie, Frank-Starling mechanism in response to the increased preload through the enhanced myocyte distension).³⁷

CONCLUSIONS

Similar to sildenafil citrate, KPE increased cardiac cGMP level via the downstream cascade from NOS in the NO signaling pathway. KPE reduces the Ca^{2+} transient, thus attenuating LV performance in rats.

STUDY LIMITATION

The present study measured cGMP levels and NOS expression in the whole ventricles. It is possible that the cGMP level, NOS expression, and the effect of KPE vary within the heart, that is, left and right ventricle or endocardium to epicardium could be different. Also, the absolute Ca^{2+} concentration in the isolated ventricular myocytes was not known in the present study because we measured the relative change of fluorescence intensity of Fura-2 as the Ca^{2+} transient. Moreover, the effects of KPE on the contractile proteins were not investigated in the present study. Because NO-cGMP signaling pathway also modulates the excitation-contraction coupling

via the desensitization mechanism of myofibril to Ca^{2+} in the heart,³⁸ it is possible that KPE may influence the coupling between Ca^{2+} and contractility.

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Low-dose dental irradiation decreases oxidative stress in osteoblastic MC3T3-E1 cells without any changes in cell viability, cellular proliferation and cellular apoptosis

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ABSTRACT

Cellular responses following low-dose irradiation have been widely debated. Several studies have revealed detrimental effects of low-dose irradiation; however, some studies have shown contrasting results. Moreover, the effects of periapical irradiation on osteoblastic cells have not yet been revealed. Therefore, in this study, we tested the hypothesis that low-dose dental irradiation of osteoblastic cells reduces reactive oxygen species (ROS) production and leads to increased cellular proliferation and high-dose dental irradiation of osteoblastic cells increases ROS production and leads to cellular apoptosis.

Methods: We irradiated MC3T3-E1 cells with various doses of periapical irradiation (0, 1, 2, 5 and 10 doses, 1.5 mGy/dose). We evaluated cell viability using MTT assay, the expression of Bax and Bcl-2, as markers for apoptosis and the expression of cyclin D1 as a marker for cell proliferation 24 h after each irradiation. We also measured ROS production 4 h following each irradiation.

Results: ROS production was significantly reduced after one dose of periapical irradiation (1.5 mGy); however, after 10 doses (15 mGy), ROS production was significantly increased ($p < 0.05$). None of the doses of dental radiation affected cell viability as determined by MTT assay, nor did they change the apoptotic marker: (the Bax/Bcl-2 ratio). However, 10 doses of dental irradiation significantly decreased the expression of cyclin D1.

Conclusions: Our findings suggest that low-dose dental radiation may help to detoxify osteoblastic cells by reducing ROS production without any changes in cell viability, cellular apoptosis or proliferation. However, high-dose dental radiation impairs osteoblastic proliferation via increase ROS production without any changes in cell viability or apoptotic responses.

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1. Introduction

It is well accepted that high-dose irradiation (>1 Gy) causes a deleterious effect on cellular organisms, such as the induction

of DNA damage and cell cycle arrest, in a dose-dependent manner, which lead to the impairment of cell proliferation and cell death through the apoptotic process.¹ However, the effects of low-dose irradiation (<1 Gy) on cellular responses remain

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unclear. Several previous studies have revealed the detrimental effects of low-dose irradiation on cells, such as increases in reactive oxygen species (ROS) formation,² DNA double-strand breaks³, chromosomal breakage⁴. However, some studies have shown contrasting results of low-dose irradiation. For example: (1) several studies have suggested that low-dose irradiation (<50 mGy) induces cell proliferation in various cell types, such as human lung fibroblasts⁵, normal human diploid cells⁶, Chinese hamster fibroblasts⁷, neuron cells⁸, and bone marrow cells⁹. (2) Ahmed and colleagues have suggested that cyclin D1 expression, an important regulator of G1 to S-phase transition in the cell cycle involving cellular proliferation, is up-regulated by low-dose ionising radiation (100 mGy) in human keratinocytes with adaptive radioresistance.¹⁰ (3) Previous *in vitro* and *in vivo* studies have reported that low-dose irradiation (250–500 mGy) can detoxify the deleterious effects of radiation-induced ROS formation by increasing intracellular glutathione and superoxide dismutase production 3–6 h post-irradiation.^{11–13}

Dental irradiation is classified as low-dose irradiation, since intraoral radiography can produce only 3.9 mGy of irradiation.¹⁴ Low-dose irradiation may be harmful to oral tissues, particularly oral bone cells. In addition, a recent clinical study has demonstrated that extra-oral panoramic radiography can induce chromosomal damage to oral mucosal cells 10 days post-irradiation.⁴ Branemark and colleagues have recommended of not performing dental irradiation procedures immediately after dental implantation due to the possibility of the detrimental effects of dental irradiation on the healing and remodelling of bone.¹⁵ However, the effects of intra-oral radiography, particularly periapical irradiation of bone cells, have not yet been studied.

Therefore, in this study, we aimed to determine the effects of low doses of dental irradiation on osteoblastic cells by measuring cell viability, ROS production, and the apoptotic process by using an apoptotic marker (the ratio of Bax and Bcl-2) as well as cellular proliferation by using a cellular proliferative marker (cyclin D1).

2. Materials and methods

2.1. Osteoblastic cell culture

Murine osteoblastic MC3T3-E1 cells were cultured in complete Dulbecco's modified Eagles medium (DMEM), containing 10% foetal bovine serum (FBS), Penicillin G (100 U/ml), Streptomycin sulphate (100 µg/ml), Amphotericin B (25 µg/ml) and L-glutamine (2 mM) at 37 °C in 95% air/5% CO₂. When the cells reached confluence, they were subcultured using 0.025% EDTA trypsin. The cells were seeded at a density of 5000 cells/well in a 96-well plate for measurement of intracellular reactive oxygen species and cell viability, and 400,000 cells/dish in 35 mm tissue culture dishes for protein extraction. The plates were incubated at 37 °C in a CO₂ incubator for 24 h before dental irradiation.

2.2. Cell irradiation

The cultures were irradiated with 0 (the controls), 1, 5 and 10 multiples of a standard dose for a periapical film radiograph,

using a portable dental X-ray machine generator (NOMAD™, Aribex, Inc., Orem, UT, USA). The radiographic parameters for one dose were 60 kVp, 2.3 mA, 0.4 s. The focal-object distance was 7.5 in. One-dose periapical irradiation was 1.5 mGy as measured by an X-ray test device (NERO mAx 8000®, Fluke Biomedical, Cleveland, OH, USA). Multiples of 1.5 mGy were delivered acutely and without any time interval between the fractions. Four hours after irradiation, the cells were collected for the intracellular reactive oxygen species analysis. Changes in cell viability and the expression of Bcl-2, Bax and cyclin D1 were determined after irradiation, as described below.

2.3. Determination of intracellular ROS

The formation of ROS was evaluated using the oxidation-sensitive dye 2,7-dichlorofluorescein diacetate (DCFH-DA). The cell cultures that had been exposed to dental radiation were treated with 5 µmol/l DCFH-DA in de-ionised (DI) water for 30 min. The fluorescence intensity was determined within 5 min thereafter using a Fluostar Optima microplate spectrofluorometer (BMG Lab Technologies, Offenburg, Germany) at 485 nm excitation and 540 nm emission.¹⁶

2.4. MTT assay for cell viability

The viability of the MC3T3-E1 cells was investigated by MTT conversion after aspiration of the culture supernatants. Monolayers of MC3T3-E1 cells were replenished with culture medium and 50 µl MTT reagent [5 mg/ml in phosphate buffered saline (PBS)] were added. Culture plates were then incubated for 4 h at 37 °C in a humidified atmosphere (5% CO₂/95% air), after which the cells were washed twice with PBS. After the supernatants were aspirated off again, 200 µl dimethyl sulfoxide was added, and the optical density was measured at 570 nm using an automated plate reader (Spectramax 340PC; Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

2.5. Bcl-2, Bax, and cyclin D1 expression by western blot analysis

Cells were suspended in 200 µl lysis buffer [10 mM Tris-HCl, pH 7.4, 0.1% (SDS)] and supplemented with a protease inhibitor cocktail (Roche complete mini-tablets, Roche Molecular Biochemicals, Indianapolis, IND, USA). Samples were left on ice for 20 min and centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatant was collected. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated by gel electrophoresis and transferred to nitrocellulose membranes. Blots were incubated in 5% nonfat dried milk-TBS for 1 h at room temperature, followed by incubation with primary antibodies to cyclin D1(1:200), Bax(1:400), Bcl-2 (1:200) and β-actin (1:400) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After washing three times in TBS, the blots were incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Bands were detected using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA) and Kodak X-Omat Blue Film (Eastman Kodak, Rochester, NY, USA). Band

intensity was quantified by the Scion Image program and the results were shown as average signal intensity (arbitrary units).

2.6. Statistical analysis

Data were expressed as mean $\pm$ standard error (SE). The data were analysed using the Mann–Whitney *U* test to compare between all different experimental groups. Significance was assessed when $p < 0.05$. Statistical analyses were performed using the SPSS software, version 13.0 (SPSS Version 13.0, SPSS, Chicago, IL).

3. Results

3.1. Dental radiation has no effect on cell viability

Osteoblastic MC3T3-E1 cells were irradiated with 0 (0 mGy), 1 (1.5 mGy), 5 (7.5 mGy), 10 (15 mGy) multiples of a standard dose for a periapical film radiograph and cell viability was determined by MTT assay 24 h post-irradiation, since radiation-induced apoptosis begins delayed to a peak and extends to over about 24 h.¹⁹ There were no statistically significant differences in MTT activity in irradiated osteoblastic cells between the individual doses and the non-irradiated controls, as shown in Fig. 1. These findings suggest that dental irradiation did not affect cell viability 24 h following irradiation.

3.2. Low-dose dental irradiation reduces ROS production; however, high dose irradiation increases ROS production

Osteoblastic MC3T3-E1 cells were irradiated with 0, 1, 5, 10 multiples of a standard dose for a periapical film radiograph and intracellular ROS production was determined by a fluorescent probe, [2',7'-dichlorofluorescein diacetate (DCFH-DA)] 4 h post-irradiation. We determined ROS production 4 h post-irradiation because it has been shown that the level of antioxidant is at maximal levels 4 h after irradiation.²⁰ We found that one dose (1.5 mGy) of dental irradiation significantly decreased intracellular ROS production, but, 10 doses (15 mGy) of dental radiation significantly increased ROS production 4 h following irradiation when compared with the non-irradiated controls, as shown in Fig. 2.

3.3. Dental irradiation has no effects on the apoptotic process, but possibly decreases the cellular proliferation in osteoblastic cells

We investigated the expression of proteins that are linked to cell proliferation (cyclin D1) and cellular apoptosis (Bax/Bcl-2) 24 h after dental irradiation. We found that 10 doses dental irradiation significantly decreased cyclin D1 in osteoblastic cells when compared with the non-irradiated controls, as shown in Fig. 3. However, the ratio of Bax/Bcl-2 expression from osteoblastic cells demonstrated no statistical difference between irradiated cells and the controls. The average ratio of Bax/Bcl-2 after 0, 1, 5 and 10 doses of dental irradiation were 1 ± 0.0 , 1.052 ± 0.33 , 0.998 ± 0.061 and 1.128 ± 0.113 , respec-

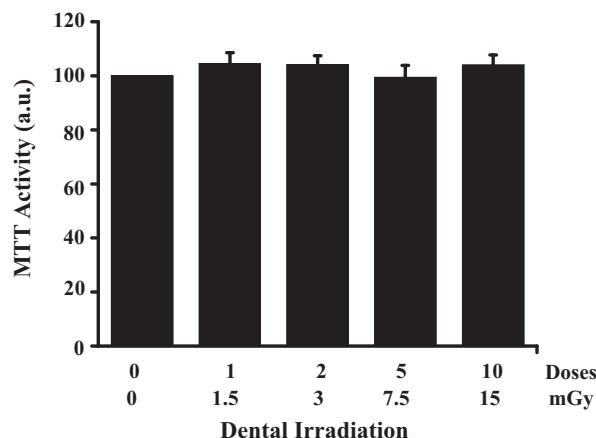


Fig. 1 – The effects of dental irradiation on cell viability. Osteoblastic cells were irradiated with 0, 1, 2, 5, 10 multiples of a standard doses of for a periapical film radiography (1.5 mGy/dose). Cell viability was assessed 24 h post-irradiation using MTT assay ($n = 5$). All doses of dental radiation did not affect the entire population of osteoblasts. Values represent the mean $\pm$ SE.

tively. These findings suggest that high doses of dental irradiation impair osteoblastic cell proliferation; however, the dental irradiation did not induce apoptotic activity in osteoblastic cells.

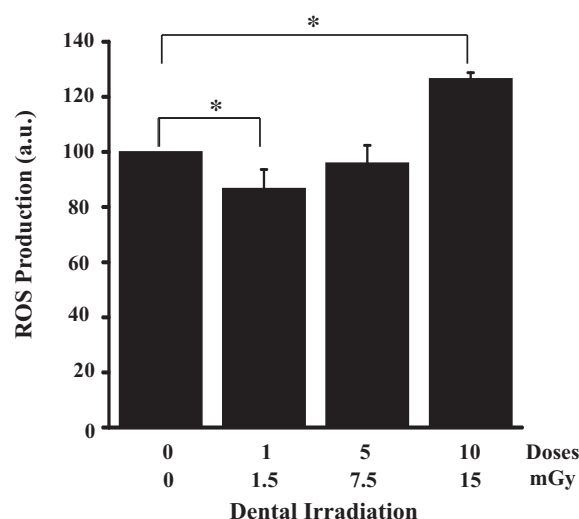


Fig. 2 – The effects of dental irradiation on intracellular ROS production. Osteoblastic cells were irradiated with 0, 1, 5, 10 multiples of a standard dose for a periapical film radiography (1.5 mGy/dose) ($n = 5$). ROS production was assessed 4 four hours post-irradiation using a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA). The ROS production was significantly reduced after one-dose of periapical radiography. However, after 10 doses of periapical radiation ROS production was significantly increased. Values represent mean $\pm$ SE. $p < 0.05$ compared to the controls (0 mGy).

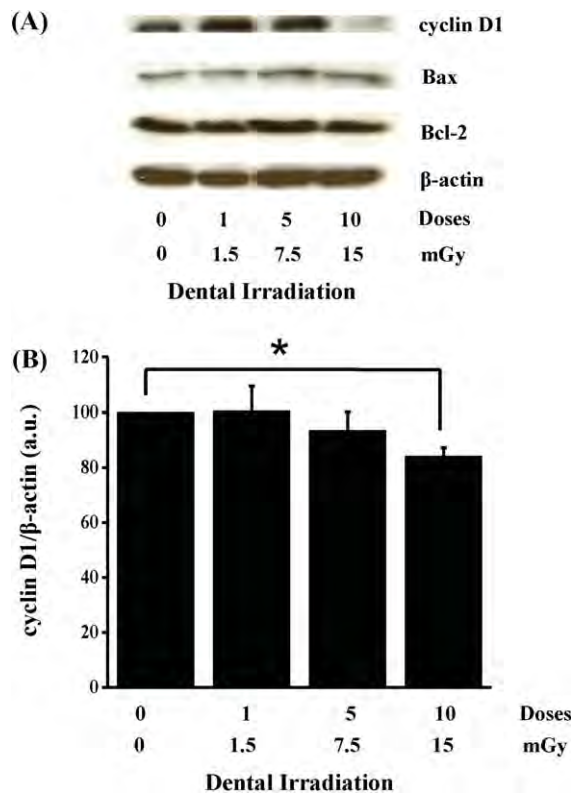


Fig. 3 – The effect of dental irradiation on cyclin D1, Bax and Bcl-2 expression. (A) A representative western blot analysis is shown. The western blot analyses were carried out on total protein lysates from cells irradiated with 0, 1, 5, 10 multiples of a standard dose for a periapical film radiography (1.5 mGy/dose) ($n = 4$). β -Actin was used as a loading control. **(B)** The levels of protein cyclin D1 significantly decreased when irradiated with 10 doses of periapical radiography (15 mGy) ($n = 4$). Values represent mean $\pm$ SE. * $p < 0.05$ compared to the controls (0 mGy).

4. Discussion

This is the first *in vitro* study demonstrating that periapical doses of irradiation affect osteoblastic cells. A previous clinical study illustrated that panoramic radiography induces chromosomal damage in oral mucosal cells.⁴ However, that finding cannot explain the various effects of dental irradiation on other types of oral cells because radiation responses depend on several factors, such as cell types, cell radiosensitivity, radiation dose and radiation dose-rate.¹⁷

The present study revealed that one dose of dental irradiation of about 1.5 mGy to osteoblastic cells significantly reduces ROS formation 4 h post-irradiation, but ROS production is significantly increased by 10 doses of periapical irradiation (15 mGy). Consistent with our findings, a previous study showed that after low-dose whole body irradiation (500 mGy) of mouse splenocytes, the level of glutathione, an antioxidant agent, increased and reached the maximal levels 4 h post-irradiation.¹⁸ Therefore, a decrease in ROS production following 1.5 mGy irradiation of osteoblastic cells may be

associated with an increase in the intracellular glutathione level. Several *in vitro* and *in vivo* studies have shown that low-dose irradiation can induce glutathione 3–6 h following irradiation.^{11–13} In contrast to 1-dose dental irradiation, the intracellular ROS production significantly increased, accompanied by decreased cell proliferation, as determined by decreasing cyclin D1 expression, when osteoblastic cells were irradiated with 10 doses of periapical irradiation (15 mGy). Cyclin D1 plays a regulatory role in cell cycle progression, especially during the G1 to S transitional phase. It has been shown that the reduction of cyclin D1 expression is associated with induction of G1 cell cycle arrest in differentiated osteoblasts.¹⁹ Thus, the 15 mGy irradiation may be high enough to cause arrest of the G1/S cell cycle in osteoblastic cells and to impair cellular proliferation, as shown by the reduction of cyclin D1 expression.

Radiation induced-apoptosis is one cellular response to severe DNA damage resulting from irradiation. The apoptosis occurs approximately 24 h following irradiation, as indicated by an increase in the Bax/Bcl-2 ratio.^{20,21} Our findings indicate that 15 mGy of dental irradiation is not high enough to induce osteoblastic cell death through the apoptotic process 24 h post-irradiation because the ratio of Bax to Bcl-2 and the MTT activity of irradiated osteoblastic cells did not differ from that of the controls. However, irradiated cells with DNA damage may not die immediately but they may undergo several cell division cycles before reaching a critical genomic instability in an apparent dose related manner, as shown in a previous study.²² Therefore, our study demonstrated a short term effect of dental irradiation on osteoblastic cells. The long-term effects of dental irradiation on osteoblastic cells should be further investigated.

The deleterious effects of dental irradiation on osteoblastic cells might be associated with the degree of intracellular ROS production by irradiation. Hydrogen peroxide is an important ROS produced from chemical changes in water after exposure to ionising radiation. Davies proposed that responses to ROS, particularly hydrogen peroxide, depend on its concentration.²³ Exposure to very low concentrations of hydrogen peroxide (3–15 μ M) causes cellular growth stimulation. One hundred and twenty to 150 μ M of hydrogen peroxide causes a temporary growth arrest in order to protect cells from excess energy use and DNA damage. A further increase to 500–1000 μ M of hydrogen peroxide causes cellular apoptosis. Our findings suggest that the level of hydrogen peroxide generated from 10 doses of periapical irradiation, 15 mGy, is enough to cause cell cycle arrest in G1/S in osteoblastic cells but it is insufficient for the induction of apoptotic cell death.

We conclude that dental irradiation causes various effects in osteoblastic cells depending on the dose of irradiation. Low-dose dental irradiation, or 1.5 mGy, seems to have a radio-adaptive response by detoxifying ROS as indicated by lowering ROS formation 4 h post-irradiation, whereas high-dose dental irradiation, or 15 mGy, impairs cellular proliferation. These results provide further support for the principle of ALARA (As Low As Reasonably Achievable). However, *in vivo* findings may be similar or different from our present study since radiation intensity is normally absorbed by soft tissues (i.e. skin and gingival) before reaching the osteoblasts. Furthermore, genotypic differences can play a role in this case due to different

radiosensitivity.²¹ Future studies in *in vivo* are needed to elucidate this hypothesis.

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Competing interest

None.

Ethical approval

Not required.

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PPAR γ Agonist Improves Neuronal Insulin Receptor Function in Hippocampus and Brain Mitochondria Function in Rats with Insulin Resistance Induced by Long Term High-Fat Diets

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We previously demonstrated that a high-fat diet (HFD) consumption can cause not only peripheral insulin resistance, but also neuronal insulin resistance. Moreover, the consumption of an HFD has been shown to cause mitochondrial dysfunction in both the skeletal muscle and liver. Rosiglitazone, a peroxisome proliferator-activated receptor- γ ligand, is a drug used to treat type 2 diabetes mellitus. Recent studies suggested that rosiglitazone can improve learning and memory in both human and animal models. However, the effects of rosiglitazone on neuronal insulin resistance and brain mitochondria after the HFD consumption have not yet been investigated. Therefore, we tested the hypothesis that rosiglitazone improves neuronal insulin resistance caused by a HFD via attenuating the dysfunction of neuronal insulin receptors and brain mitochondria. Rosiglitazone (5 mg/kg $\cdot$ d) was given for 14 d to rats that were fed with either a HFD or normal diet for 12 wk. After the 14th week, all animals were euthanized, and their brains were removed and examined for insulin-induced long-term depression, neuronal insulin signaling, and brain mitochondrial function. We found that rosiglitazone significantly improved peripheral insulin resistance and insulin-induced long-term depression and increased neuronal Akt/PKB-ser phosphorylation in response to insulin. Furthermore, rosiglitazone prevented brain mitochondrial conformational changes and attenuated brain mitochondrial swelling, brain mitochondrial membrane potential changes, and brain mitochondrial ROS production. Our data suggest that neuronal insulin resistance and the impairment of brain mitochondria caused by a 12-wk HFD consumption can be reversed by rosiglitazone. (*Endocrinology* 153: 329–338, 2012)

Insulin resistance is a pathological condition, in which target tissues cannot respond to plasma insulin at the optimal plasma insulin concentration (1). Several studies have suggested that peripheral insulin resistance is correlated with cognitive decline (2–5). In addition, a previous study by our group has shown that a 12-wk high-fat diet (HFD) consumption caused not only peripheral insulin resistance, but also neuronal insulin resistance by impairment of insulin receptor (IR) function (6).

In the central nervous system, brain mitochondria play an important role in energy-demanding neurotransmission and in controlling calcium homeostasis (7). Furthermore, mitochondria are important for ATP production via oxidative phosphorylation. Disruption of electron transport chains can lead to decreased ATP with increased reactive oxygen species (ROS) production (8, 9). Insulin resistance has been shown to dysregulate both glucose and lipid metabolisms and decrease the activity of mitochondria.

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Abbreviations: aCSF, Artificial cerebrospinal fluid; fEPSP, field excitatory postsynaptic potential; HFD, high-fat diet; HFR, HFD with rosiglitazone treatment; HFV, vehicle-treated HFD subgroup; HOMA, homeostasis model assessment; IR, insulin receptor; LTD, long-term depression; ND, normal diet; NDR, ND with rosiglitazone treatment; NDV, vehicle-treated ND subgroup; OGTT, oral glucose tolerance test; PPAR γ , peroxisome proliferator-activated receptor γ ; ROS, reactive oxygen species; SOD, superoxide dismutase.

drial oxidative phosphorylation (7, 10, 11). Recent studies have demonstrated that the consumption of a HFD leads to peripheral insulin resistance and also increases ROS production in adipocytes and liver (12–15). Those findings suggest that IR dysfunction may correlate with the dysfunction of mitochondria.

Rosiglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) agonist, has been used for the treatment of type 2 diabetes and insulin resistance by increasing insulin sensitivity (1, 16). PPAR γ is not only expressed in peripheral tissues but also in the central nervous system, including the hippocampus, an important area for learning and memory (17). Previous studies have demonstrated that rosiglitazone might improve learning and memory in both human and animal models (18–23). For example, rosiglitazone improved learning and memory as demonstrated in the Morris Water Maze test of HFD rats (22), prevented the effect of A β -induced neurodegeneration in Alzheimer's disease mouse models (17, 23), and promoted mitochondria biogenesis in mouse brain (24). However, the effect of rosiglitazone on neuronal insulin resistance and brain mitochondrial dysfunction induced by a HFD consumption has not yet been investigated. Therefore, in this study, we hypothesized that rosiglitazone improves not only peripheral insulin resistance but also neuronal insulin resistance via attenuating the dysfunction of neuronal IR, neuronal insulin signaling, and brain mitochondria, caused by HFD consumption.

Materials and Methods

Animals

Male Wistar rats weighing 180–200 g ($n = 48$) from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand, were used for this study. All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with National Institutes of Health guidelines. The animals were randomized into two groups: a control group that consumed a normal diet (ND), standard laboratory chow, which had an energy content of 4.02 kcal/g, and 19.77% of total energy (%E) of the food from fat (Mouse Food no. 082, C.P. Co., Bangkok, Thailand) and a high-fat group that received an HFD, which had an energy content of 5.35 kcal/g and contained fat mostly from lard (59.28% E) for a period of 12 wk (6). The animals were given free access to the diet and drinking water. To determine the peripheral insulin resistance, the body weight of each animal was measured every other week, and blood sampling from a tail vein was performed at wk 12 and 14 after fasting for 5 h to measure glucose, cholesterol, and insulin levels. Blood samples for glucose assay were kept on ice in NaF microcentrifugation tubes, whereas blood samples for cholesterol and insulin assay were kept on ice in

EDTA microcentrifugation tubes. All plasma was stored at -80°C for subsequent biochemical analysis.

After 12 wk, rats in both the ND and HFD groups ($n = 24/\text{group}$) were divided into two subgroups ($n = 12/\text{subgroup}$). Each subgroup received orally either 5 mg/kg $\cdot$ d of rosiglitazone (Cayman Chemical Co., Ann Arbor, MI) (dissolved in normal saline 2 ml/kg $\cdot$ d) for 14 consecutive days or a vehicle (normal saline, 2 ml/kg $\cdot$ d) for 14 consecutive days. At the end of the experimental period, the animals in each subgroup were killed to study either neuronal IR function [insulin-induced long-term depression (LTD) protocol and neuronal insulin-signaling protocol ($n = 6$)] or brain mitochondrial function (mitochondrial swelling, mitochondrial membrane potential ($\Delta\psi_m$), and mitochondrial ROS production) ($n = 6$). Before death, all animals were tested for glucose tolerance using the oral glucose tolerance test (OGTT).

Plasma analysis

Plasma glucose and cholesterol concentrations were determined using colorimetric assay, using a commercially available kit (Biotech, Bangkok, Thailand). Plasma insulin level was measured by Sandwich ELISA (LINCO Research, St. Charles, MO), with the mean intraassay variation of 1.33% and interassay variation of 6.71%.

Determination of insulin resistance [homeostasis model assessment (HOMA) index]

Insulin resistance was assessed by the HOMA index (25, 26), a mathematical model describing the degree of insulin resistance, calculated from fasting plasma insulin and fasting plasma glucose concentration. A higher HOMA index indicates a higher degree of insulin resistance. The HOMA index was determined by the following equation:

$$[\text{Fasting plasma insulin } (\mu\text{U/ml})] \times [\text{fasting plasma glucose (mmol/liter)}] / 22.5$$

OGTT

The OGTT was performed after the rats had been fasting overnight (12 h). Then the rats received a bolus of glucose (2.0 g/kg body weight) via gavage feeding, and plasma glucose of blood samples were collected from a tail vein at 0, 30, 60, 90, and 120 min after the administration of glucose in NaF microcentrifugation tubes. Plasma glucose was collected and analyzed using colorimetric assay (Biotech, Bangkok, Thailand) (6).

Preparation of brain slices and insulin stimulation

Rats were killed with isoflurane and decapitated. The brains were rapidly removed. After that, the brains were immersed in ice-cold high-sucrose artificial cerebrospinal fluid (aCSF), containing (in millimolar concentration) NaCl, 85; KCl, 2.5; MgSO $_4$, 4; CaCl $_2$, 0.5; NaH $_2$ PO $_4$, 1.25; NaHCO $_3$, 25; glucose, 25; sucrose, 75; kynurenic acid, 2; ascorbate, 0.5, saturated with 95% O $_2$ /5% CO $_2$ (pH 7.4). That solution enhanced neuronal survival during the slicing procedure. Hippocampal slices (400 μm) were cut using a vibratome (Vibratome Co., Saint Louis, MO). After a 30-min postslice incubation in high-sucrose aCSF, the brain slices were transferred to a standard aCSF solution containing (in millimolar concentration) NaCl, 119; KCl, 2.5; CaCl $_2$, 2.5; MgSO $_4$, 1.3; NaH $_2$ PO $_4$, 1; NaHCO $_3$, 26; and glucose, 10; saturated with 95% O $_2$ /5% CO $_2$ (pH 7.4) for an ad-

ditional 30 min at room temperature (22–24 °C). Some brain slices ($n = 5$ –6 brain slices per animal) were used for extracellular recording. Some brain slices ($n = 5$ –6 brain slices per animal) were used to investigate neuronal insulin signaling. In neuronal insulin-signaling protocols, brain slices with/without insulin stimulation were used by placing the brain slices into either aCSF plus insulin 500 nM (Humulin R, Eli Lilly, Giessen, Germany) or aCSF for 5 min. Then, those slices were homogenized for immunoblotting as described in a previous study (6).

Extracellular recording of hippocampal slices for insulin-induced LTD

To examine insulin-induced long term depression (LTD) as described previously, the brain slices were transferred to a submersion recording chamber and continuously perfused at 3–4 ml/min with standard aCSF warmed to 25–28 °C. Field excitatory postsynaptic potentials (fEPSP) were evoked by stimulating the Schaffer collateral-commissural pathway with a bipolar tungsten electrode, whereas the fEPSP recordings were taken from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 Mohm) filled with 2 M NaCl. A stimulus frequency of 0.033 Hz was used, and the stimulus intensity was adjusted to yield a fEPSP of 0.8–1.0 mV in amplitude, which produced less than 50% of the maximal monophasic response. The brain slices were perfused with aCSF (to establish a baseline condition) for 10 min, and then perfused with aCSF plus 500 nM insulin (to produce insulin-induced LTD) for an additional 10 min. Thereafter, the slices were perfused with aCSF again for a further 50 min and the fEPSP were recorded. Data were filtered at 3 kHz, digitized at 10 kHz, and stored in a computer using pClamp 9.2 software (Axon Instruments, Foster City, CA). The initial slopes of the fEPSP were measured and plotted against time, as described in a previous study (6).

Immunoblotting for neuronal insulin signaling

To investigate the expression of IR, Akt/PKB, and insulin-mediated Akt Ser473 phosphorylation, homogenized brain slices from each subgroup were boiled at 95 °C, for 5 min. Then, proteins were separated by electrophoresis on 10% polyacrylamide gels (SDS-PAGE) (Bio-Rad Laboratories, Inc., Hercules, CA), and transferred into polyvinylidene difluoride membranes. After blocking with 5% nonfat milk/TBST, immunoblotting was conducted with IR, Akt, Akt Ser473 antibody, and β -actin (rabbit polyclonals, 1:2000 in TBST; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight. Membranes were incubated with a secondary goat antirabbit antibody, conjugated with horseradish peroxidase (1:8000 in TBST, Bio-Rad Laboratories), and the protein bands were visualized on Amersham hyperfilm ECL using the Amersham ECL Western blotting detection reagents system (GE Healthcare, Buckinghamshire, UK). Band intensity was quantified by the Scion Image program (Scion Corp., Frederick, MD), and the results were shown as average signal intensity (arbitrary units).

Preparation of brain mitochondria

Brain mitochondria were isolated according to the protocol described in a previous study (27). The whole brain was removed and put into an ice-cold MSE solution (225 mM mannitol; 75 mM sucrose; 1 mM EGTA; 5 mM HEPES; 1 mg/ml BSA, pH 7.4) to wash out the blood rapidly. Each brain was transferred to 10 ml

ice-cold MSE-nagarse solution (0.05% nagarse in MSE solution) and homogenized at 600 rpm/min using a homogenizer. Next, the tissues were minced and centrifuged at $2000 \times g$ for 4 min, and the supernatants were then collected and centrifuged at $12,000 \times g$ for 11 min. Next, brain mitochondrial pellets were collected and resuspended in a 10-ml ice-cold MSE-digitonin solution (0.02% digitonin in MSE solution) to break down the synaptosome fraction, and the resulting brown pellets were collected. Finally, the pellets were minced with respiration buffer (150 mM KCl, 5 mM HEPES, 5 mM $K_2HPO_4 \cdot 3H_2O$, 2 mM L-glutamate, 5 mM pyruvate sodium salt) (28). Mitochondrial proteins were determined by BCA assay as described in a previous study (29).

Brain mitochondrial swelling assay

Isolated mitochondrial swelling was assessed by measuring changes in the absorbance of the suspension wavelength at 540 nm using a microplate reader. Brain mitochondria (0.4 mg/ml) were incubated in 2 ml of respiration buffer (containing 150 mM KCl, 5 mM HEPES, 5 mM $K_2HPO_4 \cdot 3H_2O$, 2 mM L-glutamate, 5 mM pyruvate sodium salt). Decreasing absorbance represents mitochondrial swelling.

Brain mitochondrial membrane potential ($\Delta\psi_m$) assay

The brain mitochondrial membrane potential was measured by fluorescent dye JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazole carbocyanine iodide), which accumulates in mitochondria. JC-1 dye, a monomer form, enters mitochondria and produces a green fluorescence (emission wavelength at 530 nm) at low mitochondrial membrane potential, whereas at high mitochondrial membrane potential, JC-1 dye changes its form to be a JC-1 aggregate form, which produces a red fluorescence (emission wavelength at 590 nm). The depolarization of brain mitochondrial membrane potential is indicated by a decreased red/green fluorescence intensity ratio.

The isolated brain mitochondria (0.4 mg/ml) were strained with JC-1 dye at 37 °C for 15 min, after which the fluorescence intensity was determined using a fluorescence microplate reader. JC-1 monomer form (green) fluorescence was excited at 485 nm, and the emission was detected at 530 nm. JC-1 aggregate form (red) fluorescence was excited at 485 nm, and emission fluorescence was detected at 590 nm (29).

Brain mitochondrial ROS assay

Isolated brain mitochondria were stained by 2',7'-dichloro-hydro-fluorescein diacetate that can pass through the cell membrane and is hydrolyzed by intracellular esterase. ROS oxidizes dichlorohydro-fluorescein and converts dichlorohydro-fluorescein to 2',7' dichlorodihydro-fluorescein diacetate, which is highly fluorescent at 485 nm, and the emission was detected at 528 nm. Therefore, ROS production causes an increase in fluorescence intensity. The isolated neuronal mitochondria (0.4 mg/ml) were stained with 2 μ M 2',7' dichlorohydro-fluorescein diacetate and incubated at room temperature for 20 min. The fluorescence intensity was detected using a fluorescence microplate reader as described in a previous study (29).

TABLE 1. Effect of rosiglitazone on body weight, visceral fat, cholesterol, glucose, insulin, HOMA index, and OGTT AUC (area under the curve) in rats fed with ND and HFD

Parameter	NDV	NDR	HFV	HFR
Body weight (g)	431.50 $\pm$ 5.68	460.33 $\pm$ 4.13 ^a	546.25 $\pm$ 12.10 ^{a,b}	603.3 $\pm$ 9.86 ^{a,b,c}
Visceral fat (g)	25.22 $\pm$ 1.11	32.57 $\pm$ 1.26 ^a	47.94 $\pm$ 2.88 ^{a,b}	61.90 $\pm$ 3.61 ^{a,b,c}
Cholesterol (mg/dl)	234.78 $\pm$ 6.38	227.67 $\pm$ 6.82	301.45 $\pm$ 9.81 ^{a,b}	251.55 $\pm$ 8.24 ^{b,c}
Glucose (mg/dl)	130.88 $\pm$ 4.26	101.39 $\pm$ 3.25 ^a	140.68 $\pm$ 3.65 ^b	122.01 $\pm$ 2.14 ^{b,c}
Insulin (ng/ml)	1.72 $\pm$ 0.13	1.84 $\pm$ 0.14	3.85 $\pm$ 0.18 ^{a,b}	2.05 $\pm$ 0.08 ^c
HOMA index	9.80 $\pm$ 0.51	8.11 $\pm$ 0.44	24.06 $\pm$ 1.26 ^{a,b}	10.8 $\pm$ 0.56 ^{b,c}
OGTT AUC (a.u.)	45,814 $\pm$ 789	39,875 $\pm$ 895 ^a	63,020 $\pm$ 1026 ^{a,b}	49,722 $\pm$ 935 ^{a,b,c}

^a $P \leq 0.05$ vs. NDV; ^b $P \leq 0.05$ vs. NDR; ^c $P \leq 0.05$ vs. HFR.

Data analysis

Data were recorded as mean $\pm$ SE. For all comparisons, the significant differences in body weight and peripheral biochemical parameters were calculated using one-way ANOVA followed by Fisher's least significant difference *post hoc* analysis. The percentages of insulin-induced LTD and brain mitochondrial experiments were calculated using Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Results

Rosiglitazone reduced peripheral insulin resistance in HFD-fed rats

The effects of rosiglitazone on body weight, visceral fat, and biochemical parameters in plasma are shown in Table 1. The 14-wk HFD-fed rats had a significantly increased body weight, visceral fat, plasma cholesterol level, plasma insulin level, and HOMA index, compared with the ND-fed rats ($P < 0.05$). Rats from both dietary groups that were treated with rosiglitazone (5 mg/kg $\cdot$ d, 14 d) had significantly increased body weight and visceral fat, compared with the vehicle groups ($P < 0.05$). Rosiglitazone significantly decreased plasma cholesterol level, plasma insulin level, and HOMA index in only HFD-fed rats ($P < 0.05$), but significantly decreased plasma glucose level in both dietary groups ($P < 0.05$).

For the glucose tolerance test, the mean area under the curve of the vehicle-treated HFD subgroup (HFV) was significantly greater than that of the vehicle-treated ND subgroup (NDV) (Table 1). The administration of rosiglitazone significantly decreased the area under the curve in both dietary groups ($P < 0.05$).

Rosiglitazone improved neuronal IR function in HFD-fed rats

The characteristics of insulin-induced LTD are shown in Fig. 1A. Insulin of various concentrations (from 0 to 1000 nM) was applied onto the hippocampal slices from the NDV rats to determine the effect of insulin-induced LTD. We found that this effect was dose dependent. In this study, 500

nM insulin was used to investigate the insulin-induced LTD in the hippocampus because it has been shown previously that 500 nM insulin is the optimal dose to determine insulin-induced LTD and to measure the IR function (6, 30, 31).

In the HFV group, an HFD could attenuate insulin-induced LTD, compared with the NDV group (Fig. 1B). In both NDV and ND with rosiglitazone treatment (NDR) groups, the application of insulin led to insulin-induced LTD, and the phenomenon was stabilized at a level of 74.43% and 80.79% of preinsulin baseline, for the NDV and NDR, respectively (Fig. 1C). In the HFD groups, the insulin-induced LTD in the vehicle-treated HFD group was significantly reduced, with the mean percentage depression of insulin-induced LTD of $5.7 \pm 4.6\%$ of the preinsulin baseline (Fig. 1D). However, rosiglitazone treatment in the HFD rats could completely abolish the impairment of insulin-induced LTD caused by the HFD found in the HFD group (insulin-induced LTD: $64.3 \pm 4.8\%$ of preinsulin baseline, Fig. 1D). These findings suggest that HFD consumption could cause the neuronal IR dysfunction and ROS could prevent this undesired effect.

To confirm whether rosiglitazone can reverse or enhance neuronal insulin signaling of neuronal insulin resistance after HFD, the Ser473 phosphorylation of Akt/PKB between both dietary groups, either with or without rosiglitazone treatment, was investigated. The serine 473 phosphorylation of Akt/PKB was used as a marker for the neuronal IR signaling activity, because we previously showed that neuronal insulin resistance after HFD consumption led to the reduction of serine 473 phosphorylation of Akt/PKB (6). In this study, we found that, without insulin stimulation, the protein levels of IR, Akt/PKB and the serine phosphorylation of Akt/PKB in all treated subgroups, *i.e.* ND with vehicle treatment (NDV), ND with rosiglitazone treatment (NDR), HFV, and HFD with rosiglitazone treatment (HFR), were not significantly different (Fig. 2, A, B, and C, respectively). However, with insulin stimulation the serine phosphorylation of Akt/PKB in the HFV subgroup was significantly decreased, com-

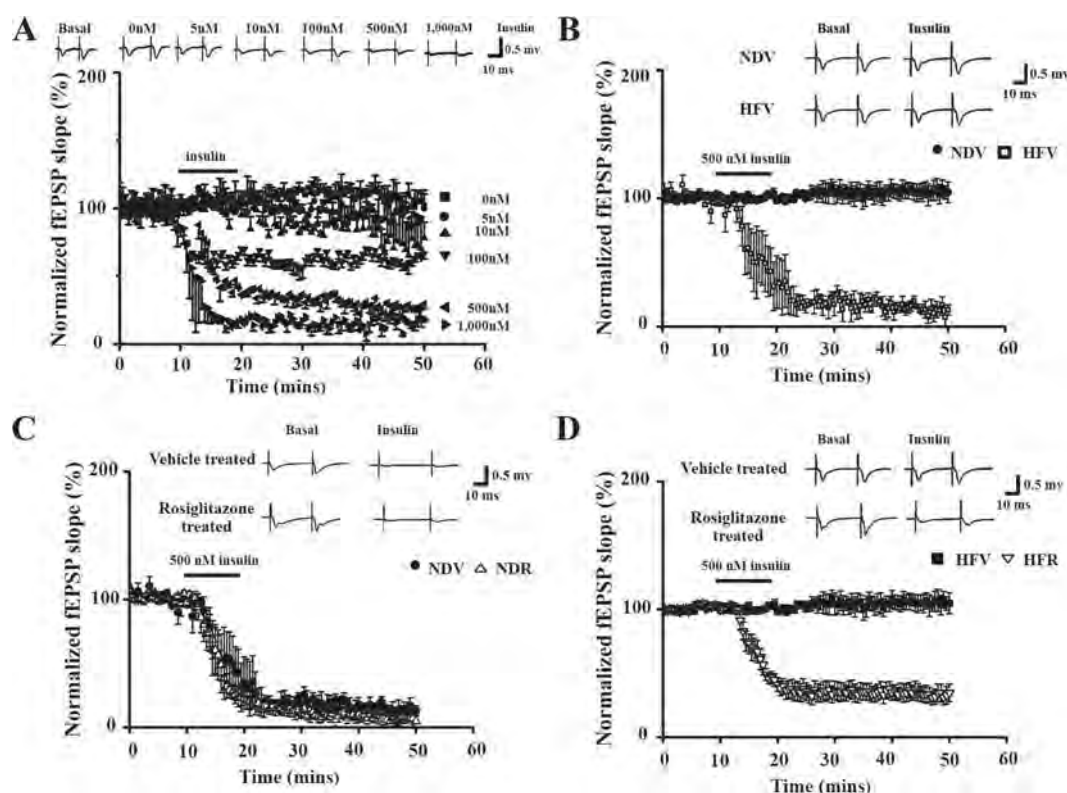


FIG. 1. HFD feeding significantly diminished the ability of insulin to induce LTD in the CA1 hippocampus. Rosiglitazone, 14 d after 12-wk HFD, significantly improved the ability of insulin to induce LTD in the CA1 hippocampus. **A**, The phenomenon of insulin-induced LTD in NDV animals ($n = 3/\text{dose}$). **B**, Insulin-induced LTD in the brain slices from NDV and HFV subgroup, confirming the impairment of insulin-induced LTD in CA1 hippocampus after HFD feeding. **C**, Insulin-induced LTD in the brain slices from NDV and NDR subgroup. **D**, Insulin-induced LTD in the brain slices from HFV and HFR subgroup. *Inset* in each panel shows examples of averages of 20 consecutive traces taken from a slice treated with aCSF (basal) and with 500 nM (insulin). **A–D**, A summary of averages of normalized fEPSP (fEPSPt/fEPSPo with fEPSP being points at which fEPSP slopes stabilized) from NDV ($n = 6–8$ independent slices), NDR ($n = 6–8$ independent slices) brain slices, HFV ($n = 6–8$ independent slices), and HFR ($n = 6–8$ independent slices) brain slices. There were no differences between the insulin-induced LTD in NDV and NDR subgroups. However, the HFR subgroup showed a significant increase in insulin-induced LTD compared with the HFV subgroup.

pared with the NDV subgroup ($P < 0.05$, Fig. 2D). The administration of rosiglitazone significantly increased Akt/PKB phosphorylation with insulin stimulation in only the HFD group ($P < 0.05$, Fig. 2D). All of these findings indicate that rosiglitazone could attenuate the neuronal insulin resistance induced by HFD consumption by preserving both neuronal IR function and neuronal IR signaling.

Rosiglitazone reduced brain mitochondrial dysfunction from HFD consumption

In addition to the effect of rosiglitazone on improving the neuronal insulin resistance induced by HFD consumption, we further investigated whether rosiglitazone can improve brain mitochondrial dysfunction after HFD consumption. We investigated the dysfunction of brain mitochondria by measuring brain mitochondrial swelling, brain ROS production, and brain mitochondrial membrane potentials ($\Delta\psi_m$). We found that brain mitochondrial morphology in the HFV subgroup was changed, with the mitochondria unfolding and swelling, whereas

rosiglitazone treatment did not change the brain mitochondrial morphology in the HFD subgroup (Fig. 3A).

To investigate the brain mitochondrial swelling, we measured the absorbance of brain mitochondria in all four treatment subgroups (NDV, NDR, HFV, HFR). The absorbance of brain mitochondria in the HFV subgroup was decreased compared with that in the NDV subgroup, indicating that brain mitochondria in the HFV subgroup were swollen (Fig. 3B). In HFD rats, we found that the absorbance of brain mitochondria in the rosiglitazone-treated (HFR) subgroup was significantly increased, compared with that in the vehicle-treated (HFV) subgroup (Fig. 3B). These findings suggest that rosiglitazone can attenuate brain mitochondrial swelling after HFD consumption. We also demonstrated that the HFV subgroup had significantly increased ROS levels after H_2O_2 application, compared with the NDV subgroup (Fig. 4A). Rosiglitazone treatment significantly reduced brain mitochondrial ROS levels in both dietary groups during oxidative stress stimulation (Fig. 4A).

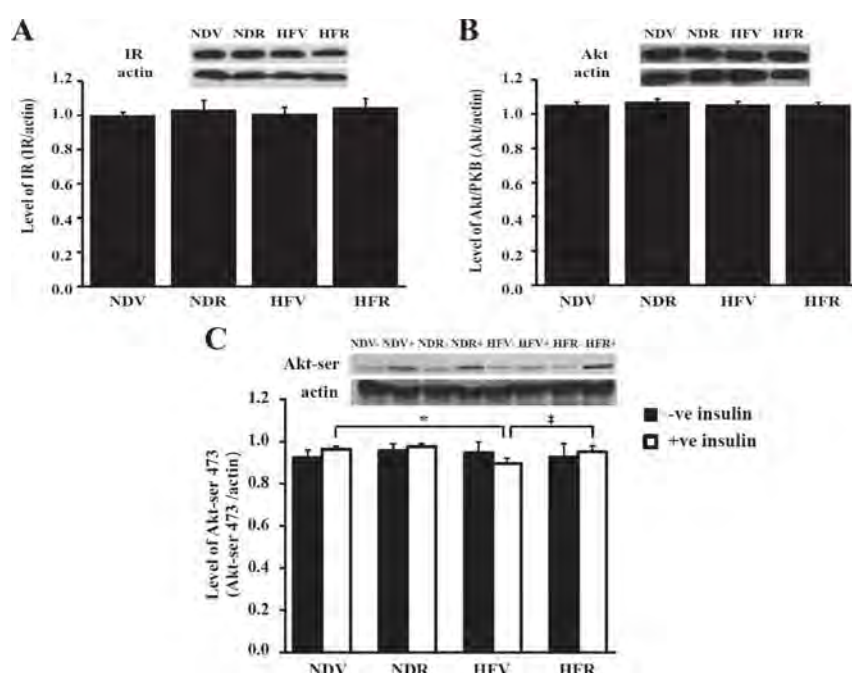


FIG. 2. A and B, There was no change in the protein levels of IR (A) and Akt/PKB (B) in any group. All immunoblot lanes were loaded with the same amount of protein (30 μ g/lane). C, The Ser473 phosphorylation did not change in any group without insulin stimulation. Rosiglitazone significantly improved insulin-induced phosphorylation of Akt/PKB at Ser473 residue in HFD consumption. Insulin-induced phosphorylation of Akt/PKB at Ser473 residue was significantly weakened in HFV group ($P < 0.05$). However, insulin-induced phosphorylation of Akt/PKB at Ser473 of the HFR subgroup significantly increased compared with the HFV subgroup ($P < 0.05$). All immunoblotting lanes were loaded with the same amount of protein (60 μ g/lane). *, $P < 0.05$ vs. NDV; #, $P < 0.05$ vs. HFV.

For brain mitochondrial membrane potential change, we found that the rate of $\Delta\psi_m$ changes in the HFV subgroup was greater than that in the NDV subgroup after H_2O_2 application (Fig. 4B). Rosiglitazone treatment decreased the rate of $\Delta\psi_m$ changes in both dietary groups (Fig. 4B). These findings suggest that brain mitochondria in the HFV subgroup were more depolarized than in the NDV subgroup during oxidative stress stimulation, and that rosiglitazone can attenuate the mitochondrial membrane depolarization caused by HFD consumption. All of these mitochondrial findings suggest that HFD consumption can lead to brain mitochondrial dysfunction and that rosiglitazone can attenuate the brain mitochondrial dysfunction caused by HFD.

Discussion

The major findings of our study are as follows: 1) rosiglitazone prevents the impairment of neuronal insulin-induced LTD in neuronal insulin-resistant rats caused by HFD consumption; 2) rosiglitazone improves IR signaling dysfunction by increasing ser 473 Akt/PKB phosphorylation in neuronal insulin-resistant rats caused by HFD consumption; 3) HFD consumption can cause brain

mitochondrial dysfunction; and 4) rosiglitazone reverses the brain mitochondrial dysfunction by preventing mitochondrial swelling, decreasing ROS production, and attenuating brain mitochondrial membrane potential changes, particularly during oxidative stress stimulation.

Consistent with our previous study (6), we also found that 14-wk HFD-fed rats (59.28% energy of fat) exhibited not only peripheral insulin resistance, as indicated by excessive body weight gain and visceral fat, hypercholesterolemia, hyperinsulinemia, increased HOMA index, and the impairment of OGTT, but also neuronal insulin resistance indicated by decreased insulin-induced LTD. Previous studies have shown that HFD consumption can impair neuronal function, such as the impairment of spatial learning in both radial arm maze and Morris Water Maze tests in rats (22, 32), the reduction of hippocampal dendritic spines (4), the reduction of long-term potentiation (4), increased brain malondialdehyde (33), and decreased brain-derived neurotrophic factor (4). In this study, we have demonstrated, for the first time, that 14-wk HFD consumption can cause brain mitochondrial dysfunction. Consumption of HFD has been shown to increase ROS production in kidney, liver, and skeletal muscle (34, 35), decrease mitochondrial sizes and numbers in liver and muscle (36), and reduce mitochondrial membrane potential in the liver (13, 37). In our study, HFD consumption disturbs brain mitochondrial functions, as indicated by mitochondrial morphological changes, increased swelling, increased ROS production, and mitochondrial depolarization.

Brain mitochondria are important organelles for maintaining intracellular Ca^{2+} levels and play an important role in synaptic transmission (38). The dysfunction of brain mitochondria has been shown to cause the impairment of synaptic plasticity (38). In our study, the brain mitochondrial dysfunction may be responsible for the impairment of insulin-induced LTD in rats fed with HFD. Increased brain mitochondrial ROS production caused by HFD consumption may cause the opening of mitochondrial permeability transition, leading to brain mitochondrial swelling and the depolarization of mitochondrial membrane potential (29, 39). These ROS-mediated mitochondrial changes can induce Akt dephosphorylation

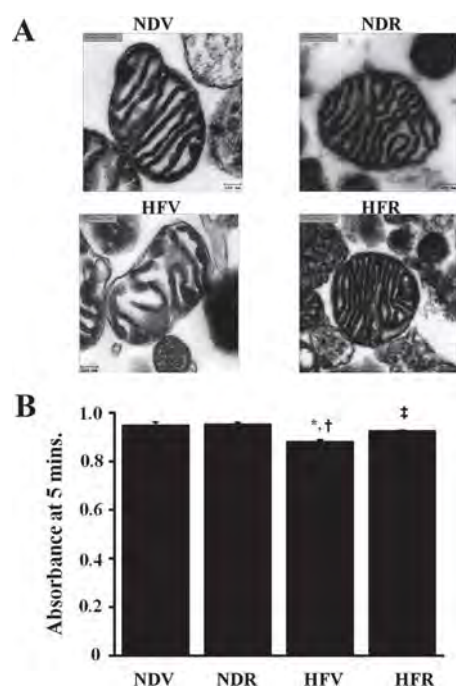


FIG. 3. A, Transmission electron microscopy (original magnification, $\times 25,000$) shows the ultrastructure of brain mitochondria in NDV, NDR, HFV, and HFR. Rosiglitazone prevented the morphology change after HFD consumption. Scale bar, 1:100 nm. B, HFD consumption induced mitochondrial swelling as measured by absorbance at 540 nm. Rosiglitazone significantly decreased mitochondrial swelling in both dietary groups. *, $P < 0.05$ vs. NDV; †, $P < 0.05$ vs. NDR; ‡, $P < 0.05$ vs. HFV.

at Ser-473 (40), indicating the disruption of downstream insulin signaling. Therefore, neuronal insulin resistance induced by HFD consumption might be developed from the brain mitochondrial dysfunction.

Rosiglitazone has been shown to increase the expression of IR and stimulate tyrosine phosphorylation of IR in the brown adipocytes of fetal rats (41). In our study, rosiglitazone increased Akt/PKB ser-473 phosphorylation in the HFD subgroup and also tended to increase that in the NDV subgroup. An increase in the phosphorylation of Akt/PKB may be responsible for the improvement of insulin-induced LTD in rosiglitazone-treated HFD rats, because increased Akt/PKB phosphorylation can lead to increased intracellular Ca^{2+} levels, thus restoring the insulin-induced LTD in the brain (31).

Because rosiglitazone can cross the blood-brain barrier rapidly after ip administration (42), the effect of rosiglitazone on brain function may be the result of direct interaction of rosiglitazone with neurons. Several studies support the direct effects of rosiglitazone on neurons. First, the administration of rosiglitazone to HFD rats can improve learning and memory, as demonstrated in the Morris Water Maze test (21, 22). Second, rosiglitazone can increase dendritic spine density in neuronal culture models (43). Third, in the Tg2576

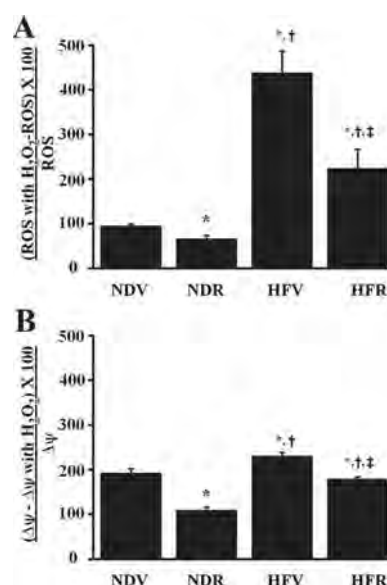


FIG. 4. A, Brain mitochondrial ROS production was measured by fluorescent dye during 2 mM H_2O_2 application onto brain mitochondria. Rosiglitazone significantly reduced ROS production after H_2O_2 application in both ND and HFD consumption. B, HFD consumption induced an increase in mitochondrial membrane potential change ($\Delta\psi_m$) during 2 mM H_2O_2 application to brain mitochondria, measured by fluorescent dye. Rosiglitazone significantly decreased brain mitochondrial membrane potential change ($\Delta\psi_m$) after H_2O_2 application in both ND and HFD consumption. *, $P < 0.05$ vs. NDV; †, $P < 0.05$ vs. NDR; ‡, $P < 0.05$ vs. HFV.

mouse model for Alzheimer's disease, rosiglitazone can reduce amyloid peptide 42 in the brain (21). Those previous reports and the findings in this study suggest that rosiglitazone passes the blood-brain barrier and may act directly in the brain to improve neuronal IR function as well as improve peripheral insulin sensitivity.

This study has also shown that rosiglitazone protects brain mitochondrial dysfunction caused by HFD by preserving conformational changes, preventing mitochondrial swelling, attenuating ROS production, and decreasing mitochondrial membrane potential dissipation. Rosiglitazone has been shown to have neuroprotective effects (44) and has beneficial effects on brain mitochondria (45–47). Rosiglitazone increases the production of at least two antioxidant agents, Cu/Zn-superoxide dismutase (SOD) and Mn-SOD, in traumatic brain injury, leading to decreased ROS production (48). It also increases SOD activity and decreases lipid peroxidation production (49), resulting in decreased ROS production. Furthermore, rosiglitazone, in a dose-dependent manner, increase mitochondrial activity by regulating ATP production and transcription of mitochondrial structural proteins and cellular antioxidant enzymes, thus attenuating ROS level (50). Moreover, rosiglitazone prevents the depolarization of mitochondrial membrane potential in brain oxygen-glucose deprivation followed by reoxygenation (51, 52).

Rosiglitazone protects hippocampal and dorsal root ganglion neurons against A β -induced mitochondrial damage and nerve growth factor deprivation-induced apoptosis by up-regulation of Bcl-2 (46). Those previous findings suggest that the role of PPAR γ agonists is to help in mitochondrial biogenesis and the repair of mitochondria during cellular injury or cell death. Consistent with those previous studies, our study showed that rosiglitazone can reduce brain mitochondrial dysfunction caused by 12-wk HFD consumption by reducing ROS production and decreasing mitochondrial membrane depolarization. It has also been shown that the reduction of ROS production can prevent the dissipation of mitochondrial membrane potential (48). In addition, keeping ROS production at a low level has been shown to improve insulin signaling (53). Therefore, the effects of rosiglitazone in reducing brain mitochondrial dysfunction may be one of the reasons that rosiglitazone improves neuronal insulin sensitivity in the brain. In addition, the improvement of brain mitochondrial dysfunction found in the rosiglitazone-treated group could also improve other metabolic control in the brain such as the hypothalamic insulin signaling, which plays an important role in maintaining normal glucose homeostasis (54).

In conclusion, our findings suggest that rosiglitazone improves not only peripheral insulin resistance but also neuronal insulin resistance in hippocampal regions caused by HFD consumption. The improvement of neuronal insulin sensitivity may occur via the effect of rosiglitazone on attenuating brain mitochondrial dysfunction. Therefore, PPAR γ agonists may be a useful medicine to ameliorate neuronal insulin resistance.

Limitation of this study

Although the direct evidence whether rosiglitazone acted directly on the brain to attenuate the neuronal insulin resistance and brain mitochondrial dysfunction induced by HFD consumption was not provided in this study, it has been clearly shown previously that rosiglitazone could pass the blood-brain barrier (42). In that study, they determined the brain concentration of rosiglitazone in normal male gerbils receiving ip injection of rosiglitazone at a dose of 3 mg/kg. They found that the mean concentration of rosiglitazone in the brain increased during the first 15 min and reached a plateau at 90–120 min, suggesting that rosiglitazone could pass blood-brain barrier even in a normal condition. Nevertheless, it is possible that rosiglitazone could also improve the peripheral insulin resistance and other systemic effects, subsequently resulting in improvement of neuronal insulin resistance caused by a HFD consumption. Furthermore, the change in the PPAR γ target gene in the brain was not investigated

in the present study. Finally, because the function of IR and the phosphorylation of Akt in response to insulin were analyzed in the hippocampal slices, the effects on other neuronal populations could be similar or different according to their anatomical areas.

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Heart Rate Variability and Exercise Capacity of Patients With Repaired Tetralogy of Fallot

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Abstract Heart rate variability (HRV) has been used as a reliable method to detect cardiac autonomic nervous system activity. Peak oxygen uptake (VO_2 peak) has been a predictor of death for adults with repaired tetralogy of Fallot (TOF). This study investigated the correlation between HRV and exercise capacity in 30 patients with TOF after surgery for total correction. The median age of the patients was 14 years (range, 9–25 years), and the median follow-up period was 11.6 months (range, 5.3–20.2 months). Low- and high-frequency-domain HRV significantly correlated with VO_2 peak ($r = 0.56$, $P = 0.001$ and $r = 0.44$, $P = 0.02$, respectively). After the 1-year follow-up evaluation, VO_2 peak and HRV analysis did not differ from those at entry to the study. However, low- and high-frequency-domain HRV still correlated significantly with VO_2 peak ($r = 0.43$, $P = 0.03$ and $r = 0.52$, $P = 0.007$, respectively). Left ventricular early diastolic myocardial velocity was most closely correlated with the VO_2 peak ($r = 0.51$, $P = 0.005$). Impaired cardiovascular autonomic control and left

ventricular diastolic dysfunction may be responsible for exercise intolerance in patients with repaired TOF. Long-term follow-up evaluation with exercise testing and 24-h Holter monitoring are warranted.

Keywords Exercise capacity · Heart rate variability · Repaired tetralogy of Fallot

The surgical repair of tetralogy of Fallot (TOF) can be performed with a low mortality rate and an excellent long-term outcome [1, 3, 6, 8, 15, 16, 19, 22, 25, 26]. However, some hemodynamic abnormalities may be found after surgery including pulmonary stenosis, pulmonary regurgitation, and myocardial dysfunction. The majority of patients have no symptoms resulting from these abnormalities. However, evaluation of exercise capacity shows cardiopulmonary compromise in some patients with repaired TOF [5, 17, 24, 27–30, 32].

Peak oxygen uptake (VO_2 peak) has been an independent predictor of death for adults with repaired TOF [13]. Heart rate variability (HRV) has been used as a reliable method to detect autonomic nervous system activity [31]. Ventricular tachycardia, progressive heart failure, and sudden cardiac death have been the late complications in the long term [12, 14, 23]. Reduced HRV is a predictor of sudden cardiac death after myocardial infarction and chronic heart failure [18, 20]. Previous studies have demonstrated that patients with TOF after complete repair have a reduction of HRV [4, 9, 10, 21].

To our knowledge, no previous studies of HRV have been correlated with exercise capacity in pediatric and adolescent patients with repaired TOF. The early recognition of patients at greater risk for exercise intolerance and

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increased morbidity is of clinical relevance for refinement in management. Therefore, in the current study, echocardiography, exercise testing, and a 24-h Holter electrocardiography (ECG) were evaluated among patients with repaired TOF. We hypothesized that heart rate variability is correlated with exercise intolerance in patients with TOF after surgical repair.

Methods

Study Patients

We prospectively studied 30 patients with TOF who underwent total surgical correction at Chiang Mai University Hospital. Electrocardiography, echocardiography, exercise testing, and a 24-h Holter ECG were performed at entry to the study and at the 1 year follow-up examination. The relationship between HRV analysis and exercise capacity was evaluated during the 1-year follow-up exam. The study protocol was reviewed and approved by the Chiang Mai University Review Board. All the patients or their parents consented to research participation.

HRV Analysis

A 24-h ECG was recorded for HRV analysis. The recordings were reviewed before the HRV was determined by the analysis software. The HRV analysis included frequency domain and time domain. The frequency-domain HRV parameters were low-frequency power (LF), high-frequency power (HF), and the LF/HF ratio. The time-domain HRV parameters included the standard deviation of all the normal sinus R-R intervals in the entire 24-h recording (SDNN) and the standard deviation of all the average normal sinus R-R intervals for all the 5-min segments in the 24-h recordings (SDANN).

Exercise Test

An exercise test was performed using an electric cycle ergometer (Corival, Lode, Groningen, Netherlands). All patients performed a maximal exercise test with a 2-min incremental bicycle protocol with a workload increment of 20 W for females and 25 W for males. The electrocardiogram, oxygen saturation, and blood pressure were monitored. The VO_2 peak, carbon dioxide production (VCO_2), minute ventilation (V_E), and respiratory exchange ratio (RER) were determined by using the breath-by-breath technique (Ultima CPX, Medgraphics, St. Paul, MN, USA). The test was terminated according to American College of Sports Medicine (ACSM) guidelines [2].

Doppler Echocardiography

Doppler echocardiographic examinations were performed using Philips Sonos 7500 (Philips Medical Systems, Bothell, WA). Echocardiographic data included left ventricular fractional shortening, pulse-wave Doppler assessment of the tricuspid valve and mitral valve, and tissue Doppler imaging. A tissue Doppler imaging signal was obtained from an apical four-chamber view at the right ventricular free wall, the ventricular septum, and the left ventricular free wall. The tissue Doppler imaging variables included systolic myocardial velocity (Sm), early diastolic myocardial velocity (Em), and late diastolic myocardial velocity (Am). The myocardial performance index was calculated by the atrioventricular valve closing-to-opening time minus the ventricular ejection time divided by the ejection time.

Statistical Analysis

All statistical calculations were assessed using commercially available software (SPSS Version 16, SPSS Inc., Chicago, IL, USA). Linear regression analysis was used to assess the correlation between the HRV and the VO_2 peak. Comparison of parameters between those at study entry and those at the 1-year follow-up assessment was performed using the Wilcoxon rank sum. Multiple linear regression analysis was used for the predictive model of VO_2 . A *P* value less than 0.05 was considered statistically significant.

Results

The study enrolled 30 patients (11 females [37%] and 19 males; median age, 14 years; range, 9–25 years) with repaired TOF. The median follow-up period was 11.6 months (range, 5.3–20.2 months). The baseline characteristics are reported in Table 1. The median time from surgical repair for tetralogy of Fallot to exercise testing was 9 years (range, 2–16 years). Six patients had a previously modified Blalock-Taussig shunt. Right ventricular outflow tract reconstruction with a transannular patch was performed for 14 patients (47%). Echocardiography, exercise testing, and HRV analysis at study entry and at the 1-year follow-up assessment are compared in Table 2. Echocardiographic parameters, VO_2 peak, and HRV analysis did not differ during the 1-year follow-up period.

Relationship of Heart Rate Variability to VO_2 Peak

The results for the relationship between the HRV and the VO_2 peak are graphically displayed in Fig. 1. Low- and high-frequency-domain HRV significantly correlated with

Table 1 Demographic and baseline characteristics of patients with repaired tetralogy of Fallot (TOF) ($n = 30$)

	TOF patients at entry (mean $\pm$ SD)
Age (years)	15.8 $\pm$ 4.4
Age at operation (years)	6.3 $\pm$ 3.0
Follow-up time from operation (years)	9.5 $\pm$ 3.0
Female: n (%)	11 (37)
BSA (m^2)	1.3 $\pm$ 0.2
Heart rate at rest (beats/min)	78 $\pm$ 11
Systolic BP (mmHg)	106 $\pm$ 13
Diastolic BP (mmHg)	65 $\pm$ 12
Previous Blalock-Taussig shunt: n (%)	6 (20)
Transannular patch: n (%)	14 (47)
QRS duration (ms)	138 $\pm$ 25
Pro-BNP (pg/ml)	198 $\pm$ 178

SD standard deviation, BSA body surface area, BP blood pressure, BNP brain natriuretic peptide

VO₂ peak ($r = 0.56$, $P = 0.001$ and $r = 0.44$, $P = 0.02$, respectively). After the 1 year follow-up period, VO₂ peak and HRV analysis did not differ from those at entry to the study. However, low- and high-frequency-domain HRV still correlated significantly with the VO₂ peak at the 1-year follow-up assessment ($r = 0.43$, $P = 0.03$ and $r = 0.52$, $P = 0.007$, respectively).

Relationship of Pulse Wave Doppler and Tissue Doppler Data to VO₂ Peak

Left ventricular early diastolic myocardial velocity was most closely correlated with the VO₂ peak ($r = 0.51$, $P = 0.005$). The ratio of peak early ventricular filling velocity to early diastolic myocardial velocity (E/Em) significantly correlated with VO₂ peak ($r = -0.50$, $P = 0.006$).

Predictors for VO₂ Peak

Using multiple regression analysis, female, reduced heart rate variability and the ratio of increased peak early ventricular filling velocity to early diastolic myocardial velocity were associated with a decreased VO₂ peak ($R^2 = 0.61$).

Discussion

The current study supports our hypothesis that HRV is correlated with exercise intolerance in patients with repaired TOF. Low- and high-frequency-domain HRV significantly correlated with VO₂ peak ($r = 0.56$,

Table 2 Echocardiography, exercise test, and heart rate variability at entry to the study and at the 1-year follow-up assessment for patients with repaired tetralogy of Fallot (TOF)

Parameter	At the entry	At the 1 year	P value
Echocardiography			
LVEF (%)	57 $\pm$ 9	60 $\pm$ 8	NS
LVSm (m/s)	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01	NS
LVE _m (m/s)	0.13 $\pm$ 0.03	0.14 $\pm$ 0.03	NS
LVA _m (m/s)	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	NS
RVFAC (%)	51 $\pm$ 7	53 $\pm$ 5	NS
RVS _m (m/s)	0.08 $\pm$ 0.02	0.08 $\pm$ 0.02	NS
RVE _m (m/s)	0.11 $\pm$ 0.03	0.12 $\pm$ 0.04	NS
RVA _m (m/s)	0.04 $\pm$ 0.01	0.08 $\pm$ 0.18	NS
Exercise test (peak)			
Heart rate (bpm)	162 $\pm$ 14	160 $\pm$ 12	NS
VO ₂ peak (ml/kg/min)	33 $\pm$ 9	31 $\pm$ 10	NS
Minute ventilation (l/min)	44 $\pm$ 14	43 $\pm$ 11	NS
V _E /CO ₂	29 $\pm$ 5	29 $\pm$ 5	NS
Gas exchange ratio	1.14 $\pm$ 0.13	1.13 $\pm$ 0.16	NS
Time domain			
SDNN (ms)	132 $\pm$ 30	139 $\pm$ 46	NS
SDANN (ms)	122 $\pm$ 31	128 $\pm$ 49	NS
Frequency domain			
Low frequency (ms^2)	20 $\pm$ 7	21 $\pm$ 8	NS
High frequency (ms^2)	14 $\pm$ 6	16 $\pm$ 7	NS
LF/HF ratio	1.6 $\pm$ 0.4	1.4 $\pm$ 0.4	NS

LVEF left ventricular ejection fraction, NS not significant, LVSm left ventricular systolic myocardial velocity, LVE_m left ventricular early diastolic myocardial velocity, LVA_m left ventricular late diastolic myocardial velocity, RVFAC right ventricular fractional area change, RVS_m right ventricular systolic myocardial velocity, RVE_m ventricular early diastolic myocardial velocity, RVA_m right ventricular late diastolic myocardial velocity, VO₂ peak peak oxygen uptake, V_E/CO₂ minute ventilation and carbon dioxide production ratio, SDNN standard deviation of all normal sinus R-R intervals in the entire 24-h recording, SDANN standard deviation of all average normal sinus R-R intervals for all 5-min segments in the 24-h recording, LF low-frequency power, HF high-frequency power

$P = 0.001$ and $r = 0.44$, $P = 0.02$, respectively). The low- and high-frequency-domain HRV still significantly correlated with VO₂ peak at the 1-year follow-up assessment ($r = 0.43$, $P = 0.03$ and $r = 0.52$, $P = 0.007$, respectively). Furthermore, left ventricular early diastolic myocardial velocity was most closely correlated with the VO₂ peak ($r = 0.51$, $P = 0.005$). The ratio of peak early ventricular filling velocity to early diastolic myocardial velocity significantly correlated with VO₂ peak ($r = -0.50$, $P = 0.006$). In fact, a decreased early diastolic myocardial velocity and an increased ratio of early ventricular filling velocity to early diastolic myocardial velocity are characteristics of left ventricular diastolic dysfunction. In the females, a reduced HRV and an

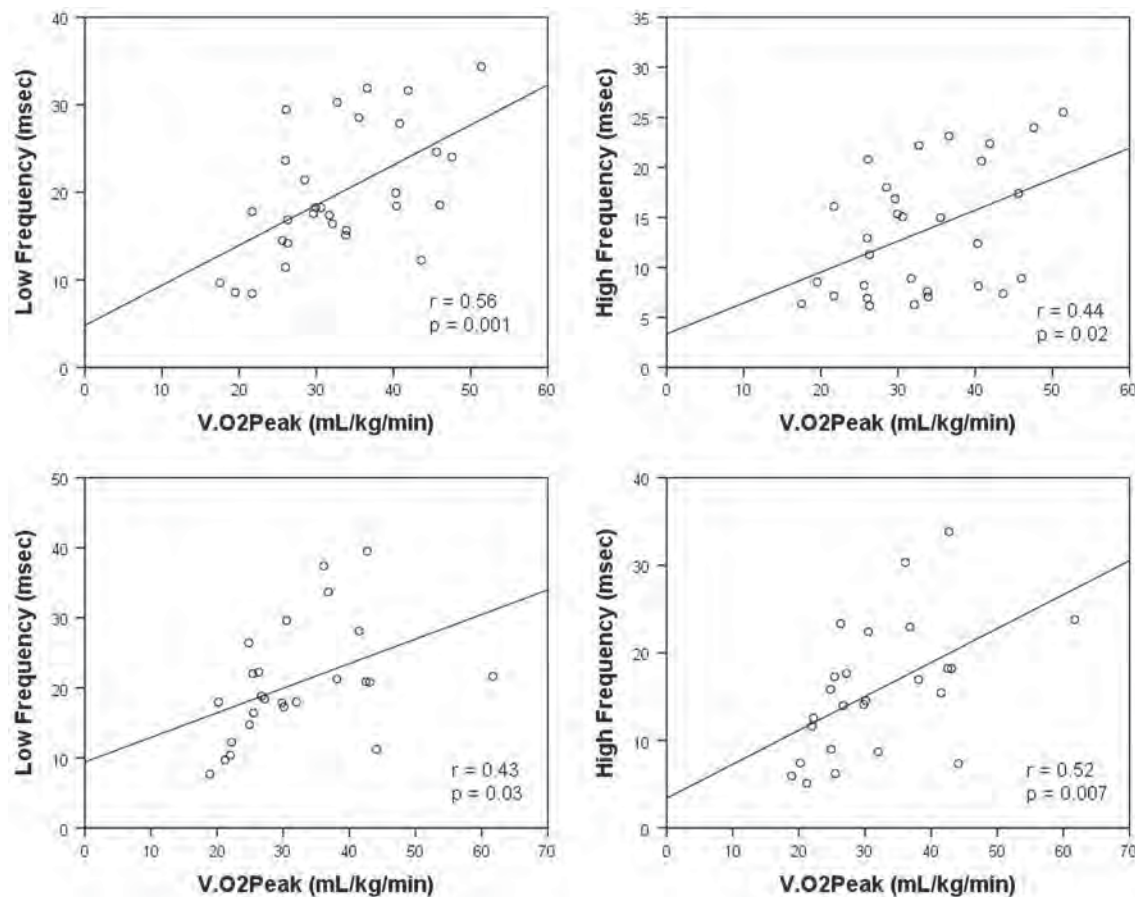


Fig. 1 Linear regression analysis of the correlation between heart rate variability and peak oxygen uptake (VO_2 peak) at study entry (*upper*) and at the 1-year follow-up evaluation (*lower*) in patients with repaired tetralogy of Fallot. VO_2 peak, peak oxygen uptake

increased ratio of peak early ventricular filling velocity to early diastolic myocardial velocity were associated with a decreased VO_2 peak.

Several studies have reported that adolescent and adult patients with repaired TOF had reduced HRV [4, 9, 10, 21]. Reduced HRV is a predictor of sudden cardiac death after myocardial infarction and chronic heart failure [18, 20]. To our knowledge, no previous studies of HRV have correlated with exercise capacity in pediatric and adolescent patients with repaired TOF. Peak oxygen uptake has been an independent predictor of death for adults with repaired TOF [13]. The current study found that low- and high-frequency-domain HRV significantly correlated with VO_2 peak at entry to the study and at the 1-year follow-up assessment.

McLeod et al. [21], and Davos et al. [9] reported that reduced HRV was associated with widening of the QRS complex, which had been identified as a risk factor for sustained ventricular tachycardia and sudden death [11]. Folino et al. [10], and Butera et al. [4] reported that patients

with ventricular tachycardia had reduced HRV. The current study did not find an association between the HRV and the QRS duration in pediatric and adolescent patients with repaired TOF. No ventricular tachycardia was found in the 24-h Holter monitoring because the patients in the current study were younger than those in previous studies.

Cheung et al. [7] reported that the impaired global left ventricular deformation due to right ventricular dilation was an independent predictor of VO_2 peak in patients with repaired TOF. The current study supported this finding that left ventricular diastolic dysfunction was correlated with VO_2 peak. Therefore, reduced HRV and left ventricular diastolic dysfunction may be responsible for exercise intolerance in patients with repaired tetralogy of Fallot.

Conclusions

Heart rate variability had a significant correlation with VO_2 peak at entry to the study and at the 1-year follow-up

assessment. Left ventricular early diastolic myocardial velocity was correlated with the VO_2 peak. Impaired cardiovascular autonomic control and left ventricular diastolic dysfunction may be responsible for exercise intolerance in patients with repaired tetralogy of Fallot. Long-term follow-up exercise testing, echocardiography, and a 24-h Holter monitoring are warranted.

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Review article

Expression of sodium channels in dental pulp

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Background: Several isoforms of voltage-gated sodium channels (VGSCs) found in peripheral nerves is associated in the pathogenesis of neuropathic and inflammatory pain. Until now, there are few studies of the distribution of VGSCs in dental pulp and its relationship to dental pain.

Objective: Perform literature review to provide update information of VGSCs in dental pulp.

Methods: We reviewed and discussed seventy-eight articles listed in MEDLINE (PubMed) database using keywords including “sodium channels” and “dental pain”. They are articles published in English from 1978 to 2010.

Results: Although several VGSCs isoforms are distributed in dental pulp, only Na_v1.7, Na_v1.8, and Na_v1.9 have been found to be upregulated in painful pulpitis.

Conclusion: Na_v1.7, Na_v1.8, and Na_v1.9 seem to have key roles in inflammatory dental pain. As a result, they might be the targets to treat dental pulp inflammation.

Keywords: Dental pulp, expression, inflammation, sodium channels

List of abbreviations

CGRP	Calcitonin gene related peptide
CNS	Central nervous system
DRG	Dorsal root ganglion
ENaC	Epithelial sodium channel
NKA	Neurokinin A
PGP9.5	Protein gene product 9.5
PNS	Peripheral nervous system
SP	Substance P
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin-resistant voltage-gated sodium channel
TTX-S	Tetrodotoxin-sensitive sodium channel
VGSCs	Voltage-gated sodium channels

Our review aims to focus on recent information regarding sodium channels, which are related to dental pain from both primary and permanent teeth, information which has not yet been thoroughly reviewed. We also include information on a variety

of fields of the pulpodentin complex, particularly the field of neural reaction to pulpal injury. Therefore, our review is divided into three parts as follows,

1. The pulpodentin complex
 - 1.1 Innervation in permanent and primary tooth pulp
 - 1.2 Sensory neuropeptides in dental pulp
 - 1.3 Neural reactions to pulpal injuries
2. The expression of sodium channels in dental pulp
3. The expression of sodium channels related to dental pain

1. The pulpodentin complex

The dental pulp is surrounded by the dental hard tissues, which form a physical barrier against pathogens and injury. The dental pulp and dentin are often discussed together as one functional unit, the pulpodentin complex. Dental pulp is responsible for dentin formation. The permeable properties of dentin regulate the diffusion rate of irritants that can initiate pulpal inflammation. Dental pulp contains a dense vascularity and nerve supply. The blood vessels in pulpal tissue are for nutrient supply and cellular recruitment, while the nerves in pulpal tissue are for

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dental sensitivity and defense response following pulpal injury, from either dental caries or trauma. The dental pulp has a low capacity for defense or repair responses because of the lack of an adequate blood supply and cellular recruitment following dental injury [1]. Several studies have shown that pulpal innervation plays an important role in both defense and repair responses [2-4]. Therefore, this review article focuses on pulpal innervation in the response to pulpal injury.

1.1 Normal innervation in primary and permanent tooth pulp

The pulpodentin complex in both primary and permanent teeth is extremely rich in innervation [5], and the innervation influences the defense reactions in the connective tissue of the dental pulp. This innervation consists of sensory, sympathetic, and parasympathetic nerve fibers.

The sensory nerve fibers are the major innervation in the dental pulp of both primary and permanent teeth. They originate from the trigeminal ganglion, and peripherally pass through the apical foramen to innervate the coronal pulp. Into the coronal pulp, they diverge, branch, and terminate as free nerve endings in the odontoblast layers, sub-odontoblastic plexus, predentin, in the inner 0.1 mm of dentin, or along blood vessels, as shown in Byers's study [6]. After stimulation, sensory nerve fibers transmit signals back via the trigeminal nerves to the trigeminal ganglion. The signals from trigeminal ganglion provide input through the spinal trigeminal tract to the spinal trigeminal nucleus and then, these signals pass through the spinothalamic tract to terminate in the somatosensory cortex of brain. There are three subgroups of sensory nerve fibers in dental pulp. They are based on size, conduction velocity, and function. First, the A- β nerve fibers are medium-sized myelinated fibers. They comprise the smallest population of sensory nerve fibers and are sensitive to mechanical stimuli such as hydrodynamic, percussion, and movement force. Second, it is the small myelinated A- δ nerve fibers. Finally, the largest population is the unmyelinated, slow conducting C fibers. Both A- δ and C fibers are classified as nociceptive, which respond to noxious stimuli. The sensory nerve fibers are also involved in dentinal fluid dynamics, vasoregulation, and protective reflexes against dental injuries [7-9]. They provide the vitality of the dental pulp by interacting with other pulpal components, such as odontoblasts, immunocompetent cells, and blood vessels. A previous study in the rat

model indicated that the sensory nerve fibers in dental pulp play an important role in the survival of pulpal tissue. In that study, the authors demonstrated that teeth with sensory denervation had greater loss of pulpal tissue than those with innervation [4].

Sympathetic nerve fibers are sparse in the dental pulp of both primary and permanent teeth. They originate in the superior cervical ganglion, are located along the blood vessels in the deeper pulp, and are involved in vasoconstriction.

The parasympathetic nerve fibers play roles in the regulation of pulpal blood flow but are much less important than either the sensory or the sympathetic fibers [10].

During maturation and aging in permanent teeth, dental pulp chamber becomes narrower with the deposition of tertiary dentin and dead tracts, which are normally not innervated. With increasing loss of primary dentin, tooth innervation decreases, as shown by the reduction in expression of neuropeptides and their receptors in the dental pulp [9, 11]. Several studies have shown the distribution of nerve fibers in dental pulp by using the expression of protein gene product 9.5 (PGP9.5), a soluble protein isolated from brains, as a marker of nerve fibers. PGP9.5 staining appears to be reliable in reacting with nerve fibers, in several studies using different techniques: immunohistochemistry [12], immunoblotting [13], immunocytochemistry [14-16], and immunofluorescence [5, 16, 17].

The sensory innervation of permanent teeth is greater than that of primary teeth [5, 14, 18]. Due to the prominent function of sensory nerve fibers in pain transmission, several investigators have hypothesized that the primary teeth have less sensitivity than the permanent teeth. This is because the primary teeth have less sensory innervation. However, another study revealed different results in sensory innervation between primary and permanent teeth [19]. In that study, the sensory nerve supply in human primary teeth differs from that in permanent teeth in two ways. First, the distribution of the innervation within the crowns of primary teeth was highest cervically, while the permanent teeth were densely supplied in the pulpal horn. Second, the primary teeth were particularly innervated at the cervical ends of the roots, but the roots of permanent dentin were virtually uninnervated. In addition, physiologic root resorption does not affect the histological structure [20] or overall innervation [21] of primary teeth.

1.2 Sensory neuropeptides in dental pulp

The sensory nerve fibers in dental pulp are afferent fibers involved predominantly in dental pain perception. The terminals of sensory nerve fibers contain neuropeptides, synthesized neurotransmitter proteins from neurons. These peptidergic neurons are associated with neurogenic inflammation, caused by extreme stimuli, such as dental caries, drilling, probing of the exposed dentin, or percussion of the teeth, in order to maintain the vitality of dental pulp [22]. Dynamic changes in peptidergic neurons occur during inflammation by extensive nerve fiber sprouting. These sprouting result in an increased number of potential sites of neuropeptide-containing fibers and, consequently, an increased quantity of neuropeptide release [3, 14, 15, 23-25]. Neuropeptides cannot cross cell membranes, so they trigger biological effects by activating their receptors located on the plasma membrane of the target cells and they are rapidly

degraded by the enzymes in pulpal tissue after exerting the effects [26]. The functions of sensory neuropeptides are multiple and varied. They can act as neurotransmitters, growth factors, hormones, vasoregulators, and immune system signaling molecules. It is known that neuropeptides contribute to promoting neurogenic inflammation to the control of pulpal blood flow and to the pain mechanisms of the pulpodentin complex [10]. Several studies demonstrated that neuropeptides could modulate vascular smooth muscles, increase vascular permeability, and modulate the immune system [8, 10, 27]. The sensory neuropeptides in primary and permanent tooth pulp consist of calcitonin gene-related peptides (CGRP), substance P (SP) and neurokinin A (NKA) [10, 28]. The origin, localization, stimulation, and biological effects of sensory neuropeptides in dental pulp are summarized in **Table 1**.

Table 1. Summary of sensory neuropeptides, receptors, and their functions (modified from [26])

Neuropeptide	Origin	Localization	Stimulus for release	Biologic effect
Calcitonin gene-related peptide	Trigeminal ganglion	C and A δ fibers	- Thermal - Mechanical - Chemical - Electrical - Caries - Capsaicin - Inflammatory mediators - Bradykinin - Prostaglandins	- Vasodilation - Plasma extravasation - Chemotaxis - T lymphocyte suppression - Hard tissue formation - Repair - Mitogen for odontoblasts - Pain - Resorption control
Substance P	Trigeminal ganglion	C and A fibers	- Thermal - Mechanical - Chemical - Electrical - Caries - Capsaicin - Inflammatory mediators - Bradykinin - Prostaglandins	- Vasodilation - Plasma extravasation - Immune system stimulation - Chemotaxis - Enhances macrophages activity - Hard tissue formation - Tissue reparation - Mitogen for T lymphocyte
Neurokinin A	Trigeminal ganglion	C and A fibers	- Thermal - Mechanical - Chemical - Electrical - Caries - Capsaicin - Inflammatory mediators - Bradykinin - Prostaglandins	- Vasodilation - Plasma extravasation - Chemotaxis - Pain

1.3 Neural reactions to pulpal injuries

When dental pulp is injured, the injury activates nerve fibers to induce neurogenic inflammation. That is a process of stimuli-induced neuropeptide release, change in vascular permeability, and the recruitment of immunocompetent cells. The neurogenic inflammation can lead to the healing process [10, 29]. Several studies have demonstrated the neurogenic inflammation occurring in the dental pulp following dental injury. For example, sensory [14, 30, 31] and sympathetic [2] nerve fiber sprouting were found in inflamed dental pulp. Byers and colleagues [32] demonstrated that the variable degrees of sensory nerve fiber sprouting is correlated with various degrees of pulpal injury in the rat model. In their study, a mild injury, e.g. shallow cavities, caused an increase in CGRP-immunoreactive fibers, and those sprouting CGRP-nerve fibers subsided within 21 days. The deeper cavities caused more injury to the dental pulp and led to microabscess formation, with more numerous branches of sensory nerve fibers sprouting underneath. The sprouting fibers took a longer time to subside and reparative dentin was substituted in the microabscesses. When the dental pulp was exposed, three defensive reactions could be found, pulp polyps, coagulation necrosis and liquefying necrosis. In those severe pulpal injuries, the CGRP-immunoreactive fibers were found sprouting adjacent to the borders of defensive reactions and the axons were found to assemble in the core of surviving pulp. As we have mentioned before, due to the increased number of potential sites of neuropeptide release and the role of sensory neuropeptides in pain transmission, the sprouting of sensory nerve fibers following inflammation may alter cytochemical reactions in the dental pulp and contribute to the altered efficacy of local anesthesia.

2. The expression of sodium channels in dental pulp

Voltage-gated sodium channels (VGSCs) are complex transmembrane pores that are responsible for depolarization of the membrane potential, or the rising phase of the action potential in the membrane. They are found in excitable cells, such as neurons, myocytes [33], and some types of glial cells [34]. VGSCs open within a millisecond in response to electrical change across the membrane to allow sodium ion influx. This causes the increased neuronal membrane potential. Then, they terminate very fast

to occlude the sodium ion flow. The neurons enter a repolarization stage by the allowance of potassium ion influx at the neuronal membrane. After closing, VGSCs return to the resting state and are available to reopen in response to new waves of electrical change. Therefore, VGSCs contribute to the determination of neuronal excitability and play a role in the propagation of nerve impulses. During injuries or inflammation, VGSCs in primary sensory neurons are continuously activated and the continuous activation of VGSCs gives rise to an unprovoked, spontaneous action potential, that finally causes continuous pain [35].

The sodium channel is a selective filter composed of one large, continuous protein, the β -subunit, and one or two smaller proteins, the α -subunits. The β -subunit, a 220-260 kD polypeptide, is a functional part of the sodium ion channel, and contains a voltage sensor, an ion pore, and activation and inactivation gates. The β -subunits modulate the functions of the α -subunits and stabilize them to the plasma membrane. In mammals, nine genes have been identified to encode VGSC β -subunits into nine isoforms, depending on amino acid sequence homology and genetic location. These isoforms include $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$, $\text{Na}_v1.5$, $\text{Na}_v1.6$, $\text{Na}_v1.7$, $\text{Na}_v1.8$, and $\text{Na}_v1.9$. Each isoform differs in function, such as tissue distribution, electrophysiological properties, pharmacological properties, and response to nerve injury and inflammation. Moreover, different isoforms aggregate to form a variety of macromolecules and to regulate the excitability of nociceptors. Therefore, there are diversified processes of nerve impulse propagation such as variation in opening thresholds, opening time length, amount of inactivation time, rate of isoform transition from the closed inactivated state to the resting, or closed state, depending on the presence of sodium channel α -subunit isoforms [36].

VGSCs can be functionally classified depending on the criteria used, as shown in **Table 2**, and the properties of each VGSC α -subunit isoform are summarized in **Table 3**.

In physiological, rather than pathological, conditions, the sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglion express both TTX-sensitive (TTX-S) and TTX-resistant (TTX-R) sodium channels. The population of sensory neurons is primarily mechanoreceptive, expressing rapidly-inactivating TTX-S sodium channels, with a small proportion being nociceptive, expressing a mixture of

Table 2. Classification of VGSCs depending on function [36]

Criteria	Classification of VGSCs
Threshold of activation	- Low threshold - High threshold
Rate of activation	- Fast activation - Slow activation
Rate of inactivation	- Fast inactivation - Slow inactivation
Sensitivity to tetrodotoxin (TTX), which is a toxin found in the liver of puffer fish	- TTX-sensitive (TTX-S) - TTX-resistant (TTX-R)

Table 3. Voltage-gated sodium channel α -subunit isoforms and their properties [35, 36]

α -subunit isoform	Site of expression	Inactivation rate	Sensitivity to blockade by TTX
Na _v 1.1	CNS and DRG sensory neurons	Fast	Sensitive
Na _v 1.2	CNS neurons	Fast	Sensitive
Na _v 1.3	Immature neurons	Fast	Sensitive
Na _v 1.4	Skeletal muscle	Fast	Sensitive
Na _v 1.5	Cardiac muscle	Slow	Intermediately sensitive
Na _v 1.6	CNS and DRG sensory neurons	Fast	Sensitive
Na _v 1.7	DRG sensory neurons and sympathetic ganglia	Fast	Sensitive
Na _v 1.8	DRG sensory neurons	Slow	Resistant
Na _v 1.9	Small DRG sensory neurons and trigeminal ganglia	Very slow (persistent)	Resistant

rapidly-inactivating TTX-S and slowly-inactivating TTX-R sodium channels. Details of studies of the expression of sodium channels in normal dental pulp are described in **Table 4**.

During the inflammatory process, inflammatory mediators can lower the threshold of activation and increase the excitability of TTX-R in primary sensory neurons, contributing to neuronal hyperexcitability [37]. Moreover, several studies have shown the alteration in the expression of both TTX-S and TTX-R VGSCs in inflamed peripheral tissues [36, 38]. These changes may lead to increased pain states.

The rapidly inactivating, TTX-S sodium currents have been detected in cultured human dental pulp cells [39]. Davidson suggested that the main source of these sodium currents is neuronal satellite cells, not odontogenic cells, because the odontoblastic processes firmly embed the odontoblasts to the dentin and do not allow these cells to be explanted. On the other hand, an *in vitro* study of Allard and colleagues [40] found that odontoblasts expressed voltage-gated

TTX-S currents which have the ability to generate action potential, but TTX-R sodium currents have not been detected.

Henry and colleagues [41] found no change in overall sodium channel expression in painful human dental pulp. However, they found that the quantity of atypical nodal sites and the expression of sodium channels at such sites were increased but the quantity of typical nodal sites and the accumulating sodium channels at those sites were decreased. That study showed that inflammation caused the demyelinating process and the remodeling of the pattern of sodium channel accumulation. Several studies supported the study of Henry and colleagues [41], revealing, for example, an increase in the expression of Na_v1.7 [17], Na_v1.8 [12, 13] and Na_v1.9 [42] in permanent human dental pulp with irreversible pulpitis compared to permanent dental pulp of non-painful teeth. Na_v1.6 has also been found in the dental pulp of both humans and rats [43], but its function in pulpal inflammation remains unclear. The expression of multiple VGSC

isoforms in inflamed dental pulp suggests the collaborative roles of various VGSC isoforms in generating spontaneous action potential, leading to pulpal pain.

Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.4, and Na_v1.5 have not been evidenced in dental pulp. Na_v1.1 and Na_v1.2 are predominantly expressed in adult central nervous system (CNS) neurons, in combination with Na_v1.6. In contrast, the expression of Na_v1.3 is particular in immature neurons. Na_v1.4 has been seen in skeletal muscle, while Na_v1.5 has been remarkably found in cardiac muscle [35]. Not only VGSCs isoforms, but also epithelial sodium channels, which are non-VGSCs, have been found in dental pulp [44]. The expression of each sodium channel isoform in permanent dental pulp is shown in **Table 4**.

3. The expression of sodium channels related to dental pain

Na_v1.6 is a TTX-sensitive VGSC isoform, remarkably expressed at the nodes of Ranvier within the myelinated PNS and CNS neurons [45] and also expressed along unmyelinated neurons of the PNS [46] and CNS [45]. Its function has been suggested to be an electrical conduction in both myelinated

and unmyelinated axons [45, 46] but the role in nociception is obscure. The expression of Na_v1.6 in human permanent tooth pulp has been reported in the study of Luo and colleagues [47] using immunocytochemistry, in which there was no significant difference in the expression of Na_v1.6 in normal and painful pulp, despite an increase in the proportion of atypical nodes of Ranvier and a decrease in typical nodal sites in painful pulp. Another study of Na_v1.6 in dental pulp, a study in rats, using immunohistochemistry and double immunofluorescence [43], found that Na_v1.6 was expressed in non-neuronal cells, such as pulpal immune cells, dendritic pulpal cells, and odontoblasts. That finding suggests that Na_v1.6 play a role in those cells. Furthermore, it might be implicated in neuro-immune interactions. In contrast to the study of Luo and colleagues [47], pulpal tissue of injured rat teeth in Byers and colleagues' study [43] showed an increase in Na_v1.6 immunoreactive cells, predominantly around the injured pulpal tissue and dilated blood vessels. The increased expression of Na_v1.6 in non-neuronal dental pulp cells of injured rats [47], despite the unchanged expression of Na_v1.6 at the nodes of Ranvier in human inflammatory pulp [43], may reflect the different

Table 4. Sodium channel expression in normal dental pulp

<i>In vitro/In vivo</i>	Models	Major findings	References
<i>In vitro</i> Whole-cell, patch-clamp methods	Human dental pulp cells	Detection of TTX-S current in human dental pulp cells	Davidson, 1994 [39]
<i>In vitro</i> Immunohistochemistry	Human dental pulp	Expression of NaV1.8 immunoreactive nerve fibers in the sub-odontoblastic layers of dental pulp	Renton, et al 2005 [11]
<i>In vitro</i> Immunocytochemistry	Human dental pulp	Expression of NaV1.8 in 16.5% of nodes of Ranvier in radicular tooth pulp	Henry, et al 2005 [61]
<i>In vitro</i> Immuno-electron microscopic methods	Rat dental pulp	Expression of and ENaC in mechanoreceptive myelinated nerve fibers	Ichikawa, et al 2005 [44]
<i>In vitro</i> Immunohistochemistry	Rat dental pulp	Expression of NaV1.9 in unmyelinated nerve fibers and suggested role of NaV1.9 in thermal pain stimuli	Padilla, et al 2007 [62]
<i>In vitro</i> immunohistochemistry	Rat dental pulp	Expression of NaV1.6 in dental pulp cells and odontoblasts	Byers et al, 2009 [43]
<i>In vitro</i> immunocytochemistry	Human dental pulp	Prominent expression of NaV1.6 at nodes of Ranvier, particularly at typical nodal sites	Luo et al, 2010 [47]

function of Na_v1.6 in different cell types. However, the difference in the expression and response mechanism of Na_v1.6 in various species and different types of pulpal tissue damage should not be ignored.

Na_v1.7 is a TTX-sensitive VGSC isoform that has been widely studied. It has been identified in the sympathetic neurons and small and medium sized sensory neurons of the DRG, including nociceptive neurons. Na_v1.7 is rapidly activated, rapidly inactivated and slowly recovers from fast activation, so it plays an important role in setting the threshold for the generation of action potentials in peripheral nociceptive neurons [35]. Na_v1.7 is markedly involved in perceiving pain sensations, as evidenced in patients with the loss-of-function mutation in the SCN9A gene, a gene that encodes Na_v1.7, or meaning that those who have loss of Na_v1.7 function are unable to experience pain [48, 49]. In addition, patients with congenital pain syndrome, who have an alteration in Na_v1.7 function, have increased pain sensitivity associated with edema, redness, and warmth, suggesting the role of Na_v1.7 in chronic inflammatory pain [50]. In the dental pulp of human permanent teeth, the upregulation of Na_v1.7 expression has also been reported in painful pulpitis studied using either immunohistochemistry [51], or immunocytochemistry [17], demonstrating the increased expression of the Na_v1.7 isoform at both typical and atypical nodal sites.

The VGSC α -subunit isoform 1.8 (Na_v1.8) and VGSC α -subunit isoform 1.9 (Na_v1.9), the slower TTX-R components, are remarkably found in small unmyelinated sensory neurons that have been identified as nociceptive neurons [36]. Na_v1.8 has a high activation threshold, slow inactivation kinetics and contributes to the electrogenesis of an action potential in C-type peripheral neurons of mice [52]. Na_v1.9 is activated at potentials near resting membrane potential and generates relatively persistent current [53]. Both TTX-R isoforms, Na_v1.8 and Na_v1.9, are believed to be involved in the prolonged duration of the action potential in response to painful stimuli and have been found to upregulate during inflammatory pain in rat [38, 54] and mouse [55] models. Therefore, both sodium channel isoforms might be new targets for treatment of inflammatory pain. The different properties of Na_v1.8 and Na_v1.9 are as follows. Na_v1.8 currents have a slow activation and inactivation rate. The slower inactivation rate of Na_v1.8 compared to those of other isoforms prolongs the action potential of neurons and may cause chronic

pain. The steady-state voltage dependence of inactivation contributes to generating an action potential even in the depolarized state. Na_v1.9 currents are unique. They can be activated at voltages near the resting membrane potential. Furthermore, they can generate persistent currents. Na_v1.9 can be easily activated. It can contribute to the setting of the threshold of activation. Finally, it can remain opening for a longer time than Na_v1.8 [36, 56]. Previous studies in rats, using oligodeoxynucleotides as antisense for Na_v1.8 [55, 57] and a study in Na_v1.8-null mice have shown that Na_v1.8 plays a role in inflammatory pain and neuropathic pain [58]. Na_v1.9 channels also have a role in inflammatory pain, but not in neuropathic pain [59, 60].

Localization of Na_v1.8 in human teeth with painful pulpitis has been investigated using immunohistochemistry [12]. It has been found that Na_v1.8-immunoreactive nerve fibers were localized in the sub-odontoblastic layer of both healthy and inflamed pulp tissue. However, the detection of Na_v1.8-immunoreactive fibers was much greater in the inflamed dental pulp. Moreover, the upregulation of Na_v1.8 has been reported using the immunoblotting method in inflamed human permanent tooth pulp compared to healthy pulp [13]. An immunocytochemical study has revealed that not only the predominant Na_v1.6, but also Na_v1.8 has presented at the nodes of Ranvier in the radicular part of healthy human permanent tooth pulp [61]. This finding suggests the coexistence of multiple sodium channel isoforms in those areas where the levels of expression may change during the inflammatory period and may contribute to an increased pain status.

For Na_v1.9, an investigation in rats has revealed the innervation of Na_v1.9-immunoreactive fibers in the lip skin and in the dental pulp of non-painful teeth, suggesting the role of this VGSC isoform in orofacial pain [62]. As well as the other sodium channel mentioned above, the immunocytochemical method has reported the increased expression of Na_v1.9 in the axons of symptomatic pulpitis of human permanent teeth [42].

Epithelial sodium channel (ENaC) protein is a member of the degenerins family (DEG), which is a large protein family of diverse functions, such as sodium ion transport, acid sensation, proprioception, and mechanosensation [63]. Differing from VGSCs, which consist of α - and β - subunits, ENaC consists of four subunits: α , β , γ and δ subunits [64]. Only

α , β and γ subunits of ENaC have been indicated in mechanoreceptors in the trigeminal ganglion of rat models with a possible function in mechanotransduction [65]. β ENaC has been identified in the terminal Schwann cells associated with the periodontal Ruffini endings in the periodontal ligament of rat incisors and is believed to be a key molecule for mechanosensation in mastication [66]. ENaC has also been found in rat dental pulp tissue, by using immunohistochemistry [44]. In that study, the β ENaC and γ ENaC-immunoreactive fibers have appeared in trigeminal ganglion neurons, periodontal ligament, and deep layer of oral mucosa, inferior alveolar nerve fibers, radicular pulp, and sub-odontoblastic plexus of rat molars pulp tissue. γ ENaC in dental pulp was mostly around myelinated nerve fibers, which are sensitive to mechanical stimuli, whereas it was mostly absent around unmyelinated nociceptive axons.

Those studies of changes in sodium channel expression within painful dental pulp are summarized in **Table 5**.

There have been attempts to discover new substances to act as sodium channel blockers for the treatment of both neuropathic and inflammatory pain. Lidocaine, a commonly used anesthetic, is a sodium channel blocker with a non-specific blocking property that can block TTX-R and TTX-S channels. Scholz and colleagues reported that TTX-R channels are more resistant to lidocaine than are TTX-S channels in A- δ and C type neurons from the dorsal root ganglion of rats [67]. In contrast, other studies reported that TTX-R channels are more sensitive to lidocaine than are TTX-S sodium channels in rat models [68] and in the mammalian dorsal root ganglion neuroblastoma hybridoma cell line0 [69]. The differences in the results of these studies may be the result of several factors. First, the ability of lidocaine to bind sodium channels depends on the status of the sodium channels. TTX-R currents were found to be blocked by lidocaine in the inactivated state more than in the resting state [67]. It was also found that TTX-S and TTX-R currents were equally sensitive to lidocaine in the

Table 5. Sodium channel expression related to dental pain

<i>In vitro/In vivo</i>	Models	Major findings	References
<i>In vitro</i> Immunohistochemistry	Inflamed human permanent tooth pulp	Significant increase in Na _v 1.8 immunoreactive nerve fibers in painful pulpitis	Renton, et al 2005 [11]
<i>In vitro</i> immunocytochemistry	Inflamed human permanent tooth pulp	Increase expression of Na _v 1.9 in axons of painful pulp	Wells et al, 2007 [42]
<i>In vitro</i> immunocytochemistry	Inflamed human permanent tooth pulp	Increased expression of Na _v 1.7 at both intact and remodeling nodal sites within the painful human dental pulp	Luo et al, 2008 [16]
<i>In vitro</i> Western blot	Inflamed human permanent tooth pulp	Increase in Na _v 1.8 density in inflamed dental pulp	Warren, et al 2008 [12]
<i>In vitro</i> immunohistochemistry	Injured rat dental pulp	Increased expression of Na _v 1.6 near dilated blood vessels beneath the injured site and nearly affected pulp	Byers et al, 2009 [43]
<i>In vitro</i> immunocytochemistry	Inflamed human dental pulp	1. Increased expression of NaCh at atypical nodal sites, but decreased expression at typical nodal sites within painful pulpitis 2. Decrease in density of NaCh expression in painful pulp but no significant difference when compared to normal pulp 3. Accumulation of NaCh at atypical nodal sites within A- fibers	Henry, et al 2009 [41]
<i>In vitro</i> immunocytochemistry	Inflamed human dental pulp	No difference in Na _v 1.6 expression at nodal sites of painful and non-painful pulp	Luo et al, 2010 [47]
<i>In vitro</i> immunohistochemistry	Inflamed human dental pulp	Increase in Na _v 1.7 immunoreactive area within sub-odontoblastic plexus of painful dental pulp	Beneng et al, 2010 [51]

resting state, while in the activated or opened state, TTX-S currents were more sensitive to lidocaine [69]. Another reason for different findings in the sensitivity of sodium channels to lidocaine may be the blocking methods used in the studies. Drug-bound TTX-R channels have a slower recovery period than do TTX-S channels [69]. Then, the use of frequency-dependent and tonic blockade of the channels by lidocaine leads to dissimilar results in comparing the sensitivity of TTX-S and TTX-R. Until now, the specific VGSC isoforms that are the problems in anesthetic failure are still controversial. The use of a combination of permanently charged lidocaine (N-ethyl-lidocaine) and capsaicin, an agonist for the transient receptor potential vanilloid 1 (TRPV1), in injured rats has been reported in the study of Kim and colleagues [70]. Those authors claimed that the advantage of that regimen over the use of plain local anesthetic agents is that it does not cause a deficit in motor and autonomic nerve function, but the authors claimed that it requires further study for clinical application. Isoflurane, an inhalation anesthetic agent, was also proved to block TTX-S and $\text{Na}_v1.8$ currents in rats [71]. Eugenol, a widely used agent in dentistry, has an ability to inhibit both TTX-R and TTX-S sodium ion currents in rats and has effect on nociceptive, as well as non-nociceptive, fibers [72, 73]. Therefore, eugenol may be another good choice to be an analgesic and anesthetic agent in dental treatment. In addition to the sodium channel blockers mentioned above, the sodium channel blocking efficacy of variety opioid derivatives has been studied and it has been found that tramadol, fentanyl and sufentanil have sodium channel blocking ability, especially in slow-activation sodium channel isoforms, while morphine does not [74]. The specific sodium channel blockers have been improved but they are limited to specific $\text{Na}_v1.8$ blockers, such as O -conotoxin MrVIB from *Conus Marmoreus* [75], a small molecule antisense oligonucleotide (A-803467) [76, 77] and 5-Aryl-2-furfuramides [78]. Unfortunately, despite much research on sodium channel blockers, none of the sodium channel blocking agents is considered to be effective and safe enough to use in humans. Further studies on the new generation of pain treatments, particularly in the field of dentistry, are still needed.

Conclusions

Dental pain is a significant health problem. Although several voltage-gated sodium channel

isoforms, as well as an epithelial sodium channel, have been identified in dental pulp with different location and function, only $\text{Na}_v1.7$, $\text{Na}_v1.8$, and $\text{Na}_v1.9$ play a key role in inflamed pulp. These sodium channel isoforms are suggested as potential targets for novel treatments of pain from pulpal inflammation and as options for novel anesthetics in the treatment of painful pulpitis.

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Effects of estrogen in preventing neuronal insulin resistance in hippocampus of obese rats are different between genders

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ABSTRACT

Aim: The effects of estrogen on the prevention of impaired insulin-induced long-term depression in the hippocampus and neuronal insulin signaling caused by high-fat diet (HF) were studied in male and female rats. **Main methods:** Both male and female rats were fed with a normal diet (ND; 19.7% energy from fat) or a high-fat diet (HF; 59.3% energy from fat) for 12 weeks. Then, rats were divided into four subgroups: ND, ND + E, HF and HF + E. The subgroups with + E were given 50 µg/kg estrogen subcutaneously once a day for 30 days. At the end of the experimental period, blood and brain samples were collected to determine the peripheral insulin resistance and neuronal insulin resistance, respectively.

Key findings: Both male and female rats fed with HF developed peripheral insulin resistance as indicated by increased body weight, visceral fat, plasma insulin and HOMA index. Estrogen administration decreased those parameters, indicating improved peripheral insulin sensitivity, in both male and female HF rats. HF diet consumption also caused impaired insulin-induced long-term depression in hippocampus and impaired neuronal insulin receptor function and signaling, indicating neuronal insulin resistance, in both male and female rats. Estrogen treatment could attenuate these neuronal impairments only in HF female rats.

Significance: The activation of the estrogen pathway could preserve insulin sensitivity in the peripheral tissue in both male and female rats. In neuronal tissue, however, the benefit of estrogen could be found only in female rats.

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Introduction

Several studies demonstrated that high-fat (HF) feeding in animal models could cause obesity and insulin resistance in peripheral tissues, which are features of the metabolic syndrome (Cani et al., 2007; Shoelson, 2006). Our previous study has shown that a 12-week HF diet consumption in male rats could lead not only to peripheral insulin resistance but also to neuronal insulin resistance, indicating an important role of diet on neuronal insulin sensitivity (Pratchayasakul et al., 2011). In addition, it is known that the loss of ovarian hormones, such as estrogen, can cause a significant increase in visceral fat deposition and insulin resistance, which can then easily develop into type 2 diabetes (Alonso and Gonzalez, 2008; Asthana et al., 2001; Asthana et al., 1999; Gonzalez et al., 2008). Estrogen has been shown to contribute to glucose homeostasis (Louet et al., 2004). The beneficial effect of estrogen on insulin activity has been found to be greater in women before menopause than in age-matched men (Desrocher and Rovet, 2004; Nuutila et al., 1995). A

number of studies have also demonstrated several beneficial effects of estrogen replacement on the insulin resistance model, such as the improvement of peripheral insulin sensitivity, the increase in the rate of IRS-1 and Akt phosphorylation of insulin receptors in skeletal muscle, the reduction of central body fat, the lowering of lipid and cholesterol levels, and the reduced risk of the development of type 2 diabetes (Alonso and Gonzalez, 2008; Godslund, 2005; Palin et al., 2001). Nevertheless, only two studies have demonstrated those beneficial effects of estrogen on improving insulin sensitivity in peripheral tissues induced by HF consumption (Bryzgalova et al., 2008; Riant et al., 2009). Both studies showed that the estrogen treatment exerted anti-diabetic and anti-obesity effects in rats which received a HF diet. Their findings suggest that the pathway of estrogen acts as an effective target to protect against HF-induced metabolic imbalance.

Previous studies also demonstrated the beneficial effects of estrogen on improving memory function in human and animal models (Daniel et al., 1997; Luine et al., 1998; Sherwin, 2005). We have shown previously that a significant modification of important neuronal insulin receptor signaling can be induced by a fat-enriched diet in male rats (Pratchayasakul et al., 2011). Being fed the HF diet for 12 weeks, clearly induces neuronal insulin resistance, which is identified as a significant reduction in the ability of insulin to induce long-

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term depression (LTD), and a reduction in the stimulated phosphotyrosine activity of IR, IRS-1 and Akt/PKB in brain slices (Pratchayasakul et al., 2011). Those findings indicated that the impairment of neuronal insulin resistance was induced by a HF diet. Nevertheless, the effects of estrogen administration on neuronal insulin signaling in HF-fed rats have never been investigated. In the present study, we tested the hypothesis that the administration of estrogen in both male and female rats can reverse the impairment of both insulin-induced LTD in the hippocampus and neuronal insulin signaling caused by a 12-week HF diet consumption.

Materials and methods

Animals and dietary protocols

All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Male ($n = 40$) and female ($n = 40$) Wistar rats weighing ~180–200 g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were individually housed in a temperature-controlled environment with a 12:12 light–dark cycle. One week after arrival, all rats were randomly assigned to one of the two dietary groups ($n = 40$ in a HF diet group, 20 male:20 female and $n = 40$ in a normal diet group, 20 male:20 female). The normal-diet (ND) group received a standard laboratory chow, in which 19.7% of total energy (%E) was from fat, with energy content calculated at 4.02 kcal/g (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand). The HF group consumed a fat-enriched diet, containing fat, mostly from lard (59.3% E), with energy content calculated at 5.35 kcal/g, for 12 weeks. The animals were maintained in individual cages with unrestricted access to food and water. Body weight and food intake were recorded daily. Blood samples were collected from the tail at weeks 0 and 12 after fasting for at least 5 h and kept at -80°C for subsequent biochemical analyses, such as plasma glucose, triglyceride, and insulin assay. After 12 weeks, each dietary group was divided into two subgroups and each subgroup was given either vehicle (90% sesame oil + 10% ethanol) or 17- β estradiol (50 $\mu\text{g/kg}$) subcutaneously for 30 days (Bryzgalova et al., 2008). This dose and duration were chosen since it has been shown to improve the peripheral insulin resistant condition (Bryzgalova et al., 2008). At the end of the experimental periods, animals were deeply anesthetized with isoflurane after fasting for at least 5 h and decapitated. The brain was rapidly removed for brain slice preparation. Visceral fat was removed and weighed. Plasma collected from animals was stored at -80°C for further biochemical analysis.

Analytical procedure

Fasting plasma glucose and triglyceride were determined by colorimetric assay using commercially available kits (Biotech, Bangkok, Thailand). Fasting plasma insulin level and plasma estrogen were measured by Sandwich ELISA kits (LINCO Research, St. Charles, Missouri, USA).

Determination of insulin resistance (HOMA index)

Insulin resistance was assessed by Homeostasis Model Assessment (HOMA) (Appleton et al., 2005; Haffner et al., 1997) as a mathematical model describing the degree of insulin resistance, calculated from fasting plasma insulin and fasting plasma glucose concentration.

Brain slice preparation

After decapitation, the brain was removed and immersed in ice-cold “high sucrose” aCSF containing (mM): NaCl 85; KCl 2.5; MgSO_4 4; CaCl_2 0.5; NaH_2PO_4 1.25; NaHCO_3 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95% O_2 /5% CO_2 (pH 7.4). This solution enhanced neuronal survival during the slicing procedure (Chattipakorn and McMahon, 2002). Hippocampal slices were cut using a vibratome (Vibratome Company, St. Louis, Missouri, USA). Following a 30-minute post-slice incubation in high sucrose aCSF, slices were transferred to a standard aCSF solution containing (mM): NaCl 119; KCl 2.5; CaCl_2 2.5; MgSO_4 1.3; NaH_2PO_4 1; NaHCO_3 26; and glucose 10, saturated with 95% O_2 /5% CO_2 (pH 7.4) for an additional 30 min, before being used for the extracellular recordings and immunoblotting.

Extracellular recording of hippocampal slices

To investigate insulin-induced LTD, the hippocampal slices were transferred to a submersion recording chamber and continuously perfused at 3–4 ml/min with standard aCSF warmed to $25\text{--}28^{\circ}\text{C}$. Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collateral–commissural pathway with a bipolar tungsten electrode, while recordings were gathered from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 Mohm) filled with 2 M NaCl. Stimulus frequency was 0.033 Hz. Stimulus intensity was adjusted to yield a fEPSP of 0.8–1.0 mV in amplitude. Hippocampal slices were perfused with aCSF (as baseline condition) for 10 min and then perfused with aCSF plus 500 nM insulin (as insulin stimulation) for 10 min, after which the hippocampal slices were perfused with aCSF again (wash out) and fEPSPs were recorded for the next 30 min.

All data were filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using pClamp 9.2 software (Axon Instruments, Foster City, California, USA). The initial slope of the fEPSPs was measured and plotted versus time using Origin 8.0 software.

Preparation of brain homogenates for immunoprecipitation, immunoblotting, and neuronal estrogen levels

To examine the alteration of neuronal insulin-mediated phosphorylation of the Akt/PKB following vehicle and estrogen treatment of two dietary regimens, six brain slices per animal were placed into either aCSF or aCSF plus 500 nM insulin (Humulin R, Eli Lilly, Giessen, Germany) for 5 min. Then, three brain slices in each conditioned group were homogenized in 500 μl of ice-cold brain slice lysis buffer [1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% Triton X-100 and supplemented with a protease inhibitor cocktail, Roche complete mini-tablets, (Roche Molecular Biochemicals, Indianapolis, Indiana, USA)]. Next, the homogenates were centrifuged at 9000 g for 30 min at 4°C and the protein concentration was measured using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Hercules, California, USA). These homogenates were then stored at -80°C for further biochemical analysis of neuronal estrogen levels and the western blot analysis of the serine phosphorylation of Akt/PKB.

To determine the level of Akt/PKB protein expression in the brain, another set of three brain slices in aCSF was homogenized over ice in non-ionizing lysis buffer containing: 100 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% Triton X-100, 1% NP-40 supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals).

Immunoprecipitation and immunoblotting

Akt/PKB at serine 473 kinases phosphorylation was electrophoresed and immunoblotted with rabbit antibodies for Akt/PKB at serine 473. Examination of the levels of Akt/PKB protein was conducted with

homogenates prepared from another set of three brain slices. Both proteins were resolved by the immunoprecipitation and immunoblot assay conducted with rabbit anti-Akt/PKB (1:1000 in TBST, Santa Cruz Biotechnology, Santa Cruz, California, USA). For loading control, immunoblotting for each membrane was incubated with anti- β -actin (1:400; rabbit polyclonal; Sigma, St. Louis, Missouri, USA).

All membranes for visualizing the phosphorylation and the protein levels of Akt/PKB were incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (1:8000 in TBST, Bio-Rad Laboratories). The protein bands were visualized on Amersham hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) using Amersham ECL western blotting detection reagents (GE Healthcare). Band intensities were quantified by Scion Image and the results were shown in average signal intensity (arbitrary) units.

Statistical analysis

Data were presented as means $\pm$ SE. All statistical analyses were performed using the program SPSS (version 16; SPSS, Chicago, Ill., USA). The significance of the difference between the means was calculated by two way ANOVA and posthoc analysis with Fisher's test with $p < 0.05$ for the level of phosphorylated Akt/PKB at serine 473 site in all animals. Pearson's correlation analysis was used to determine the relationship among the plasma parameters, liver triglyceride content, visceral fat, body weight, HOMA index, plasma estrogen and brain estrogen in all animals.

Results

β -Estradiol (E2) administration improved peripheral insulin sensitivity in both male and female HF-fed rats

After 12-weeks of HF feeding, both male and female rats demonstrated the characteristics of peripheral insulin resistance, such as increased body weight, visceral fat, liver triglyceride, fasting plasma insulin and HOMA index (Table 1). After 1 month of E2 administration, these parameters were significantly decreased in both male and female HF-fed rats, compared to the vehicle-treated group. These parameters were not altered in ND-fed rats (Table 1). Furthermore, the circulating estrogen level in the vehicle-treated female HF-fed rats was also significantly reduced, compared to the vehicle-treated female ND rats. This finding is consistent with a previous study demonstrating that high body fat in female rats was associated with decreased estradiol levels (Ziomkiewicz et al., 2008). In contrast;

the circulating estrogen levels were not significantly changed between the HF and ND groups that received E2 in either gender (Table 1).

E2 administration prevented neuronal insulin resistance in female HF-fed rats

The neuronal insulin receptor function or insulin-induced LTD was impaired following HF consumption in both male and female rats as indicated by the disappearance of insulin-induced LTD in the hippocampus of HF-fed rats (Fig. 1A). E2 administration could significantly prevent the impairment of insulin-induced LTD only in female HF rats (Fig. 1B). In E2-treated female HF-fed rats, the percentage depression of fEPSPs after insulin administration was $31.18 \pm 9.93\%$, compared to $2.37 \pm 3.18\%$ from E2-treated male HF-fed rats. These findings indicated that administration of E2 decreased the occurrence of HF-induced neuronal insulin resistance in female HF-fed rats, but not in male HF-fed rats. The body weight, fasting insulin level and HOMA index were also decreased by E2 treatment in both male and female rats fed with HF ($p < 0.05$, Table 1).

E2 administration improved neuronal insulin signaling in female HF-FED rats

To study the mechanism of E2 on neuronal insulin resistance, we determined whether the E2 improves insulin signaling, particularly Akt/PKB in the brain. As shown in our previous study (Pratchayasakul et al., 2011), 12-week HF consumption led to deleterious effects on insulin receptor signaling, such as phosphorylated IR, phosphorylated IRS-1 and phosphorylated Akt/PKB. In the present study, the impairment of neuronal insulin signaling was examined using immunoblot Akt/PKB phosphorylation. We also investigated whether estrogen can improve neuronal insulin signaling, particularly Akt/PKB phosphorylation to investigate the mechanism of E2 in neuronal insulin signaling in both male and female rats. Our results demonstrated that the level of Akt/PKB protein was not different in both male and female rats receiving either ND or HF diet. Furthermore, E2 administration did not alter Akt/PKB protein concentration in the brain of both male and female rats of either dietary group (Fig. 2A). However, the amounts of the phosphorylated form of Akt/PKB at the serine 473 site were significantly decreased in both vehicle-treated male and female rats in the HF group, compared to the vehicle-treated rats in the ND group (Fig. 2B). However, the amounts of the

Table 1
The effects of estrogen on peripheral insulin sensitivity parameters in rats fed with normal and high-fat diet.

Parameters	Vehicle-treated				Estrogen-treated			
	Normal diet		High-fat diet		Normal diet		High-fat diet	
	Male	Female	Male	Female	Male	Female	Male	Female
Body weight (g)	482.50 $\pm$ 12.50	282.50 $\pm$ 10.31 ^a	595.00 $\pm$ 47.*	380.00 $\pm$ 4.* ^a	407.50 $\pm$ 14.93*	254.00 $\pm$ 6.00 ^a	514.00 $\pm$ 6.00 ^{*,#}	340.00 $\pm$ 17.03 ^{*,#}
Visceral fat (g)	22.41 $\pm$ 2.72	10.16 $\pm$ 0.42 ^a	43.81 $\pm$ 7.95*	37.71 $\pm$ 2.02*	17.69 $\pm$ 2.20	13.33 $\pm$ 0.83	40.99 $\pm$ 3.76*	29.93 $\pm$ 2.09 ^{*,#}
Glucose (mg%)	121.70 $\pm$ 4.70	119.78 $\pm$ 2.54	134.36 $\pm$ 12.72	130.75 $\pm$ 19.11	99.43 $\pm$ 4.89	102.28 $\pm$ 5.86	118.52 $\pm$ 9.07	108.58 $\pm$ 9.57
Triglyceride (mg%)	86.99 $\pm$ 20.58	47.12 $\pm$ 1.29	77.16 $\pm$ 17.71	128.18 $\pm$ 3.18 ^{*,a}	126.50 $\pm$ 24.11	53.75 $\pm$ 11.43 ^a	79.89 $\pm$ 7.19	61.25 $\pm$ 8.11 [#]
Free fatty acid (mM)	0.44 $\pm$ 0.17	0.47 $\pm$ 0.07	0.42 $\pm$ 0.07	0.46 $\pm$ 0.04	0.57 $\pm$ 0.13	0.53 $\pm$ 0.05	0.55 $\pm$ 0.10	0.38 $\pm$ 0.01
Insulin (ng/ml)	1.61 $\pm$ 0.48	1.32 $\pm$ 0.23	4.88 $\pm$ 0.34*	3.40 $\pm$ 0.25 ^{*,a}	0.93 $\pm$ 0.15	1.29 $\pm$ 0.13	2.50 $\pm$ 0.46 [#]	2.43 $\pm$ 0.42 ^{*,#}
Liver triglyceride (mg/g tissue)	39.31 $\pm$ 4.39	24.21 $\pm$ 1.80	103.00 $\pm$ 3.56*	109.28 $\pm$ 8.42*	24.37 $\pm$ 3.91	23.43 $\pm$ 3.53	61.61 $\pm$ 6.52 ^{*,#}	75.69 $\pm$ 6.69 ^{*,#}
HOMA index	11.57 $\pm$ 2.87	9.35 $\pm$ 1.64	40.04 $\pm$ 4.76*	26.41 $\pm$ 4.15 ^{*,a}	5.51 $\pm$ 0.92	7.69 $\pm$ 0.55	16.72 $\pm$ 2.69 ^{*,#}	14.89 $\pm$ 1.99 ^{*,#}
Plasma estrogen (pg/ml)	56.66 $\pm$ 4.60	133.38 $\pm$ 10.32 ^a	44.80 $\pm$ 6.85	112.91 $\pm$ 24.43 ^a	240.23 $\pm$ 27.91 [#]	266.89 $\pm$ 37.87 [#]	247.74 $\pm$ 23.61 [#]	260.01 $\pm$ 12.36 [#]
Brain estrogen (pg/mg protein)	64.87 $\pm$ 14.17	75.33 $\pm$ 7.32	65.11 $\pm$ 7.74	82.03 $\pm$ 15.44	74.25 $\pm$ 6.81	149.06 $\pm$ 16.78 ^{*,a}	68.80 $\pm$ 9.20	128.54 $\pm$ 22.99 ^{*,a}

^a $p < 0.05$ from male.

* $p < 0.05$ from diet.

[#] $p < 0.05$ from vehicle.

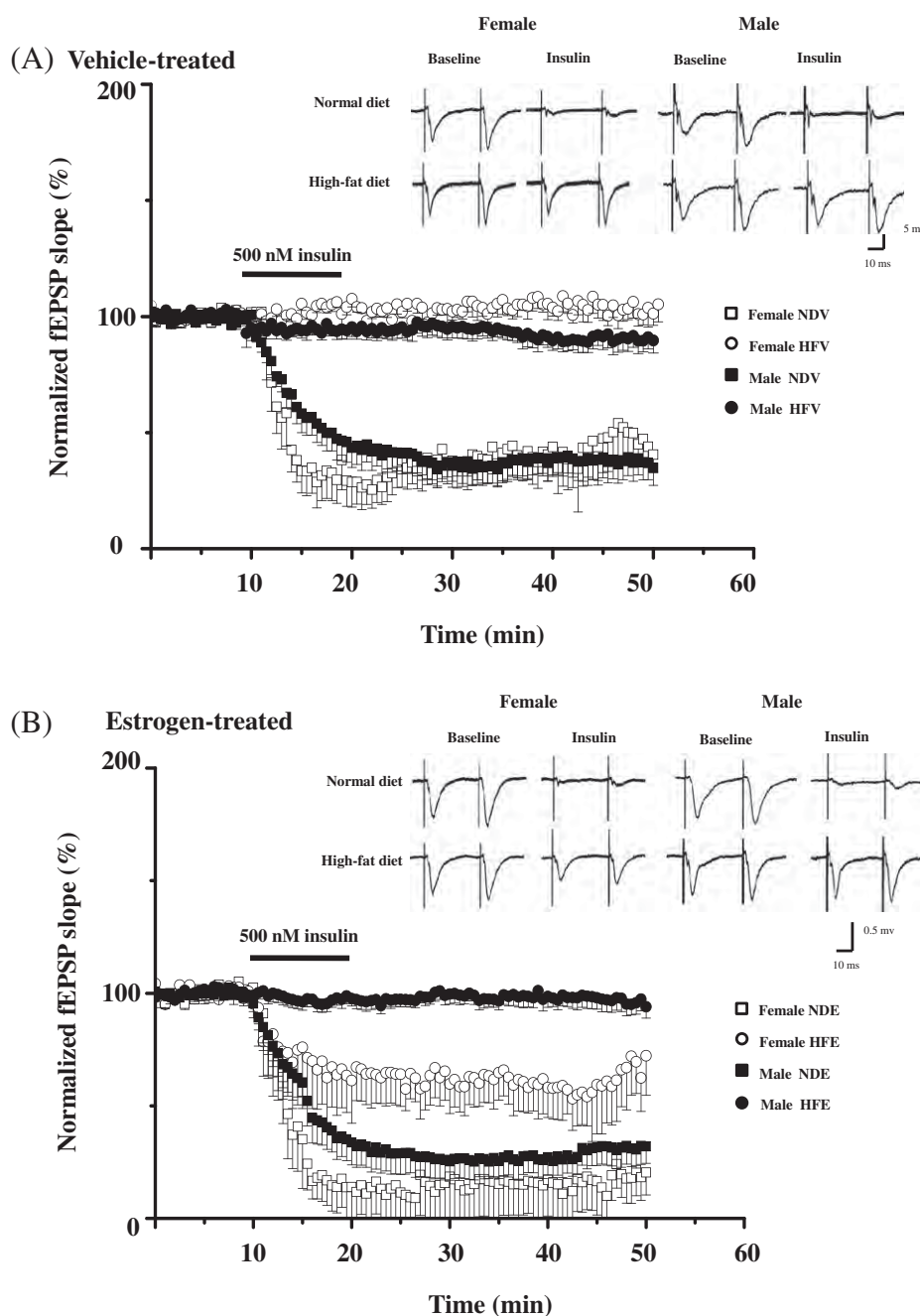


Fig. 1. E2 administration for 30 days significantly improved the impairment of insulin-mediated long term depression (LTD) in the CA1 hippocampus following HF consumption in female rats. *Panel A:* A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from NDV male and female rats ($n = 8-9$ independent slices, $n = 4-5$ animals/group) and HFV male and female rats ($n = 8-9$ independent slices, $n = 4-5$ animals/group) brain slices. It shows that bath application of 500 nM insulin for 10 min produced a depression of fEPSPs in male and female NDV brain slices and the fEPSPs did not fully recover after washout of insulin. However, 500 nM insulin-mediated LTD was significantly attenuated in both male and female HFV rats. *Panel B:* summary of average normalized fEPSPs from estrogen-treated ND (NDE) male and female rats ($n = 8-9$ independent slices, $n = 4-5$ animals/group) and estrogen-treated HF (HFE) male and female rats ($n = 8-9$ independent slices, $n = 4-5$ animals/group) brain slices. NDV = normal diet group with vehicle treatment, HFV = high-fat diet group with vehicle treatment, NDE = normal diet group with E2 treatment, HFE = high-fat diet group with E2 treatment.

phosphorylated form of Akt/PKB at the serine 473 site were significantly increased in the brains of E2-treated female rats compared to the vehicle-treated female rats in the HF group, suggesting that E2 could protect neuronal insulin signaling from the deleterious effect of HF feeding in female rats (Fig. 2B). In male brains, the phosphorylated form of Akt/PKB at the serine 473 site was still decreased after E2 treatment (Fig. 2B). Furthermore, in the E2 treatment group, the amount of the phosphorylated form of Akt/PKB at the serine 473 site in the ND and HF-fed groups was not different in female rats. However, in male rats that received E2 treatment, the level of phosphorylated Akt/PKB

at the serine 473 site of the HF group was significantly reduced compared to that in the ND group (Fig. 2B).

Discussion

Our study demonstrated that a 30-day administration of estrogen reduces the occurrence of HF-induced peripheral insulin resistance in male and female rats. Our findings confirmed the therapeutic effect of exogenous estrogen on peripheral insulin resistance induced by HF consumption as shown in previous studies (Kumagai et al., 1993;

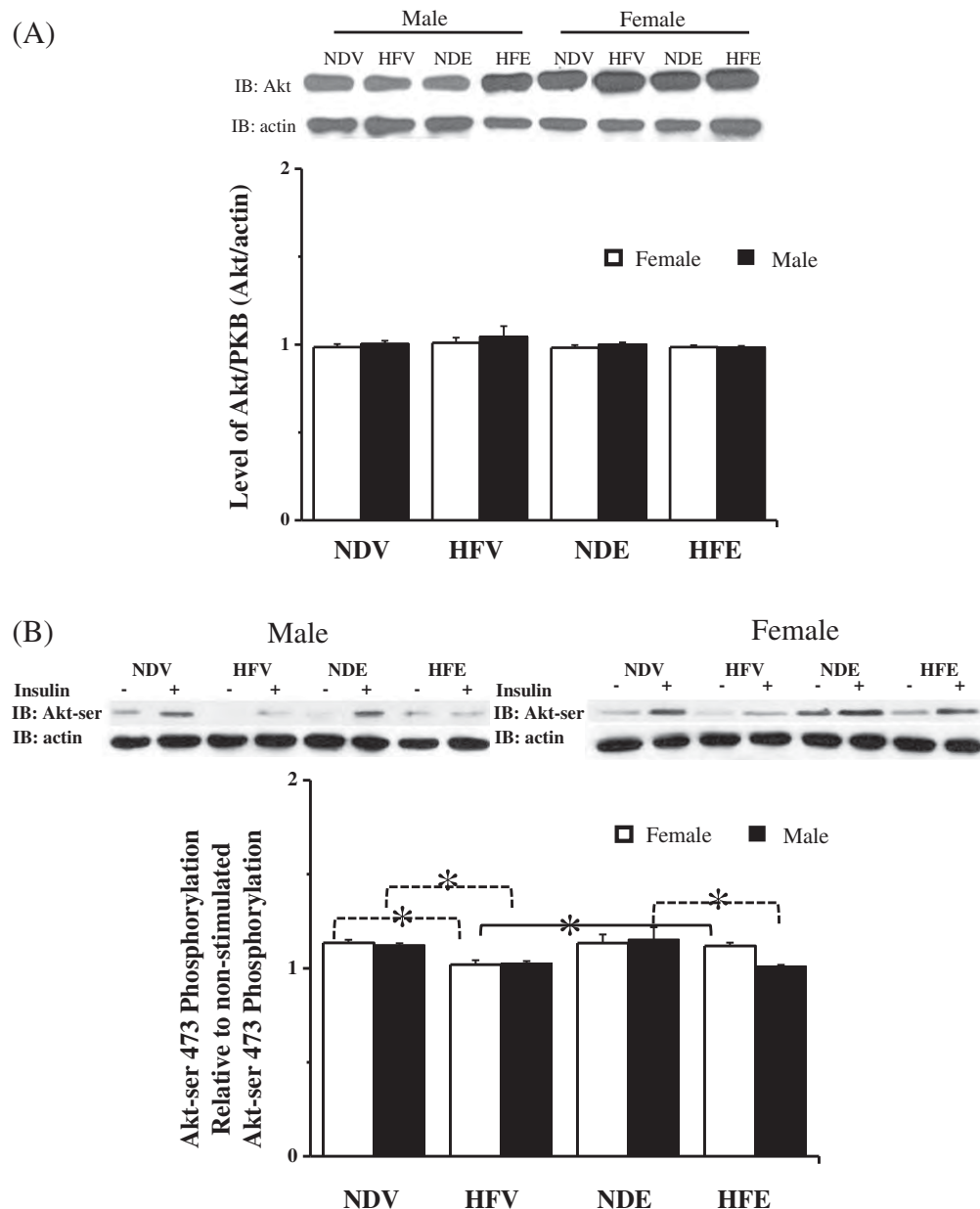


Fig. 2. Insulin-induced serine phosphorylation of neuronal Akt/PKB was decreased in both male and female HFV groups. *Panel A:* Representative blots of protein level of Akt/PKB in brain slices harvested from the NDV, NDE, HFV and HFV groups ($n = 4-5/\text{group}$) compared between male and female. The densitometric quantitation of blots from all groups was not different. *Panel B:* Representative blots of serine 473 kinase of Akt/PKB phosphorylation in brain slices harvested from the NDV, NDE, HFV and HFV groups compared between male and female rats. Densitometric quantitation of blots from insulin-stimulated Akt/PKB in the female HFE group was significantly greater than in the female HFV group. All immunoblotting lanes were loaded with equal amounts of protein (40 $\mu\text{g}/\text{lane}$). *, $p < 0.05$; NDV = normal diet group with vehicle treatment, HFV = high-fat diet group with vehicle treatment, DE = normal diet group with E2 treatment, HFE = high-fat diet group with E2 treatment; -, no insulin stimulation; +, insulin stimulation.

Riant et al., 2009). Furthermore, the present study was the first to demonstrate the beneficial effect of exogenous estrogen on neuronal insulin resistance in ND- and HF-fed rats. Our results demonstrated that estrogen treatment could attenuate the impairment of neuronal insulin receptors and neuronal insulin signaling dysfunction, particularly the Akt/PKB phosphorylation, caused by HF diet consumption only in female rats. These findings support the hypothesis that estrogen could exert its beneficial effect on neuronal insulin receptor function via improved downstream neuronal insulin receptor signaling. Our findings also indicated that estrogen could enhance Akt/PKB phosphorylation in insulin-stimulated neuronal tissues, suggesting that estrogen is directly involved in the insulin signaling pathway in neuronal-sensitive tissues. Moreover, the present study also demonstrated an important role of

estrogen receptor activation in the central nervous system which could play an important role in the metabolic effect of insulin signaling in the brain.

Estrogen has been shown to play an important role in regulating neuronal structure and function. Previous studies have demonstrated that estrogen can either prevent or improve the cognitive deficits of Alzheimer's disease (AD) (Shang et al., 2010; Simpkins et al., 1997) that regulate synaptic density in hippocampal regions (Woolley et al., 1996). In addition, chronic estradiol (90 days) treatment in female rats has been shown to induce the activation of the insulin receptor substrate-1 signaling pathway in the cerebral cortex and diencephalon of the brain (Alonso and Gonzalez, 2008). Our findings demonstrated that estrogen could improve the impairment of Akt/PKB phosphorylation. All of these beneficial effects of estrogen help explain the

improvement of insulin-induced LTD in CA1 hippocampus in the HF-fed female rats.

In the present study, the finding that estrogen could not improve the impairment of insulin-induced LTD in the HF-fed male rats could be due to the difference in the distribution of estrogen receptors in the hippocampus between male and female rats. Estrogen receptors consist of estrogen receptor alpha (ER- α) and estrogen receptor beta (ER- β). Both ER- α and ER- β are found in rat hippocampus, suggesting that this receptor plays an important role in learning and memory (Shughrue and Merchenthaler, 2000). In addition, previous studies have shown that the ERs are important regulators for metabolic disorders such as insulin resistance (Alonso and Gonzalez, 2008; Godsland, 2005; Palin et al., 2001). Furthermore, a previous study has clearly shown gender differences in the ER- β immunoreactivity in rats (Zhang et al., 2002). In that study, the authors found that the expression of ER- β in the hippocampus of female rats is greater than that in male rats. Therefore, the low numbers of ERs in the hippocampus of male rats could be insufficient for estrogen to act to attenuate the neuronal insulin resistance found in male HF rats.

Conclusion

In conclusion, the present study demonstrated that the activation of the estrogen pathway could preserve insulin sensitivity in the peripheral tissue in both male and female rats, whereas it could preserve the neuronal insulin sensitivity only in female rats. With the development of selective ER modulators, future use of the beneficial action of estrogen on neuronal insulin receptor function could be clinically significant and the undesired effects of estrogen could be avoided.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

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Effects of Left Ventricular Function on the Exercise Capacity in Patients with Repaired Tetralogy of Fallot

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Background: Tissue Doppler imaging has been recently used to evaluate ventricular function. Peak oxygen uptake ($\dot{V}O_{2\text{peak}}$) has been demonstrated as a predictor for death in adults with repaired tetralogy of Fallot (TOF). The aim of this study was to determine which Doppler parameters correlated with $\dot{V}O_{2\text{peak}}$ in patients with repaired TOF. **Method and Results:** Doppler echocardiography, tissue Doppler imaging, and exercise test were performed in 30 patients with TOF after surgical repair. In 30 patients with repaired TOF (median age 14 years, range 9–25 years), 11 patients (37%) were female. Seven patients (median age 12 years) had normal left ventricular diastolic function, whereas the rest of the patients were classified as diastolic dysfunction grade II (median age 15 years; $n = 15$) and III and IV (median age 18 years; $n = 8$). The oxygen uptake at anaerobic threshold ($\dot{V}O_{2\text{AT}}$) and peak exercise in patients with left ventricular diastolic dysfunction was significantly lower than that in those with normal diastolic function. Also, $\dot{V}O_{2\text{AT}}$ and $\dot{V}O_{2\text{peak}}$ in patients with diastolic dysfunction grade III and IV were significantly lower than that in those with diastolic dysfunction grade II. Left ventricular early diastolic myocardial velocity was most closely correlated to $\dot{V}O_{2\text{peak}}$ ($r = 0.51$; $P = 0.005$). Peak early ventricular filling velocity to early diastolic myocardial velocity ratio was significantly correlated with $\dot{V}O_{2\text{peak}}$ ($r = -0.50$; $P = 0.006$). **Conclusion:** Left ventricular diastolic dysfunction is correlated with $\dot{V}O_{2\text{peak}}$. Left ventricular diastolic function should be a routine echocardiographic assessment in patients with repaired TOF. (Echocardiography 2011;28:1019-1024)

Key words: left ventricular diastolic dysfunction, exercise, repaired tetralogy of Fallot

Tetralogy of Fallot (TOF) is the most common form of cyanotic congenital heart disease occurring in 4–9% of all congenital heart defects.¹ TOF is comprised of the four cardiac findings: large ventricular septal defect (VSD), overriding of the aorta, right ventricular outflow tract obstruction, and right ventricular hypertrophy. The surgical goal is to achieve adequate complete relief of right ventricular outflow obstruction with closure of the VSD. Surgical repair can be performed with low mortality rate and good long-term outcome.^{2–11} Nevertheless, the surgical repair leaves the patient with some degree of hemodynamic abnormalities because of residual pulmonary stenosis, pulmonary regurgitation, or myocardial dysfunction. These abnormalities cause no clinical symptom in the majority of patients. However, the careful assessment of exercise capacity demonstrates cardiopulmonary abnormality in some patients.^{12–19} Patients repaired at an older age or those followed for a longer

time tended to be the most limited.¹³ Patients with branch pulmonary stenosis,¹⁴ significant pulmonary regurgitation,^{15–21} and left^{22,23} or right ventricular dysfunction^{24–28} have reduced exercise capacity. New echocardiographic techniques may be promising for early diagnosis of right and left ventricular dysfunction. Tissue Doppler imaging has been recently used to evaluate early myocardial dysfunction. In some patients with myocardial dysfunction, diastolic ventricular dysfunction preceded the onset of systolic impairment.²⁹ Therefore, in this study, conventional pulse-wave Doppler and tissue Doppler echocardiography were evaluated in patients with repaired TOF. We hypothesized that pulse-wave Doppler and tissue Doppler imaging patterns of diastolic ventricular dysfunction are correlated with exercise intolerance in patients with TOF after a complete repair.

Methods:

Study Patients:

We prospectively studied 30 patients with TOF who underwent surgery for total correction at Chiang Mai University Hospital. Echocardiography, electrocardiography, and exercise test were

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performed. The study protocol was reviewed and approved by the Chiang Mai University Review Board. All patients or parents had consented to research participation.

Doppler Echocardiography:

Doppler echocardiographic examinations were performed using Philips Sonos 7500 (Philips Medical Systems, Bothell, WA, USA). Doppler echocardiogram and tissue Doppler imaging were performed to evaluate systolic and diastolic ventricular function. Echocardiographic data included left ventricular fractional shortening, pulse-wave Doppler assessment of mitral and pulmonary venous flows, tissue Doppler imaging, the degree of pulmonary stenosis and pulmonary regurgitation, right and left myocardial performance index, and right ventricular fractional area change. Pulse-wave Doppler of mitral valve flow measured peak early ventricular filling velocity (E), peak atrial contraction velocity (A), A-wave duration, and deceleration time. Pulse-wave Doppler of pulmonary venous flow were systolic forward flow velocity, diastolic forward flow velocity, atrial reversal flow velocity, and atrial reversal flow duration. Tissue Doppler imaging signal was obtained from an apical four-chamber view at the right ventricular free wall, ventricular septum, and left ventricular free wall. Tissue Doppler imaging variables included systolic myocardial velocity, early diastolic myocardial velocity (E_m), and late diastolic myocardial velocity. Myocardial performance index was calculated by the atrioventricular valve closing to opening time minus ventricular ejection time and divided by the ejection time. Right ventricular fractional area change (ΔFA) was calculated in apical four-chamber views; $\Delta FA = 100 \times (EDA - ESA)/EDA$, where end-diastolic area (EDA) was measured at the maximum ventricular area, coincident with the R-wave of the electrocardiogram, and end-systolic area (ESA) was measured at the minimal area in the same cycle used for EDA. Diastolic left ventricular dysfunction was graded according to pulse-wave Doppler of mitral and pulmonary venous flows and tissue Doppler imaging.³⁰

Exercise Test:

Exercise test was performed on an electric cycle ergometer (Corival, Lode, Groningen, The Netherlands). All patients performed a maximal exercise test with a two-minute incremental bicycle protocol with a workload increment of 20 watts for female and 25 watts for male. Electrocardiogram, oxygen saturation, and blood pressure were monitored. Peak oxygen uptake ($\dot{V}O_{2peak}$), carbon dioxide production ($\dot{V}CO_2$), minute ventilation ($\dot{V}_E$), and respiratory exchange ratio (RER) were measured by using

breath-by-breath technique (Ultima CPX, MED-GRAPHICS, St. Paul, MN, USA). The test was terminated according to ACSM guidelines.³¹

Statistical Analysis:

All statistical calculations were assessed using commercially available software (SPSS Version 16, SPSS Inc., Chicago, IL, USA). Comparison of $\dot{V}O_{2peak}$ between groups of diastolic ventricular dysfunction was performed using Wilcoxon rank sum. Linear regression analysis was used to assess the correlation between the Doppler parameters and the $\dot{V}O_{2peak}$. A P-value less than 0.05 was considered statistically significant.

Results:

Thirty patients with repaired TOF were studied (median age 14 years, range 9–25 years). Eleven patients (37%) were female. Seven patients (median age 12 years) had normal left ventricular diastolic function, 15 patients (median age 15 years) had diastolic dysfunction grade II, and 8 patients (median age 18 years) had diastolic dysfunction grade III and IV. Baseline clinical characteristics were compared in Table I. The patients with normal diastolic function were younger at the time of operation than the other groups. The patients with diastolic dysfunction grade III and IV had more body surface area and more females than the other groups. However, follow-up time, previous modified Blalock–Taussig shunt, heart rate, and blood pressure were not statistically different among these three groups. The patients had mild residual pulmonary stenosis (median gradient 8 mmHg, range 5–32 mmHg).

Exercise Parameters:

Observed exercise values stratified by left ventricular diastolic function were summarized in Table II. The oxygen uptake at anaerobic threshold ($\dot{V}O_{2AT}$) and $\dot{V}O_{2peak}$ in patients with left ventricular diastolic dysfunction was significantly lower than that in those with normal diastolic function. Also, $\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$ in patients with diastolic dysfunction grade III and IV were significantly lower than that in those with diastolic dysfunction grade II (Fig. 1). However, heart rate, oxygen pulse, $\dot{V}_E$, $\dot{V}_E/\dot{V}CO_2$, RER, and submaximal exercise were not significantly different among these three groups.

Relationship of Pulse-Wave Doppler and Tissue Doppler Data to $\dot{V}O_{2peak}$:

Results of the relationship between the Doppler data and $\dot{V}O_{2peak}$ are graphically displayed in Figure 2. Left ventricular early diastolic myocardial velocity obtained at the left ventricular free wall was most closely correlated to the $\dot{V}O_{2peak}$ ($r = 0.51$; $P = 0.005$). Peak early ventricular filling

TABLE I

Demographic and Characteristics of Patients According to Left Ventricular Diastolic Dysfunction (N = 30)

	Normal (N = 7)	Diastolic Dysfunction Grade II (N = 15)	Diastolic Dysfunction Grade III and IV (N = 8)	P Value
Age (years)	12 ± 2	16 ± 4	18 ± 6	0.02
Age at operation (years)	4 ± 1	6 ± 2	8 ± 5	0.04
Follow-up time (years)	8 ± 1	10 ± 3	10 ± 4	0.20
NYHA class (N)				0.06
I	7	14	5	
II	0	1	3	
Modified Blalock–Taussig shunt, N (%)	2 (29%)	2 (13%)	2 (25%)	0.65
BSA (m ²)	1.1 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	0.02
Heart rate at rest (beats/min)	75 ± 10	77 ± 11	84 ± 13	0.40
Systolic BP at rest (mmHg)	98 ± 7	110 ± 14	106 ± 13	0.09
Diastolic BP at rest (mmHg)	59 ± 8	69 ± 13	63 ± 12	0.14
Severity of pulmonary regurgitation, N				0.89
Mild	2	3	1	
Moderate	2	7	4	
Severe	3	5	3	

TABLE II

Comparison of the Exercise Data Stratified by Left Ventricular Diastolic Function

Parameters	Diastolic Dysfunction	Rest	Exercise	
			Anaerobic Threshold	Peak
Heart rate (beats/min)	Normal	86 ± 15	108 ± 10	164 ± 16
	Grade II	85 ± 12	99 ± 14	164 ± 13
	Grade III and IV	89 ± 11	102 ± 11	153 ± 12
V̇O ₂ (mL/kg per min)	Normal	11 ± 2	21 ± 4	41 ± 9
	Grade II	8 ± 2*	16 ± 4*	33 ± 7*
	Grade III and IV	8 ± 1*	12 ± 2*#	25 ± 6*#
V̇O _{2AT} /V̇O _{2max} (%)	Normal			51 ± 4
	Grade II			49 ± 7
	Grade III and IV			51 ± 13
Oxygen pulse (mL/min per beats)	Normal	3.9 ± 1.3	6.0 ± 1.6	7.6 ± 1.6
	Grade II	4.0 ± 1.1	7.1 ± 2.4	8.8 ± 3.4
	Grade III and IV	4.2 ± 1.1	5.7 ± 1.1	7.7 ± 2.5
Minute ventilation (L/min)	Normal	10 ± 2	19 ± 4	42 ± 8
	Grade II	11 ± 3	19 ± 6	47 ± 16
	Grade III and IV	12 ± 3	15 ± 3	39 ± 14
V̇ _E /CO ₂	Normal	38 ± 4	33 ± 5	32 ± 6
	Grade II	36 ± 5	29 ± 5	27 ± 4
	Grade III and IV	40 ± 6	33 ± 5	29 ± 6
Respiratory exchange ratio	Normal	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
	Grade II	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
	Grade III and IV	0.8 ± 0.1	0.8 ± 0.1	1.1 ± 0.2
Submaximal exercise, N (%)	Normal			2 (29%)
	Grade II			2 (13%)
	Grade III and IV			1 (13%)

V̇O₂ = oxygen uptake; V̇O_{2AT} = oxygen uptake at anaerobic threshold; V̇_E/CO₂ = minute ventilation and carbon dioxide production ratio.

*P < 0.05 versus normal diastolic function.

#P < 0.05 for grade II versus grade III and IV.

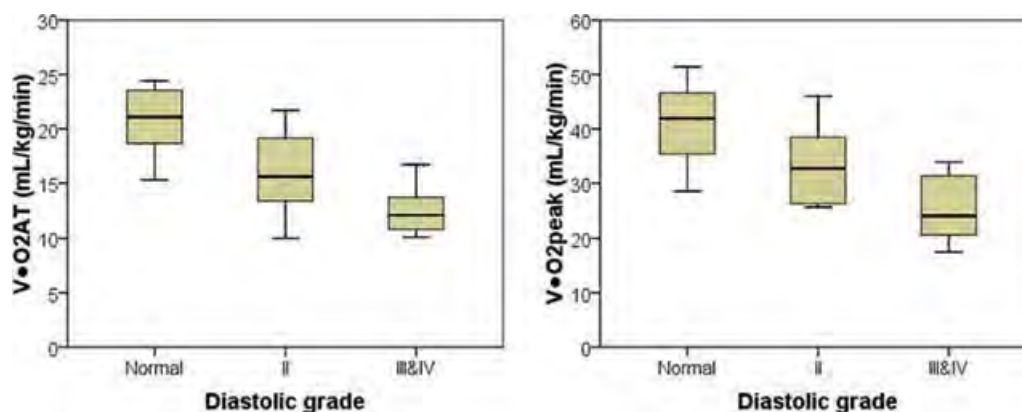


Figure 1. Graphic display comparing the peak oxygen uptake according to grade of left ventricular diastolic function. $\dot{V}O_{2AT}$ = oxygen uptake at anaerobic threshold; $\dot{V}O_{2peak}$ = peak oxygen uptake.

velocity to early diastolic myocardial velocity ratio (E/Em) significantly correlated with $\dot{V}O_{2peak}$ ($r = -0.50$; $P = 0.006$). Left ventricular ejection fraction, myocardial performance index, systolic myocardial velocity, and late diastolic myocardial velocity were not correlated with $\dot{V}O_{2peak}$. Right ventricular echocardiographic parameters including myocardial performance index, fractional area change, and tissue Doppler imaging were not significantly correlated with $\dot{V}O_{2peak}$. Also, pulmonary stenosis gradient and severity of pulmonary regurgitation were not correlated with $\dot{V}O_{2peak}$.

Discussion:

This study supports our hypothesis that pulse-wave Doppler and tissue Doppler patterns of left ventricular diastolic dysfunction correlated with $\dot{V}O_{2peak}$. $\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$ in patients with repaired TOF, who have left ventricular diastolic dysfunction, was significantly lower than that in those with normal diastolic left ventricular func-

tion. Furthermore, $\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$ in patients with diastolic dysfunction grade III and IV was significantly lower than that in those with diastolic dysfunction grade II. Left ventricular early diastolic myocardial velocity obtained at the left ventricular free wall was significantly correlated to $\dot{V}O_{2peak}$. Peak early ventricular filling velocity to early diastolic myocardial velocity ratio also correlated with $\dot{V}O_{2peak}$. In fact, decreased early diastolic myocardial velocity and increased early ventricular filling velocity to early diastolic myocardial velocity ratio are characteristics of diastolic left ventricular dysfunction with increased ventricular end-diastolic pressure and increased left atrial pressure. These findings are of clinical relevance, because tissue Doppler imaging is a readily available tool, and early diastolic myocardial velocity is easier and faster to obtain than exercise capacity testing, especially in young children.

Cheung et al. reported that global left ventricular deformation because of right ventricular dilation was an independent predictor of $\dot{V}O_{2peak}$

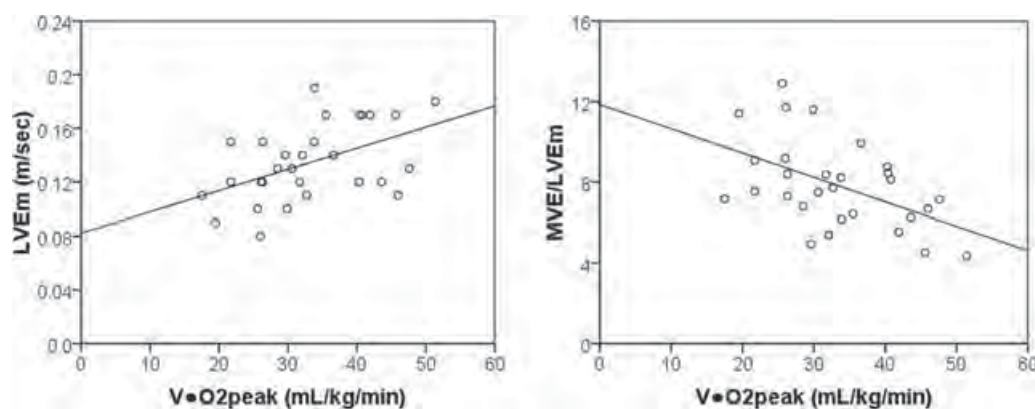


Figure 2. Linear regression analysis comparing the pulse-wave Doppler and tissue Doppler data and peak oxygen uptake in patients with repaired tetralogy of Fallot. LVEEm = left ventricular free wall early diastolic myocardial velocity; MVE/LVEEm = mitral valve early ventricular filling velocity to left ventricular early diastolic myocardial velocity ratio; $\dot{V}O_{2peak}$ = peak oxygen uptake.

in patients with repaired TOF.³² This study supported this finding that diastolic left ventricular dysfunction was correlated with $\dot{V}O_2$ peak. Right ventricular dilation might affect the ventricular–ventricular interaction resulting in left ventricular diastolic dysfunction. Norozi et al.³³ and Samman et al.³⁴ reported that biventricular dysfunction was associated with diminished exercise capacity. $\dot{V}O_2$ peak was correlated with left ventricular Tei index.^{33,34} In fact, Tei index has been shown as an echocardiographic parameter of global systolic and diastolic ventricular function.³⁵

Several studies demonstrated right ventricular dysfunction was associated with decreased exercise capacity in adult patients after repaired TOF.^{24,25} However, Cheung et al. reported right ventricular function including myocardial performance index, ejection fraction, and tricuspid annular velocity was not a predictor of $\dot{V}O_2$ peak in adolescents after repaired TOF.³⁶ Our findings in this study supported their finding that right ventricular function in children and adolescents was not correlated with $\dot{V}O_2$ peak. Right ventricular function in children and adolescents may be still preserved for pressure and volume overload. We do believe that residual severe pulmonary stenosis and severe pulmonary regurgitation in long-term follow-up might affect right ventricular function, left ventricular diastolic function, and the exercise capacity in adult patients with repaired TOF. The correction of the residual heart defect and medical treatment of left ventricular diastolic function might improve the exercise tolerance in the adult patients after repaired TOF.

The diastolic grading system provides a semi-quantitative approach to classify the severity of ventricular function using mitral valve flow, pulmonary venous flow, and tissue Doppler imaging.³⁰ Ommen et al. demonstrated that the combined parameter of E/Em provided a better estimate of left ventricular filling pressure than other variables.³⁷ In this study, the grade of diastolic left ventricular function was correlated to $\dot{V}O_2$ peak. Also, Doppler variables of diastolic dysfunction including left ventricular early diastolic myocardial velocity and increased E/Em ratio were significantly correlated with $\dot{V}O_2$ peak.

Aortic root dilatation and aortic regurgitation were correlated with reduced left ventricular systolic function in the adult patients with repaired TOF.³⁸ In this study, five patients had aortic root dilatation. Only two patients had mild aortic regurgitation in children and adolescents with repaired TOF. Therefore, the number of patients is not enough to be correlated with left ventricular function. We believe that these sequelae might have significant impact on left ven-

tricular function in adult patients after repaired TOF.

There is a limitation in our study for evaluation of right ventricular function using echocardiography. Magnetic resonance imaging is the gold standard for evaluation of right ventricular function, but this method is expensive. However, tissue Doppler imaging has been recently used to evaluate right ventricular dysfunction.^{24,39,40} Long-term study of the echocardiographic assessment for systolic and diastolic ventricular function in a large number of patients with repaired TOF including older ages is warranted.

Conclusions:

The severity of the left ventricular diastolic dysfunction was inversely correlated with $\dot{V}O_2$ peak. Left ventricular diastolic dysfunction could be responsible for exercise intolerance. Left ventricular diastolic function should be a routine part of the echocardiographic assessment in patients with repaired TOF. Treatment of left ventricular diastolic dysfunction might improve exercise intolerance in children and adolescents after repaired TOF.

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Changes in peripheral innervation and nociception in reticular type and erosive type of oral lichen planus

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ABSTRACT

Background: Oral lichen planus (OLP) is a chronic inflammatory lesion in oral mucosa. Reticular (OLP-R) and erosive (OLP-E) types of OLP are the common forms that have been found in dental clinics. The aim of this investigation is to determine the correlation between neurogenic inflammation and nociception associated with OLP-R and OLP-E.

Materials and Methods: The oral mucosal lesions from six patients with OLP-E, four with OLP-R and three with noninflamed oral mucosa, which represent normal mucosa, were identified by morphometric analysis of nerve fibers containing immunoreactive protein gene product (PGP) 9.5. The level of inflammation was measured with hematoxylin and eosin staining and the level of nociception was analyzed with visual analog scale measurement.

Results: We found that 1) an increase in peripheral innervation was related to the size of the area of inflammatory cell infiltration from both OLP-R and OLP-E; 2) the pattern of PGP 9.5-immunoreactivity among OLP-R and OLP-E was not significantly different ($P=0.23$); and 3) the correlation between nociception and an increase in PGP 9.5-immunoreactivity was not found in OLP-E and in OLP-R.

Conclusions: Our findings suggest that an increase in peripheral innervation may lead to increased inflammation, which is part of the immunopathogenesis of OLP. Differences in nociception between OLP-R and OLP-E arise from the pathogenesis of each lesion, not from the differences in peripheral innervation.

Key words: Neurogenic inflammation, nociception, oral lichen planus

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During the past few years, substantial evidence for a relationship between the immune and the nervous systems has been reported. For example, 1) antigen-presenting dendritic cells or immune cells of the epidermis have been shown to be in close contact with cutaneous nerve fibers^[1] and 2) neuropeptides, i.e. mediators released by cutaneous nerves, have been demonstrated to influence antigen-presenting cells or immune cells in skin.^[2] There is evidence that changes in the skin innervation observed in dermatological diseases contribute to the inflammatory process and to the associated painful sensation. An increase

in cutaneous nerve fibers has been reported in itching and painful cutaneous diseases including neurofibroma,^[3] nostalgia paraesthetica,^[4] prurigo nodularis,^[5] vulvodynia^[6] and psoriasis.^[7,8] In contrast, a reduction of cutaneous nerve fibers can be found in conditions associated with decreased cutaneous nociception, such as diabetic neuropathy,^[9] leprosy,^[10] or HIV-infected patients suffering from xerosis.^[11] The current theory of persistent pain following tissue injury or inflammation is that sensitization involves both the central^[12-15] and peripheral nervous systems.^[16] A number of studies suggest that the interaction between the inflammation and neural system exists and may lead to painful sensation.^[16]

Oral lichen planus (OLP) is a chronic inflammatory disease of the oral mucosa.^[17,18] Clinically, OLP is commonly found bilaterally and may present as striated, plaque, atrophic or erosive lesions. OLP is histologically characterized by a well-defined, band-like lymphocytic subepithelial inflammation. OLP usually presents with burning sensation or persistent pain. Although OLP is classified into six types, the reticular type (OLP-R) and erosive type of OLP (OLP-E) are the two most common types. Each type has different clinical features. OLP-R presents without a painful sensation,

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whereas pain is the most commonly found symptom in OLP-E. Although it has been shown that cutaneous innervation or peripheral sensitization is increased following chronic inflammation in dermatological diseases^[3-8] and oral lichen planus,^[19] information regarding the correlation between neurogenic inflammation and nociception associated with oral mucosal disease such as OLP-R and OLP-E is limited. The present study sought to determine whether 1) two different types of OLP with different levels of nociception, OLP-R and OLP-E, express different patterns of nerve sprouting or peripheral sensitization, and 2) a correlation of an increase of peripheral innervation, inflammatory reaction, and nociception in both OLP lesions exists.

MATERIALS AND METHODS

Patient selection

Thirteen patients with oral mucosal lesions were included in this study. None of the patients had a history of either systemic disease or corticosteroid injections to their lesions. All patients had been treated for *Candida* infection before biopsy. Clinical details of these patients are shown in Table 1.

All patients were asked to rank the painful sensation of oral lesions before biopsy using a visual analog scale (VAS) system, in which "0" meant no pain at all and "10" meant as painful as imaginable. Biopsies from the oral mucosa of these thirteen patients were obtained. From histological examination, six patients were diagnosed with OLP-E, four with OLP-R and three with noninflamed oral mucosa from the operculum of a third molar after surgical removal of the tooth. The lesions were classified histologically using WHO criteria.^[20]

All biopsies came from the buccal mucosa under local anesthesia (2% lidocaine with 1000,000 units norepinephrine). In this study, the biopsy of the OLP-E lesions was performed at the edge of erosive area with the white-striated lesions and the biopsy of the OLP-R lesions was performed at the white-striated area. Some slices of all tissue samples were used for routine histological evaluation including hematoxylin and eosin (H and E) staining. Other slices of all samples were used for protein gene product 9.5 (PGP 9.5) immunohistochemical analysis.

Informed consent was obtained from all patients, and the research plan was accepted by the Ethics Committee of the Faculty of Dentistry, Chiang Mai University, Thailand.

Table 1: Clinical data for patients with OLP-R, OLP-E and noninflamed oral mucosa

	<i>n</i>	Male	Female	Age mean/ range (years)
OLP-R	4	1	3	55/38-76
OLP-E	6	3	3	47/26-63
Noninflamed oral mucosa	3	0	3	30/21-47

OLP-R: Reticular type of oral lichen planus; OLP-E: Erosive type of oral lichen planus

Immunohistochemistry

Three-micron thickness paraffined sections were mounted on 3-aminopropyltriethoxysilane (Dako, Glostrup, Denmark)-coated slides. Sections were deparaffinized with xylene and ethanol and then placed in a boiling solution of freshly prepared citrate buffer (pH 6.0) for 10 min in a pressure cooker before immunostaining. Endogenous peroxidase was inhibited by soaking the sections in 0.3% H₂O₂ in 0.1 M phosphate-buffer saline (PBS) for 30 min at room temperature. The sections were incubated sequentially in a humidified chamber with 1) 3% normal goat serum in 0.1 M PBS (3% NGS, Sigma, St. Louis, MO, USA) as a blocking serum at room temperature for 1 h; 2) primary mouse monoclonal antisera PGP 9.5 (Novacastra Co, Wetzlar, Germany) diluted 1: 20 in 3% normal goat serum overnight at + 4°C; 3) biotinylated goat anti-mouse IgG (Santa Cruz Biotech, Santa Cruz, CA, USA) diluted 1:200 in 0.1 M PBS for 1 h at room temperature; and 4) avidin-biotin-peroxidase complex (Santa Cruz Biotech, Santa Cruz, CA, USA) for 45 min at room temperature. After each step, the sections were rinsed three times in PBS for 5 min. Staining was amplified with the glucose oxidase-3, 3-diaminobenzidine (DAB)-nickel method for 10 min. Sections were washed three times with water for 5 min. The slides were dehydrated in alcohol and xylene, before being mounted in permount (Sigma, St. Louis, MO, USA).

Microscopic evaluation and qualitative assessment

Microscopic examination was performed with an Olympus microscope coupled for morphometric assessment with a digital camera and linked to a semiautomatic Kontron image analysis and processing system. Morphometry of PGP 9.5 immunoreactivity (PGP 9.5-ir) and H and E staining were performed under ×200 magnification. Nerve fiber densities or PGP 9.5-positive fibers (linear intercept number/mm² tissue) were performed from the subepithelial area (i.e. an area extending 100 μm deep from the basement membrane area to the lamina propria) in each section as described in a previous study.^[19] In each patient, nerve fibers densities were counted in five microscopic fields, in which were covered the whole area in each slide and counted three slides per sample. The epithelium was excluded from the measurement.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). For comparison between multiple groups, nonparametric analysis with Kruskal–Wallis test and Pearson correlation coefficient were used for data analysis.

RESULTS

Overall innervation as assessed by PGP 9.5-ir as a marker

To determine the pattern of cutaneous innervation of OLP-E, OLP-R and the noninflamed oral mucosa, representing normal mucosa, PGP 9.5-immunoreactivity (PGP 9.5-ir)

was used. The specificity of this primary PGP 9.5 antiserum was previously tested in previous studies.^[19,21] An absence of staining was found when the primary antibody was omitted [Figures 1a, c, e and g]. The peripheral nerves visualized by the general neural marker PGP 9.5-ir were easily noticeable. In noninflamed oral mucosa (OM), nerve fibers were located in the deep lamina propria. They were rarely found in close contact with basement membrane. Nerve trunks and rich perivascular innervation appeared in the deeper parts of lamina propria [Figures 1b, d]. However, in both types of OLP, PGP 9.5-positive nerve fibers were concentrated in the superficial subepithelial tissue, and nerve fibers in OLP were found

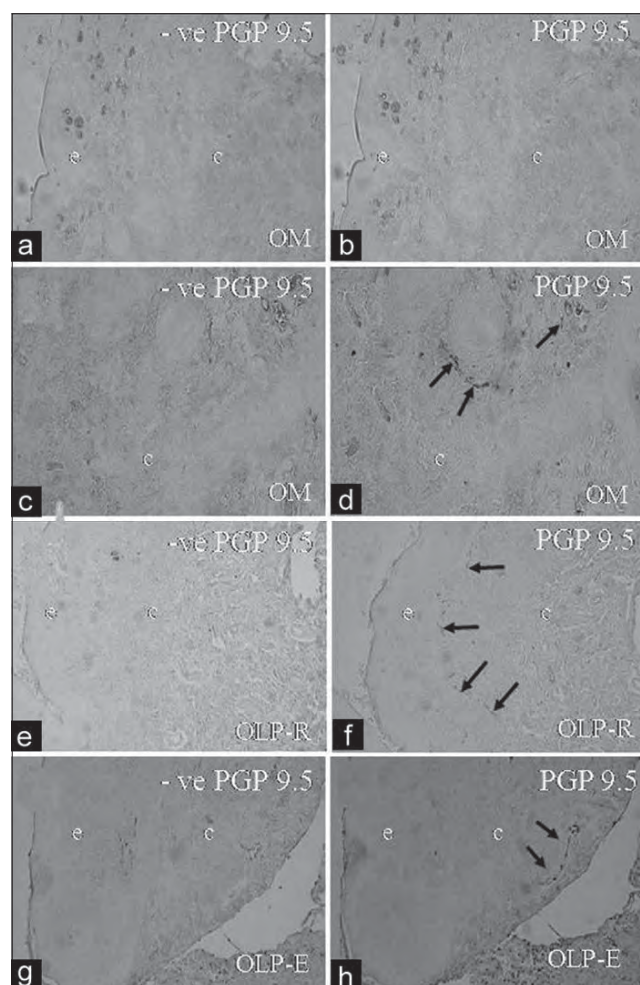


Figure 1: Pattern of overall innervation in noninflamed oral mucosa (OM), reticular type of oral lichen planus (OLP-R) and erosive type of oral lichen planus (OLP-E). Avidin-biotin peroxidase complex (ABC) staining of PGP 9.5 amplified with glucose oxidase-nickel ammonium sulfated methods in all panels (a-h), magnification $\times 200$. a, c, e, f represent the negative control (omitting primary antibody) from adjacent sections of PGP 9.5-positive nerve fibers. No PGP 9.5-positive nerve fibers are seen in any control sections. B and D show the PGP 9.5-positive nerve fibers in noninflamed oral mucosa. Innervation in noninflamed oral mucosa is distributed completely in the deep lamina propria surrounding the blood vessels (arrows) (d). No subepithelial neural network is found (b). OLP-R (f) and OLP-E (h) show a dense subepithelial neural network in OLP (arrows). Nerve fibers are concentrated close to the basement membrane. e: Epithelium; c: Connective tissue

close to the basement membrane [Figures 1f, h]. As shown in Figure 2, the pattern of PGP 9.5-positive nerve fibers in OLP-R and OLP-E was similar. Means of PGP 9.5-positive nerve fibers per each subepithelial area of OM (1.0 ± 1.0 linear intercept/ mm^2), OLP-R lesions (2.4 ± 0.8 linear intercept/ mm^2) and OLP-E lesions (3.5 ± 1.2 linear intercept/ mm^2) were not significantly different ($P=0.39$) [Figure 2c]. Furthermore, PGP 9.5-positive nerve fiber density of both types of OLP lesions was high at the superficial subepithelial areas. Underneath this area, i.e. connective tissue layer, high lymphocytic infiltration was seen where no PGP 9.5-positive fibers were found [Figures 3a-d]. By contrast, neither PGP 9.5-positive nerve fibers nor inflammatory infiltration at the superficial subepithelial areas were found in noninflamed oral mucosa tissue [Figures 3e, f]. Hence, the density of PGP 9.5-positive nerve fibers was greatest in the subepithelial inflamed area of both types of OLP lesions, suggesting the existence of a neuroinflammatory process or peripheral sensitization in this disease as in other inflammatory dermatological diseases. In addition, the pattern of peripheral innervation between the two different clinical features of OLP-R and OLP-E were not different.

No correlation of innervation and pain levels as assessed by means of VAS in OLP-E and OLP-R

To determine whether correlation between peripheral sensitization or nerve sprouting and nociception exists, VAS

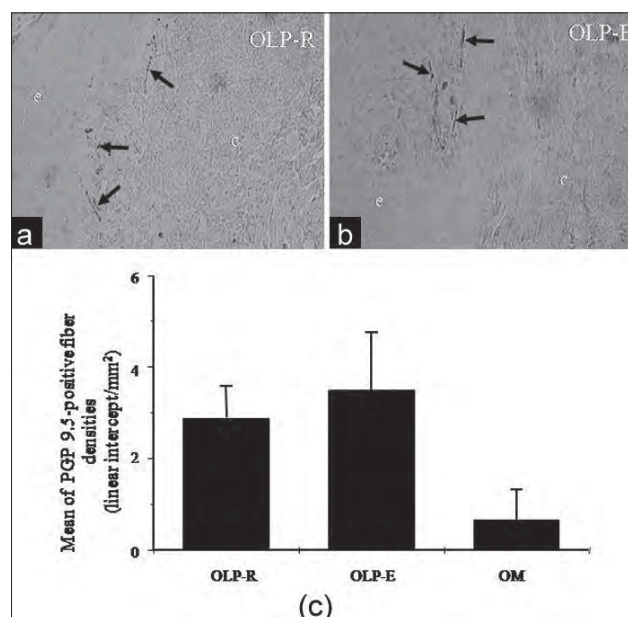


Figure 2: No differences in the pattern of PGP9.5-positive nerve fibers between reticular type of oral lichen planus (OLP-R) and erosive type of oral lichen planus (OLP-E). (a) A sample of PGP 9.5-positive nerve fibers from OLP-R lesions. (b) A sample of PGP9.5-positive nerve fibers from OLP-E lesions. The innervation of both OLP lesions is distributed at the subepithelial networks (arrows). Magnification $\times 200$. (c) A bar graph represents the mean number of PGP 9.5-positive nerve fiber densities in each pathological lesion: Reticular type of oral lichen planus (OLP-R), erosive type of oral lichen planus (OLP-E) and noninflamed oral mucosa (OM)

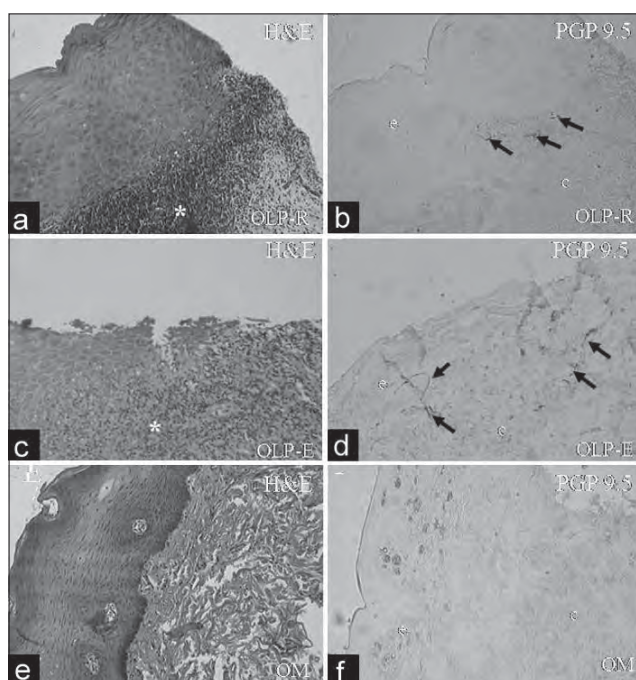


Figure 3: An increase in innervation in the areas of prominent subepithelial lymphocytic infiltration in reticular type of oral lichen planus (OLP-R) and in erosive type of oral lichen planus (OLP-E). H and E staining of OLP-R, OLP-E and noninflamed oral mucosa show in a, c and e, respectively. Avidin-biotin peroxidase complex (abc) staining of PGP 9.5 amplified with glucose oxidase-nickel ammonium sulfated methods from adjacent tissue sections of H and E sections from OLP-R (a), OLP-E (c) and noninflamed oral mucosa: OM (e) presented in b, d and f, respectively. Both PGP9.5-positive nerve fibers (arrows) and lymphocytic infiltration areas (asterisks) are concentrated in the subepithelial areas, in both types of OLP lesions. However, there is no subepithelial innervation with any inflammatory infiltration in noninflamed oral mucosa (e and f). Magnification $\times 200$

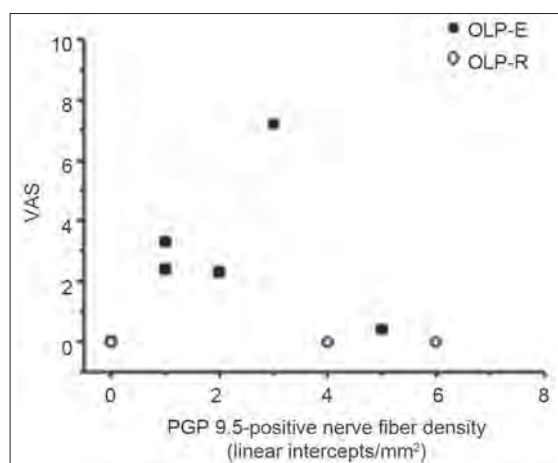


Figure 4: There is no correlation between PGP 9.5-positive nerve fiber densities and nociception in the reticular type of oral lichen planus (OLP-R) or in the erosive type of oral lichen planus (OLP-E). Nociception in each patient is measured with a visual analog scale (VAS) before biopsy

was used as a pain sensation measurement. VAS and PGP 9.5-positive nerve fiber densities were compared in each

patient. We found that the average VAS scores in patients with OLP-E (3.12 ± 1.12 , $n=6$) were significantly greater than those in patients with OLP-R (0 ± 0 , $n=4$, $P=0.005$). However, a correlation between means of PGP 9.5-positive nerve fiber density and VAS scores existed in patients with OLP-R ($r=0.0$, $P=0$) and in patients with OLP-E ($r=0.21$, $P=0.7$) was not found [Figure 4]. Although an increase in innervation of OLP-E lesions were similar in quality and quantity to that of OLP-R lesions, the pathophysiological changes of epithelial lesions from both types of lesion were different. Loss of epithelium was the characteristic of OLP-E; in contrast, parakeratinized epithelium was distinct in OLP-R. These data imply that it is the epithelial changes in the lesions, not the number of peripheral nerve fibers, which are related to nociception in OLP lesions.

DISCUSSION

Our findings have confirmed the earlier findings that 1) the pattern of innervation in the normal oral mucosa is described as a discontinuous network in the deep lamina propria with no appearance in the superficial lamina propria;^[22] 2) the increase in PGP 9.5-positive nerve fibers at the superficial lamina propria was seen in OLP; and 3) the pathophysiological changes in innervation are associated with chronic inflammation.^[19] In addition, we have demonstrated that 1) the pattern of innervation in OLP-R and OLP-E is similar, despite the fact that the painful sensations in each is different; and 2) nociception and an increase of nerve sprouting in the superficial lamina propria is not correlated in either OLP-E lesions or in OLP-R lesions.

Nerve sprouting and chronic inflammation in OLP

An increase in PGP 9.5-positive nerve fibers was always observed in both OLP lesions but not in the normal mucosa. These results could be due to both nerve sprouting and collateral formation in OLP lesions. The regeneration of nerve fibers in OLP lesions may occur after tissue injury and may follow angiogenesis of inflamed tissue as demonstrated in previous studies.^[23] Sprouting has been demonstrated in other inflammatory models such as: 1) in adjuvant-induced arthritis at the border zone of inflammation^[24,25] and 2) in the wound healing of skin lesions from chronic constrictive injury of sciatic nerves or capsaicin-induced depletion of sensory C-fibers in animal models.^[26] The induction factors for the existence or elongation of PGP 9.5-positive nerve fibers in lichen planus may be the local mediators secreted by inflammatory cells as reported in previous studies.^[24,25] It is also possible that the elongation process of PGP 9.5 positive nerve fibers in lichen planus may act as an attempt at regeneration after chronic inflammation.

The pattern of sprouting from OLP-R and OLP-E was seen to follow the pattern of lymphocytic inflammation. In OLP lesions, the band-like lymphocytic inflammation was found in the subepithelial layers or superficial lamina propria, and

the nerve sprouting was also restricted to the superficial lamina propria. This finding suggests the existence of the reaction between neurogenic and immune systems. In addition, our findings support the theory of neurogenic inflammation in chronic inflammatory diseases.^[18,23,27]

Increased peripheral innervation and nociception in OLP

PGP 9.5-immunoreactivity (PGP 9.5-ir) was used to locate single nerve fibers, nerve bundles, and nerves surrounding vessels and innervating muscles and glands.^[21] Although PGP 9.5-ir reflects the general innervation pattern of the tissue, several studies have shown that PGP 9.5-positive nerve fibers were correlated with nociception. For example, 1) the increase of PGP 9.5-positive nerve fibers at skin lesions was found with an increase in painful sensation such as thermal hyperalgesia and mechanical allodynia in a chronic constrictive injury in an animal model of painful partial nerve injury;^[28,29] 2) loss of cutaneous pain sensation with a reduction of PGP 9.5-positive nerve fibers in leprosy skin lesions^[30] and 3) PGP 9.5-positive nerve fibers were reduced in the capsaicin-induced dermal lesion in a rat model, in which capsaicin induces depletion of sensory C-fiber neuropeptides.^[26] All of the findings from these previous reports suggest that PGP 9.5 positive nerve fibers are very well correlated with nociceptors. In this study, we found that the mean number of PGP 9.5-positive nerve fibers was not correlated with nociception in OLP-E or in OLP-R. The difference in nociception in both OLP types may be the result of pathophysiological changes in both types of lesion. OLP-E is characterized by loss of mucosal epithelium, which leads to the exposure of lamina propria to the surrounding environment in the oral cavity. OLP-R, however, has parakeratinized epithelium covering the mucosal lesion. Although the pattern and number of PGP 9.5-positive nerve fibers from both lesions are not significantly different, the pathophysiological nature of epithelium from both lesions is totally distinguishable. The epithelial differences in the pathophysiology of OLP-R and OLP-E may be the reason for the difference in pain sensation, because sensory afferents may receive stronger and/or more frequent nociceptive input in OLP-E than in OLP-R. The reason that being no difference in lymphocytic infiltration or PGP 9.5-positive nerve fibers were seen between OLP-E and OLP-R could be due to the fact that extensive lymphocytic inflammation could lead to the loss of nerve fibers in that area, as seen in previous study.^[19]

Limitation of this study

Although the pattern of sprouting in OLP lesions in our study was similar to the pattern in a previous study,^[19] the number of PGP 9.5-positive nerve fibers was much less than in the previous report.^[19] This difference may be due to different techniques used in each study. We used paraffined sections with pressure-cooking techniques as antigen-unmasking techniques, whereas the previous study

used frozen sections without antigen-unmasking. Our technique could have led to the loss of some tissue during the immunochemical process.

CONCLUSIONS

Chronic subepithelial inflammation is a diagnostic feature of OLP. Although OLP-E and OLP-R have similar inflammatory responses, the nociception or stressful sensations in patients with the respective OLP lesions are different. The present work suggests that the difference in nociception between both OLP lesion types relates to the structural changes in the epithelium rather than the increase of nerve sprouting from the lesions. In addition, this study supports the relationship between associated neurogenic factors and inflammatory processes in OLP lesions.

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Research Paper

Effect of rosiglitazone on cardiac electrophysiology, infarct size and mitochondrial function in ischaemia and reperfusion of swine and rat heart

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Rosiglitazone, a peroxisome proliferator-activated receptor γ agonist, has been used to treat type 2 diabetes. Despite debates regarding its cardioprotection, the effects of rosiglitazone on cardiac electrophysiology are still unclear. This study determined the effect of rosiglitazone on ventricular fibrillation (VF) incidence, VF threshold (VFT), defibrillation threshold (DFT) and mitochondrial function during ischaemia and reperfusion. Twenty-six pigs were used. In each pig, either rosiglitazone (1 mg kg^{-1}) or normal saline solution was administered intravenously for 60 min. Then, the left anterior descending coronary artery was ligated for 60 min and released to promote reperfusion for 120 min. The cardiac electrophysiological parameters were determined at the beginning of the study and during the ischaemia and reperfusion periods. The heart was removed, and the area at risk and infarct size in each heart were determined. Cardiac mitochondria were isolated for determination of mitochondrial function. Rosiglitazone did not improve the DFT and VFT during the ischaemia–reperfusion period. In the rosiglitazone group, the VF incidence was increased (58 *versus* 10%) and the time to the first occurrence of VF was decreased (3 ± 2 *versus* 19 ± 1 min) in comparison to the vehicle group ($P < 0.05$). However, the infarct size related to the area at risk in the rosiglitazone group was significantly decreased ($P < 0.05$). In the cardiac mitochondria, rosiglitazone did not alter the level of production of reactive oxygen species and could not prevent mitochondrial membrane potential changes. Rosiglitazone increased the propensity for VF, and could neither increase defibrillation efficacy nor improve cardiac mitochondrial function.

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Rosiglitazone is a synthetic agonist of the peroxisome proliferator-activated receptor γ , which has been used to reduce insulin resistance in the treatment of type 2 diabetes (Guo & Tabrizchi, 2006). Rosiglitazone acts as a potent insulin sensitizer by improving glycaemic control (Sauer *et al.* 2006). Data from various studies in the past decade have suggested that rosiglitazone also has cardioprotective effects, such as improvement of cardiac contractile dysfunction (Yue *et al.* 2005), inhibition of the inflammatory response by reducing accumulation of neutrophils and macrophages (Mersmann *et al.* 2008), and protection from myocardial injury during periods of

ischaemia–reperfusion in both animal (Yue *et al.* 2001; Khandoudi *et al.* 2002; Molavi *et al.* 2006; Gonon *et al.* 2007; Mersmann *et al.* 2008) and clinical studies (Sidhu *et al.* 2003; Yu *et al.* 2007; Li *et al.* 2008).

Despite these beneficial effects, growing evidence has indicated that rosiglitazone could be harmful to the heart. A recent study in a porcine model demonstrated that treatment with rosiglitazone decreased the time to onset of spontaneous ventricular fibrillation (VF) during ischaemia (Lu *et al.* 2008). Moreover, reports on the effects of rosiglitazone on the reduction of infarct size have been inconsistent (Lautamaki *et al.* 2005; Yue *et al.* 2005; Gonon

et al. 2007; Graham *et al.* 2010). Growing evidence in clinical trials also indicates possible adverse cardiac effects in patients treated with rosiglitazone (Monami *et al.* 2008; Graham *et al.* 2010; Kaul *et al.* 2010; Loebstein *et al.* 2011), including increased risk of stroke, heart failure and mortality. Regardless of these controversial findings, the effect of rosiglitazone on cardiac electrophysiology, including VF inducibility and defibrillation efficacy during ischaemia–reperfusion periods, is not known.

In the present study, we aimed to determine the effect of rosiglitazone on cardiac electrophysiology during ischaemia–reperfusion periods in swine. The VF threshold (VFT), the incidence of VF occurrence, the defibrillation threshold (DFT) and the effective refractory period (ERP) during periods of ischaemia–reperfusion were determined. We tested the hypothesis that rosiglitazone attenuates the occurrence of VF by increasing the VFT, improves the defibrillation efficacy by decreasing the DFT, and reduces the infarct size during ischaemia–reperfusion periods in swine hearts. We also determined the effect of rosiglitazone on cardiac mitochondria isolated from the ischaemic and remote areas of each heart. We tested the hypothesis that rosiglitazone prevents mitochondrial damage in ischaemia–reperfusion of swine hearts by reducing production of reactive oxygen species (ROS) and preventing mitochondrial membrane potential changes. Moreover, we tested the effects of various concentrations of rosiglitazone on the function of isolated rat cardiac mitochondria exposed to oxidative stress induced by hydrogen peroxide (H_2O_2).

Methods

Ethical approval

All animal study protocols were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Animal preparation

Twenty-six domestic pigs (20–25 kg) of either sex were used in this study. Pigs were anaesthetized by intramuscular injection of a combination of atropine (0.04 mg kg^{-1} ; T.P. Drug Laboratories Co., Ltd., Bangkok, Thailand), zoltil (5 mg kg^{-1} ; Vibbac Laboratories, Carros, France) and xylazine (2.2 mg kg^{-1} ; Laboratorios Calier, S.A., Barcelona, Spain), and maintained by 1.5–3.0% isoflurane (Abbott Laboratories Ltd., Queenborough, UK) delivered in 100% oxygen. After cuffed endotracheal intubation, mechanical ventilation (volume controlled, tidal volume = 12 ml kg^{-1} , respiratory rate = $10\text{--}15 \text{ cycles min}^{-1}$) was started with pigs in a restrained dorsally recumbent position. Pancuronium (2 mg kg^{-1} loading, $0.5 \text{ mg kg}^{-1} \text{ h}^{-1}$ maintenance) was administered

intravenously to minimize skeletal muscle contraction. The vital signs, including the surface electrocardiogram (lead II), femoral arterial blood pressure (BP), heart rate (HR), respiratory rate and core temperature, were continuously monitored. Blood gases and electrolytes were also monitored every 30 min and maintained within acceptable physiological ranges (Kanlop *et al.* 2008). Under fluoroscopic guidance, catheters with 34 and 68 mm platinum-coated titanium coil electrodes (Guidant Corp., St Paul, MN, USA) were inserted into the right ventricular apex and the junction between right atrium and superior vena cava, respectively. These two electrodes were used to deliver the stimulus during VFT and DFT determination (Kanlop *et al.* 2008; Sungnoon *et al.* 2008). The chest was opened through a median sternotomy, and the heart was suspended in a pericardial cradle. The pacing electrodes were affixed to the epicardium at the right ventricle (RV) outflow tract and left ventricle (LV) apex for an ERP and diastolic pacing threshold (DPT) determination. The left anterior descending coronary artery (LAD) was identified and dissected from surrounding tissues.

Study protocol

In the ischaemia–reperfusion study, pigs were divided into two groups. Group I ($n = 12$) received rosiglitazone, and group II ($n = 10$) received normal saline solution. In each pig, the cardiac electrophysiological parameters, including DPT, ERP, corrected QT interval (QTc), VFT and DFT, were determined at the beginning of the study. Then, pigs were randomly assigned to receive either rosiglitazone potassium salt (Cayman Chemical, Ann Arbor, MI, USA; 1 mg kg^{-1} dissolved in 30 ml sterile normal saline solution) or the same volume of sterile normal saline injected intravenously over 60 min. After that, the LAD was ligated with sterile 4–0 silk suture material 5 cm above a distal branch to perform a regional occlusion (Kanlop *et al.* 2011). During the first 20 min of occlusion, if spontaneous VF occurred, the defibrillation shock was delivered to determine the DFT. If VF did not occur within 20 min, VF was electrically induced, and the DFT and VFT were determined using a three-reversal up/down protocol (Kanlop *et al.* 2011). The ischaemic period was sustained for 60 min and then the LAD ligation was released to promote reperfusion for 120 min. All studied parameters were determined again beginning at 60 min after reperfusion.

At the end of experiment, the heart was removed for the determination of the myocardial infarction size. Furthermore, isolated cardiac mitochondria taken from the ischaemic and non-ischaemic areas in each heart were used to determine cardiac mitochondrial function ($n = 4$ in each group). In this study, another group of four pigs without LAD occlusion were also studied for

the electrophysiological effects of rosiglitazone in normal hearts. In this group, haemodynamic parameters as well as the DPT, ERP and DFT were determined before and after rosiglitazone administration.

Diastolic pacing threshold determination

The DPT testing was performed by delivering a train of 10 S1 stimuli of a 5 ms square pulse via the electrode at the tip of the RV catheter at 400 ms intervals. The current was started at 0.1 mA and was increased in 0.1 mA steps until all drive trains elicited a ventricular response (capture). The DPT was defined as the minimal current strength necessary to capture the ventricle (Shinlapawittayatorn *et al.* 2006; Kanlop *et al.* 2008).

Effective refractory period determination

In each train of 10 S1 stimuli, an S2 stimulus ($2 \times$ DPT) was introduced in late diastole of the last S1-paced beat (350 ms after the R wave) to elicit a capture. The basic S1–S2 coupling interval was decreased in 10 ms steps until S2 failed to elicit a capture. The ERP was defined as the longest S1–S2 interval at which an S2 stimulus fails to elicit a ventricular response (Kanlop *et al.* 2008).

Ventricular fibrillation threshold determination

The heart was paced three times. The interval between the last S1 and the mid-T wave was determined each time and the average of S1–mid-T wave interval was used as a coupling interval between the last S1 and S2 shock. The VFT testing was performed by delivering S2 shocks which started at 100 V. If this shock induced VF, decrements of 10 V steps were used for each successive shock until VF was no longer induced. If 100 V did not induce VF, increments of 10 V steps were used for each successive shock until VF was induced. The lowest shock strength that could induce VF was the VFT (Kanlop *et al.* 2008).

Defibrillation threshold determination

The defibrillation shock was delivered after 10 s of VF to determine the DFT by using a three-reversal up/down protocol (Kanlop *et al.* 2011). The DFT was defined as the lowest energy required for successful defibrillation of VF after three reversal points, when the next lower setting failed to defibrillate the heart. During the VF event, if the tested shock failed to defibrillate, a rescue shock (600–700 V) was delivered to successfully defibrillate the heart. A period of 4 min was allowed between each VF induction so that the heart could recover (Kanlop *et al.* 2008).

Infarct size and area at risk (AAR) measurement

At the end of each experiment, the heart were removed and irrigated with normal saline to wash out blood from chambers and vessels. The LAD was occluded again at the

Table 1. Basic electrophysiological and haemodynamic parameters in normal hearts without left anterior descending coronary artery occlusion

Parameter	Baseline	After rosiglitazone administration
Heart rate (beats min ⁻¹)	110 ± 9	112 ± 5
Systolic blood pressure (mmHg)	88 ± 3	86 ± 2
Diastolic blood pressure (mmHg)	58 ± 4	57 ± 2
Mean arterial blood pressure (mmHg)	68 ± 4	67 ± 2
Effective refractory period (ms)		
Right ventricular apex (endocardium)	263 ± 19	265 ± 13
Right ventricular outflow tract (epicardium)	265 ± 19	255 ± 13
Left ventricular apex (epicardium)	255 ± 10	255 ± 10
Diastolic pacing threshold (mA)		
Right ventricular apex (endocardium)	0.18 ± 0.05	0.20 ± 0.00
Right ventricular outflow tract (epicardium)	0.18 ± 0.05	0.20 ± 0.08
Left ventricular apex (epicardium)	0.13 ± 0.05	0.15 ± 0.06
Corrected QT interval (ms)	530 ± 68	481 ± 36
QRS duration (ms)	62 ± 9	59 ± 7
Defibrillation threshold		
Peak voltage (V)	340 ± 41	287 ± 32
Total energy (J)	9 ± 2	5 ± 3
Impedance (Ω)	63 ± 6	58 ± 7
Pulse width (ms)	17 ± 1	16 ± 2

same site previously done during the ischaemic period. The catheters were inserted into the right and left coronary ostia for Evans Blue dye infusion. The area which could not be infused by Evans Blue was defined as the area of no blood flow during the ischaemic period. The heart was frozen and cut horizontally into 5-mm-thick slices, starting from the apex until 5 mm above the occluding site. Then, each slice was immersed in triphenyltetrazolium chloride for at least 25 min, after which the area with viable tissue could be seen in red. The area that was not stained with Evans Blue was defined as the AAR. The area which demonstrated neither blue nor red was defined as the infarct site (Kanlop *et al.* 2011). The area measurement was performed with Image tool software version 3.0 (The University of Texas Health Science Center at San Antonio, Texas, USA). The infarct size was calculated depending on the weight of each slice as described previously (Kanlop *et al.* 2011).

Cardiac mitochondrial isolation

Cardiac mitochondria were isolated from the ischaemic and non-ischaemic regions, using the technique described

Table 2. Basic electrophysiological and haemodynamic parameters in hearts during ischaemia–reperfusion protocol

Parameter	Saline group			Rosiglitazone group		
	Baseline	Ischaemia	Reperfusion	Baseline	Ischaemia	Reperfusion
Heart rate (beats min ⁻¹)	107 ± 7	112 ± 8	117 ± 9	103 ± 17	113 ± 18	116 ± 21
Systolic blood pressure (mmHg)	104 ± 8	97 ± 11	93 ± 10	96 ± 15	91 ± 10	92 ± 14
Diastolic blood pressure (mmHg)	65 ± 7	63 ± 9	55 ± 8	60 ± 8	53 ± 5	53 ± 12
Mean arterial blood pressure (mmHg)	70 ± 25	67 ± 25	61 ± 23	66 ± 23	61 ± 20	60 ± 22
Corrected QT interval (ms)	463 ± 45	492 ± 55	495 ± 33	461 ± 47	455 ± 51	459 ± 48
QRS duration (ms)	55 ± 10	55 ± 9	58 ± 10	61 ± 13	61 ± 8	59 ± 10
Effective refractory period (ms)						
Right ventricular apex (endocardium)	242 ± 17	252 ± 23	246 ± 27	260 ± 13	259 ± 7	248 ± 38
Right ventricular outflow tract (epicardium)	234 ± 28	241 ± 25	240 ± 21	253 ± 16	263 ± 16	257 ± 45
Left ventricular apex (epicardium)	226 ± 22	219 ± 38	238 ± 29	247 ± 20	239 ± 32	257 ± 45
Diastolic pacing threshold (mA)						
Right ventricular apex (endocardium)	0.20 ± 0.05	0.51 ± 0.27	0.52 ± 0.18	0.25 ± 0.16	0.60 ± 0.61	0.60 ± 0.29
Right ventricular outflow tract (epicardium)	0.21 ± 0.14	0.26 ± 0.10	0.24 ± 0.09	0.25 ± 0.12	0.31 ± 0.16	0.32 ± 0.17
Left ventricular apex (epicardium)	0.19 ± 0.09	0.54 ± 0.44	1.03 ± 0.90	0.22 ± 0.13	0.58 ± 0.75	1.13 ± 1.06

Table 3. Ventricular fibrillation threshold and defibrillation threshold

Parameter	Saline group			Rosiglitazone group		
	Baseline	Ischaemia	Reperfusion	Baseline	Ischaemia	Reperfusion
Ventricular fibrillation threshold						
Peak voltage (V)	36 ± 12	30 ± 12	26 ± 5	32 ± 9	40 ± 16	42 ± 22
Total energy (J)	0.10 ± 0.07	0.07 ± 0.07	0.04 ± 0.02	0.04 ± 0.01	0.13 ± 0.11	0.15 ± 0.18
Impedance (Ω)	70 ± 7	65 ± 6	67 ± 6	74 ± 10	72 ± 8	68 ± 5
Pulse width (ms)	16 ± 3	15 ± 2	14 ± 2.65	17 ± 2	17 ± 3	16 ± 3
Defibrillation threshold						
Peak voltage (V)	387 ± 160	382 ± 74	349 ± 92	486 ± 133	470 ± 112	384 ± 125
Total energy (J)	14 ± 7	11 ± 4	10 ± 5	19 ± 10	18 ± 9	12 ± 9
Impedance (Ω)	63 ± 10	61 ± 7	60 ± 8	63 ± 8	62 ± 9	61 ± 9
Pulse width (ms)	17 ± 2	16 ± 1	16 ± 2	17 ± 2	17 ± 2	16 ± 2

previously (Thummasorn *et al.* 2011), and the protein concentration was determined according to bicinchoninic acid assay (Thummasorn *et al.* 2011).

Reactive oxygen species measurement

The fluorescent dye dichlorohydro-fluorescein diacetate (DCFDA) was used to determine the level of ROS production in cardiac mitochondria (Thummasorn *et al.* 2011). The DCFDA could pass through the mitochondrial membrane, and was oxidized by ROS in the mitochondria into DCF. Fluorescence was determined at $\lambda_{\text{excitation}}$ 485 nm and $\lambda_{\text{emission}}$ 530 nm using a fluorescence microplate reader. The ROS level was expressed as arbitrary units of fluorescence intensity of DCF.

Measurement of mitochondrial membrane potential changes ($\Delta\psi_m$)

The dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to determine the change in the mitochondrial membrane potential (Thummasorn *et al.* 2011). JC-1

was characterized as a cation and remained in the mitochondrial matrix as a monomer (green fluorescence) form. However, it could interact with anions in the mitochondrial matrix to form an aggregate (red fluorescence) form. JC-1 monomer fluorescence (green) was excited at 485 nm and the emission detected at 530 nm. JC-1 aggregate fluorescence (red) was excited at 485 nm and the emission fluorescence recorded at 590 nm. Mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio.

Isolated rat cardiac mitochondria study protocol

To investigate the dose-dependent effect of rosiglitazone on mitochondrial function, male Wistar rats (300–350 g, ~2 months old) were used for cardiac mitochondrial isolation. Rats were deeply anesthetized by intraperitoneal injection of thiopental (0.5 mg/kg; Research institute of antibiotics and biotransformations, Roztoky, Czech Republic), after which the heart was removed. Cardiac mitochondria were obtained using the differential centrifugation technique as previously described (Thummasorn *et al.* 2011). Isolated cardiac

mitochondrial morphology was confirmed by using an electron microscope. Isolated cardiac mitochondria were divided into eight groups ($n=5$ in each group) as follows: (1) control group; (2) 2 mM H_2O_2 -treated group; (3) 10 μM rosiglitazone pretreated for 15 min; (4) 25 μM rosiglitazone pretreated for 15 min; (5) 50 μM rosiglitazone pretreated for 15 min; (6) 10 μM rosiglitazone pretreated for 15 min, followed by

5 min 2 mM H_2O_2 application; (7) 25 μM rosiglitazone pretreated for 15 min, followed by 5 min 2 mM H_2O_2 application; and (8) 50 μM rosiglitazone pretreated for 15 min, followed by 5 min 2 mM H_2O_2 application. Production of ROS and $\Delta\Psi_m$ were determined in all groups. The 2 mM H_2O_2 was used because it has been shown to be an optimal dose to cause cardiac mitochondrial dysfunction (Thummasorn *et al.* 2011).

Statistical analysis

Data were expressed as means $\pm$ SD. Comparisons of variables at baseline, ischaemic and reperfusion periods (comparison within group) were performed using the Wilcoxon signed ranks test. Comparisons among groups were performed using the Mann–Whitney U test. Comparisons of the incidence of VF among groups were evaluated by χ^2 test. A value of $P < 0.05$ was considered statistically significant.

Results

The basic electrophysiological and haemodynamic parameters in normal swine heart (i.e. without ischaemia–reperfusion) are shown in Table 1. Acute intravenous administration of rosiglitazone (1.0 mg kg^{-1}) altered neither hemodynamics nor cardiac electrophysiological parameters.

For the ischaemia–reperfusion experiment, the basic electrophysiological and haemodynamic parameters prior to and during ischaemia–reperfusion are shown in Table 2. Neither saline nor rosiglitazone altered any of the haemodynamic parameters during the ischaemia–reperfusion period. The ERP during the ischaemic phase tended to decrease in the ischaemic region (i.e. LV apex); however, it did not reach statistical significance in either the saline or the rosiglitazone group.

For the VFT, peak voltage and total delivered energy during ischaemic as well as reperfusion periods were not statistically different from the baseline values when treated with rosiglitazone (Table 3). Similar to the VFT, the DFT determined during ischaemic and reperfusion periods were not different compared with the baseline value in both saline- and rosiglitazone-treated groups (Table 3).

The incidence of spontaneous VF was also significantly increased in rosiglitazone-treated pigs (seven of 12 pigs) compared with the saline group (one of 10 pigs; Fig. 1A). Moreover, acute intravenous administration of rosiglitazone significantly reduced the time interval from LAD ligation to the onset of the first spontaneous VF occurrence during ischaemia–reperfusion compared with that in the saline group (Fig. 1B). However, the dispersion of refractoriness as shown by the standard deviation of the three ERPs measured at three different sites was not different between the saline- and the rosiglitazone-treated group (Fig. 2). Unlike the effect on VF incidence, the areas

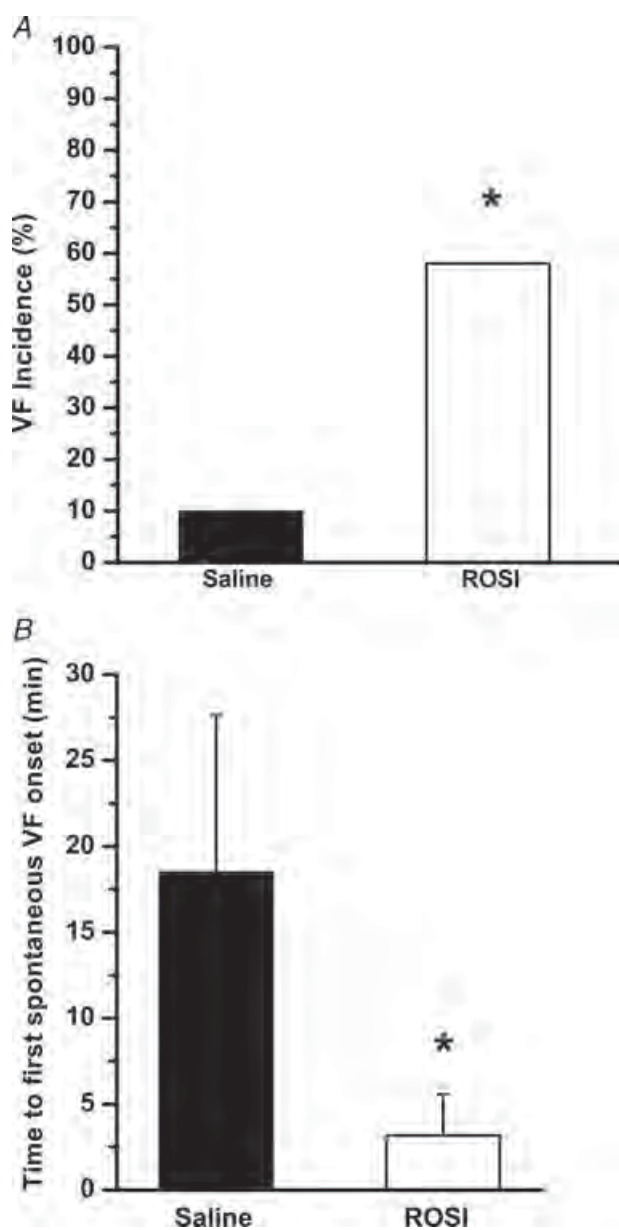


Figure 1. Rosiglitazone (ROSI) and the occurrence of ventricular fibrillation (VF)

Rosiglitazone significantly decreased the time interval from left anterior coronary artery occlusion to the first spontaneous VF onset ($n = 12$; A; * $P < 0.05$ versus saline group, Mann–Whitney U test) and increased the incidence of VF ($n = 12$; B), in comparison with the saline group. * $P < 0.05$ versus saline group (χ^2 test).

of myocardial infarction (i.e. the ratio of infarct size to AAR) in the rosiglitazone group were decreased ($P < 0.05$) compared with the saline group (Fig. 3).

In isolated cardiac mitochondria, rosiglitazone could not prevent mitochondrial membrane potential changes (Fig. 4A). Moreover, rosiglitazone could not reduce the

level of ROS production in the ischaemic myocardium (Fig. 4B).

In rat isolated cardiac mitochondria treated with rosiglitazone at 10, 25 and 50 μM , the drug did not alter cardiac mitochondrial ROS level or membrane potential changes, in comparison to the control group

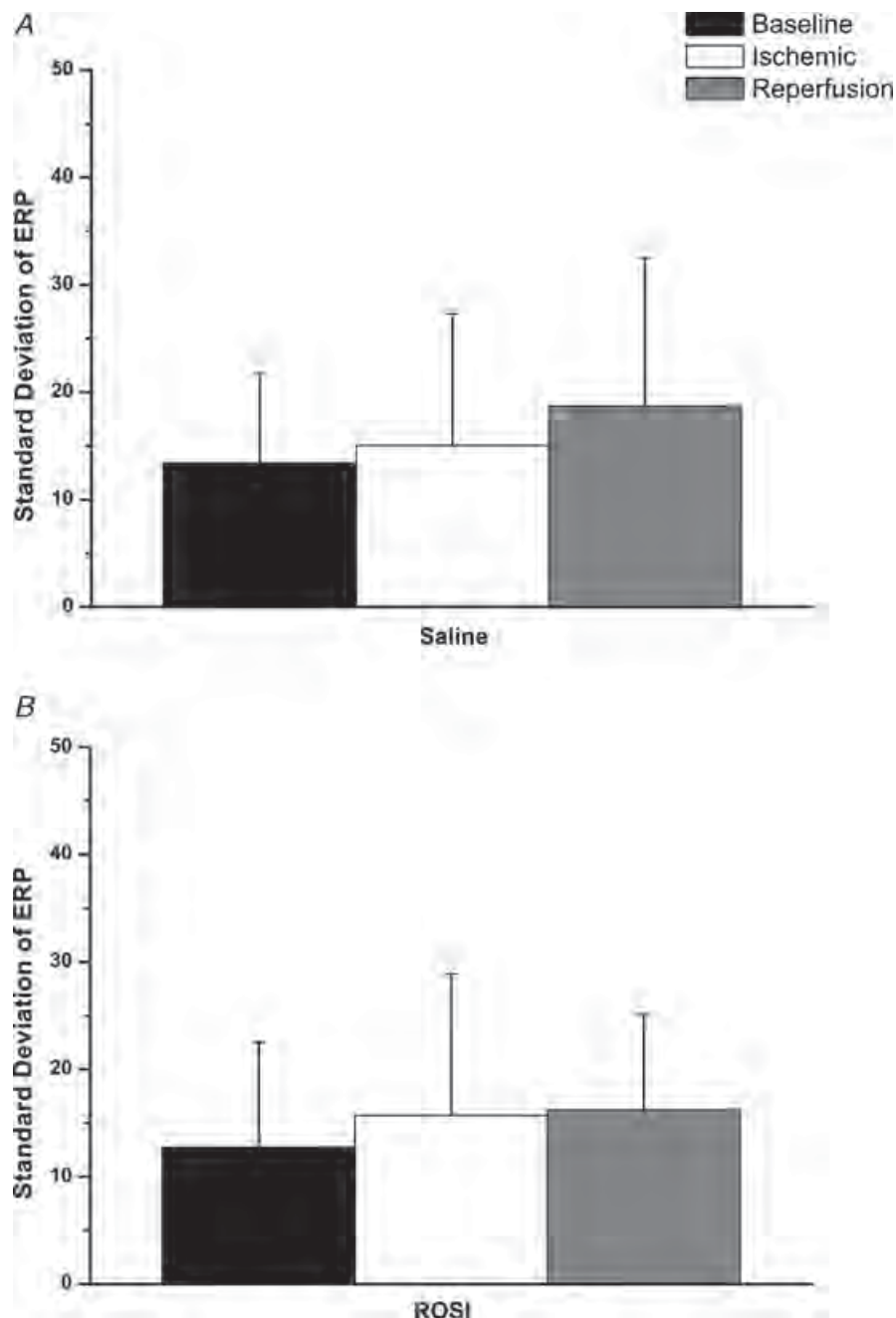


Figure 2. Effect of rosiglitazone on the dispersion of the effective refractory period (ERP) during ischaemia–reperfusion periods

Standard deviation of ERP at baseline and during ischaemia and reperfusion periods was not different in saline group ($n = 10$; A) and the rosiglitazone group ($n = 12$; B), indicating that rosiglitazone did not cause any change in the dispersion of refractoriness compared with the saline group.

(Fig. 5). When cardiac mitochondria were under severe oxidative stress caused by H_2O_2 , i.e. conditions that mimic ischaemia–reperfusion injury, cardiac mitochondrial dysfunction was observed as indicated by a decrease in the red/green fluorescence ratio, indicating cardiac mitochondrial membrane depolarization (Fig. 5A) and an increase in ROS level (Fig. 5B). None of the three concentrations of rosiglitazone could prevent or attenuate cardiac mitochondrial dysfunction caused by oxidative stress in these isolated cardiac mitochondria (Fig. 5).

Discussion

The major findings of the present study are as follows. In ischaemia–reperfusion of the swine heart, acute intravenous administration of rosiglitazone had the following effects: (1) it facilitated the occurrence of ventricular fibrillation; (2) it reduced the myocardial infarction area; and (3) it failed to prevent cardiac mitochondrial ROS production and mitochondrial depolarization caused by ischaemia–

reperfusion injury. Furthermore, acute intravenous administration of rosiglitazone did not alter the basic cardiac electrophysiology and haemodynamic parameters both in normal hearts and in hearts during ischaemia–reperfusion.

In the present study, our finding that acute administration of rosiglitazone did not alter haemodynamic parameters in swine is consistent with a previous report that rosiglitazone did not alter the blood pressure (Lu *et al.* 2008). In addition to haemodynamics, our study also demonstrated for the first time that the basic cardiac electrophysiological parameters as well as the defibrillation efficacy and the VFT were not altered by rosiglitazone in both normal conditions and ischaemia–reperfusion in swine hearts.

During the ischaemic period, our results clearly demonstrated that pigs treated with rosiglitazone had a higher incidence of VF than in the vehicle group. This novel finding indicates that rosiglitazone could facilitate VF initiation in the heart during ischaemia. This adverse effect of rosiglitazone could be responsible for the increased mortality associated with the use of rosiglitazone reported in previous clinical trials (Diamond *et al.* 2007; Nissen & Wolski, 2007; Cobitz *et al.* 2008). Moreover, our results also demonstrated that pigs treated with rosiglitazone also had a shorter time interval from LAD occlusion to the onset of the first spontaneous VF. This latter finding is also consistent with a previous report which demonstrated that rosiglitazone decreased the time to onset of VF during complete coronary occlusion (Lu *et al.* 2008). Both the increased VF incidence and the shorter interval to VF onset suggest that rosiglitazone is proarrhythmic in this model of ischaemia–reperfusion in swine hearts.

It has been proposed that rosiglitazone blocks K_{ATP} channels, and thus alters the action potential shortening during ischaemia and may promote arrhythmia by causing a greater dispersion of refractoriness in myocardial tissue (Janse, 1998). However, our findings do not support this hypothesis, because the dispersion of refractoriness observed in the present study was not different between pigs treated with rosiglitazone and vehicle, suggesting that the profibrillatory effect of rosiglitazone may not be due to facilitating the increased dispersion of ERP during ischaemia.

One possible mechanism of the profibrillatory effect of rosiglitazone could be due to its effects on the cardiac mitochondria. Increased ROS production and oscillation of the mitochondrial membrane potential ($\Delta\Psi_m$) have been shown to play an important role in the genesis of cardiac arrhythmias (Aon *et al.* 2009). In the present study, cardiac mitochondria isolated from ischaemic and non-ischaemic areas of swine hearts demonstrated that the clinically relevant dose of rosiglitazone used in this study could not prevent the collapse of $\Delta\Psi_m$

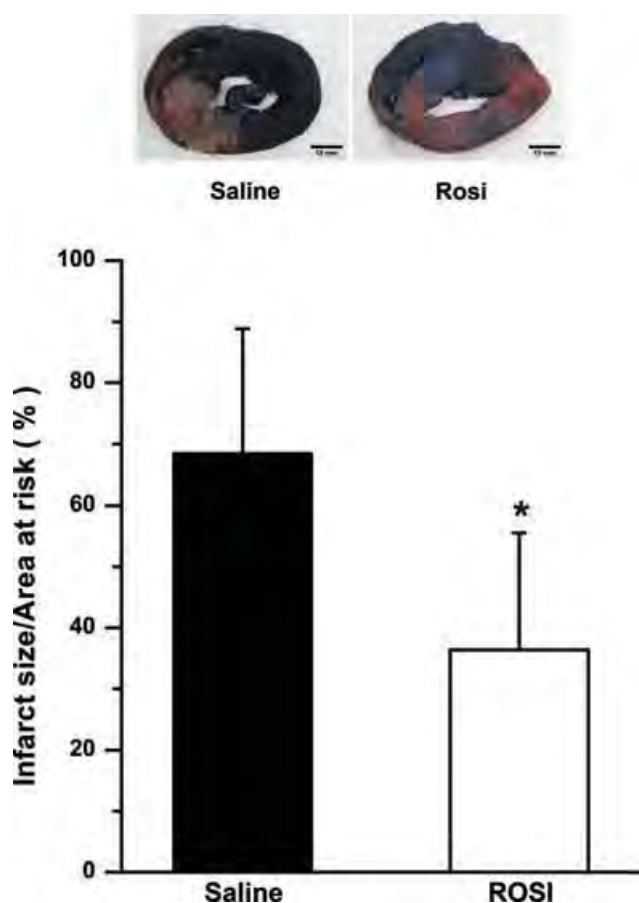


Figure 3. Rosiglitazone and the infarct size

The infarct size in the rosiglitazone group ($n = 12$) was significantly smaller than that in the saline group ($n = 10$). * $P < 0.05$ versus saline group (Mann–Whitney U test).

and did not reduce the mitochondrial ROS level during ischaemia–reperfusion injury. In rat isolated cardiac mitochondria, our results demonstrated that rosiglitazone at any tested concentrations could neither prevent mitochondrial membrane potential depolarization nor reduce mitochondrial ROS level against oxidative stress caused by H_2O_2 , which mimics ischaemia–reperfusion injury. As cardiac mitochondrial ROS production and

mitochondrial depolarization have been shown to be responsible for fatal arrhythmias (Aon *et al.* 2009), our findings indicate that rosiglitazone could not prevent the deterioration of mitochondrial function during ischaemia–reperfusion injury.

The only beneficial effect of rosiglitazone found in the present study is that it could significantly reduce the myocardial infarct size. This finding is consistent

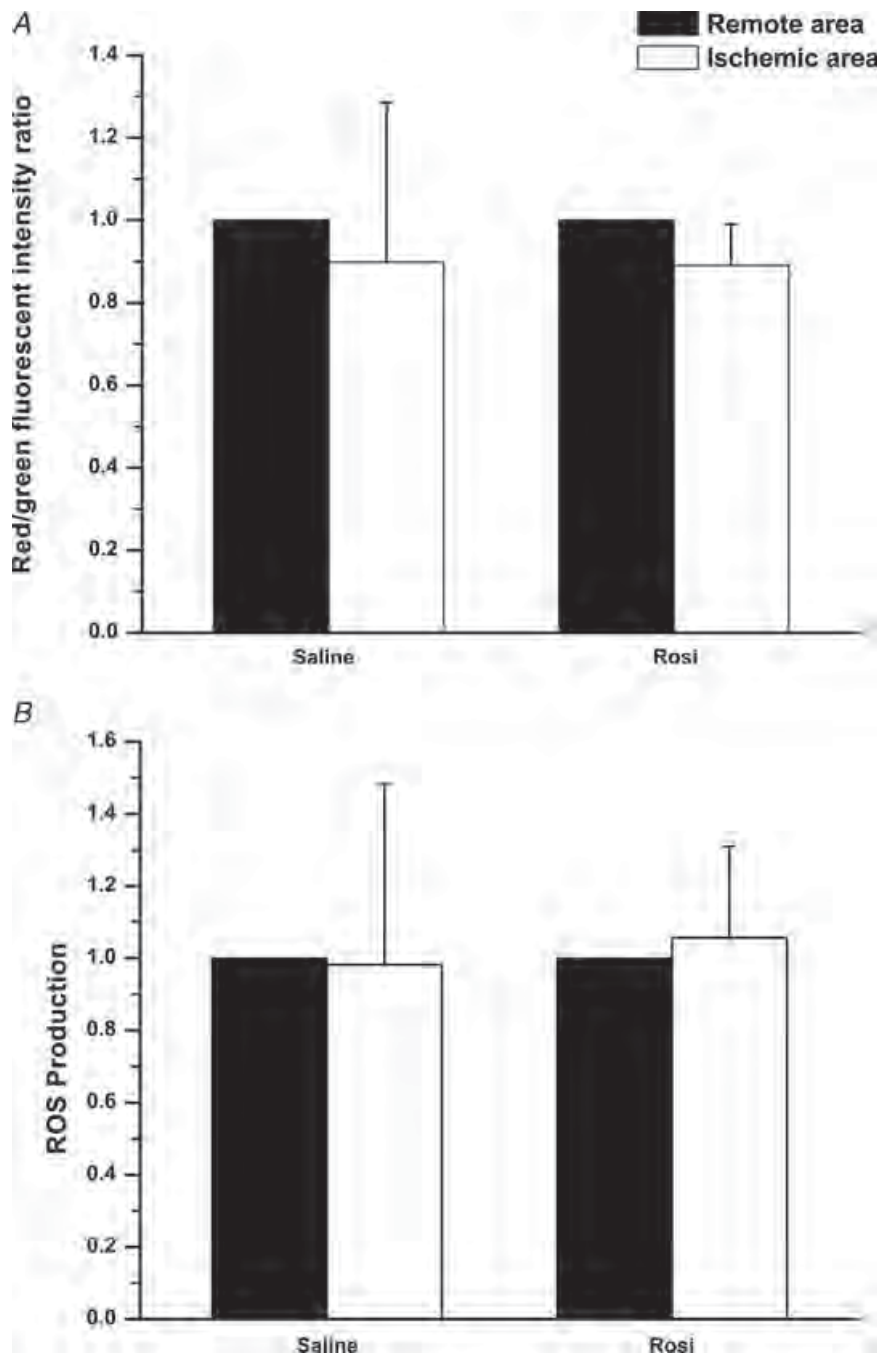


Figure 4. Effect of rosiglitazone on cardiac mitochondria after ischaemia–reperfusion in swine. Rosiglitazone neither prevented mitochondrial membrane depolarization (A) nor decreased mitochondrial ROS production (B) in the ischaemic myocardium, in comparison to the saline group ($n = 4$).

with previous *in vivo* studies showing that rosiglitazone reduced the ratio of infarct size to the area at risk (Sidell *et al.* 2002; Liu *et al.* 2004; Yue *et al.* 2005; Tao *et al.* 2010; Zhang *et al.* 2010), suggesting that rosiglitazone has an anti-apoptotic effect in myocardium during ischaemia–reperfusion. As mitochondrial ROS production is involved in cell apoptotic pathways (Loor

et al. 2011) and rosiglitazone could not prevent ROS production, our findings suggest that anti-apoptotic effect of rosiglitazone may not be direct, via the mitochondria, but that it could be involved in the extrinsic apoptotic signalling pathway, which is a mitochondria-independent apoptosis pathway that mediates cellular apoptosis by activating death receptors and transmitting

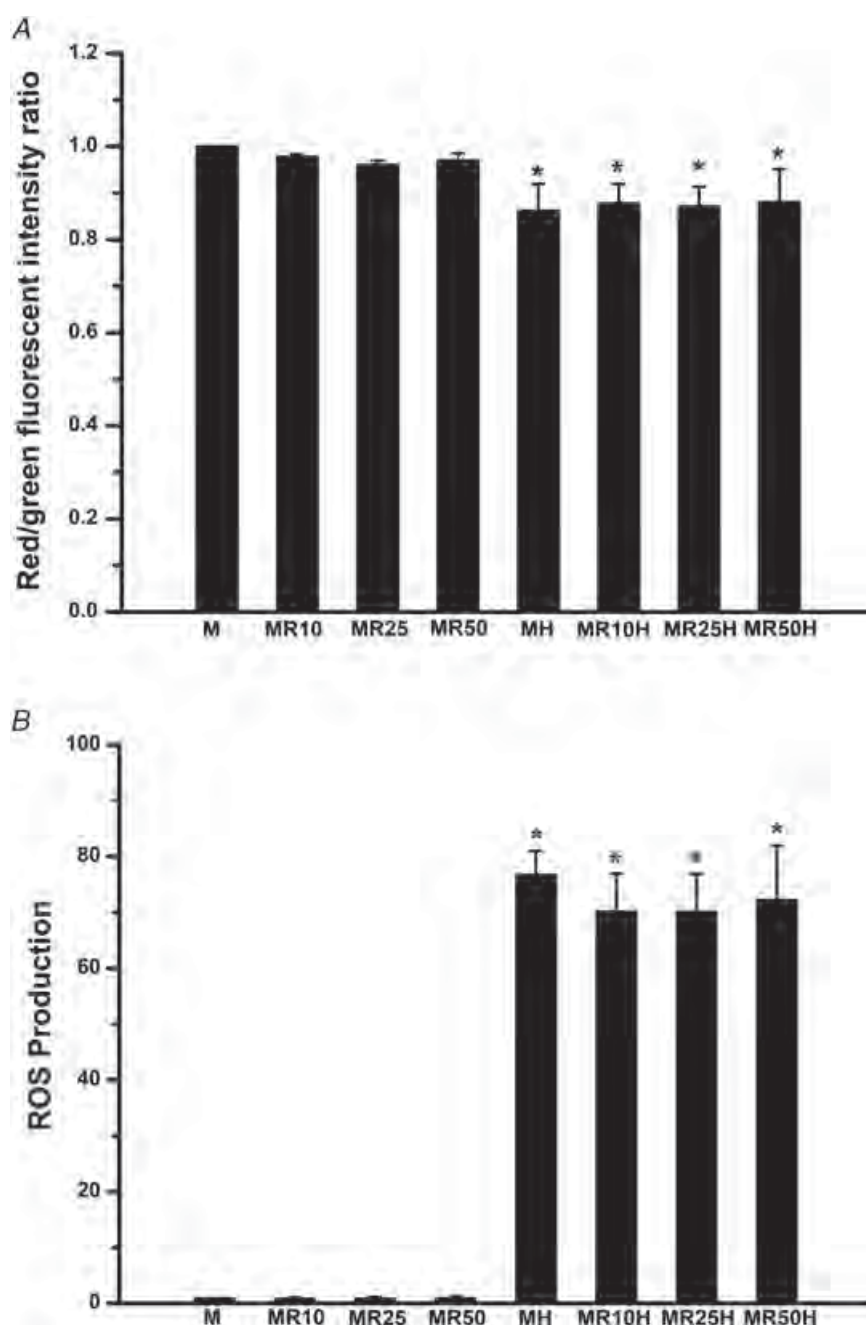


Figure 5. Effect of various concentrations of rosiglitazone on rat isolated cardiac mitochondrial function. Rosiglitazone neither prevented mitochondrial membrane depolarization (A) nor decreased mitochondrial ROS production (B) under severe oxidative stress induced by H_2O_2 , compared with the mitochondrial group. The eight columns in the figure correspond to the eight groups described in the Methods.

apoptotic signals after ligation with specific ligands (Ashe & Berry, 2003; Delhalle *et al.* 2003). However, the inflammatory response could also be responsible for the infarct size reduction observed in this study. Previous studies demonstrated that inflammation could play an important role in myocardial infarction during ischaemia–reperfusion (Werns & Lucchesi, 1987; Bonvini *et al.* 2005); therefore, inhibition of the inflammatory response might reduce myocardial infarct size (Werns & Lucchesi, 1987). As rosiglitazone has been shown to inhibit the inflammatory response by reducing the accumulation of neutrophils and macrophages and the expression of monocyte chemoattractant protein-1 (MCP-1) in the ischaemic heart (Yue *et al.* 2001) and by inhibiting the inflammatory cytokines tumour necrosis factor- α and nuclear factor- κ B (Shah *et al.* 2005), the infarct size reduction found in this study could be due to the anti-inflammatory effect of rosiglitazone. Future studies are required to elucidate the anti-apoptotic mechanism of rosiglitazone. Nevertheless, the controversy regarding the cardioprotective effect of rosiglitazone by its ability to decrease the infarct size (Sidell *et al.* 2002; Liu *et al.* 2004; Yue *et al.* 2005; Tao *et al.* 2010; Zhang *et al.* 2010) and the increased mortality with its use (Lygate *et al.* 2003; Lu *et al.* 2008; Blasi *et al.* 2009) have converged into the present study showing that rosiglitazone, despite its infarct size reduction effect, facilitates the occurrence of fatal arrhythmia during ischaemia–reperfusion injury, and could be responsible for increased mortality in patients using this drug, as shown in clinical trials (Diamond *et al.* 2007; Home *et al.* 2007; Lipscombe *et al.* 2007; Nissen & Wolski, 2007).

Study limitation

In the present study, we did not assess the anti-inflammatory signalling, cardiac function or structural changes of cardiomyocytes. However, previous studies demonstrated that rosiglitazone could improve cardiac function during ischaemia–reperfusion (Geng *et al.* 2006; Gonon *et al.* 2007), suggesting that rosiglitazone could influence cardiac function during ischaemia–reperfusion injury.

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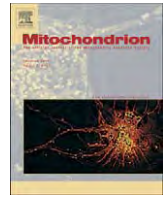
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Granulocyte-colony stimulating factor attenuates mitochondrial dysfunction induced by oxidative stress in cardiac mitochondria

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ABSTRACT

During cardiac ischemia–reperfusion injury, reactive oxygen species (ROS) level is markedly increased, leading to oxidative stress and mitochondrial dysfunction. Although granulocyte-colony stimulating factor (G-CSF) is known to be cardioprotective, its effects on cardiac mitochondria during oxidative stress have never been investigated. In this study, we discovered that G-CSF completely prevented mitochondrial swelling and depolarization, and markedly reduced ROS production caused by H₂O₂-induced oxidative stress in isolated cardiac mitochondria. Its effects were similar to those treated with cyclosporine A and 4'-chlorodiazepam. These findings suggest that G-CSF could act directly on cardiac mitochondria to prevent mitochondrial dysfunction caused by oxidative stress.

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1. Introduction

Ischemic heart disease has been a major health problem in most countries around the world (Melberg et al., 2010). Pharmacological intervention in ischemic heart disease is one of several means that has been investigated extensively in an attempt to decrease myocardial damage, infarct size, cardiac dysfunction, and fatal arrhythmia. In the heart, mitochondria are known as the principal source of energy as well as reactive oxygen species (ROS) production (Sucher et al., 2009). Under stress condition such as ischemia–reperfusion injury, this condition has been demonstrated to markedly cause severe oxidative stress, an imbalance between the production of reactive oxygen species and a biological system's ability to defend cell damage, leading to mitochondrial dysfunction (Correa et al., 2008). Normally, the defense mechanism is preserved by enzymes that decrease ROS production in cardiac mitochondria. Disturbances in normal oxidative phosphorylation of the electron transport chain during ischemia–reperfusion have been shown to cause toxic effects through the

production of ROS that will eventually damage all components of the cells.

A growing body of evidence has demonstrated the important roles of mitochondria in the heart during ischemia–reperfusion injury (Farber et al., 1981; Xiao et al., 2010). During ischemia, the energy production is decreased due to reduced oxidative phosphorylation rate at the inner mitochondrial membrane, causing a rapid increase in ROS production (Niizuma et al., 2009). When ROS produced by the electron-transport chain is accumulated up to a threshold level, it triggers the opening of the inner membrane anion channel (IMAC) (Aon et al., 2003). The opening of the IMAC allows the release of O₂^{•−} from the mitochondrial matrix, resulting in mitochondrial membrane depolarization. Recently, the mitochondrial permeability transition pore (mPTP) has been proposed to have an important role in cell apoptosis and necrosis (Lemasters et al., 1998). The mitochondrial permeability transition (MPT) caused by the opening of mPTP determines not only whether cells will live or die, but also whether cell death occurs by apoptosis or necrosis (Hirsch et al., 1997; Susin et al., 1997). The burst of ROS production could cause further inhibition of oxidative phosphorylation (Nulton-Persson and Szwedda, 2001) and increased permeability of the inner mitochondrial membrane via the opening of mPTP (Zoratti and Szabo, 1995). Such events are responsible for the uncoupling of oxidative phosphorylation, mitochondrial membrane potential changes ($\Delta\Psi_m$), and mitochondrial swelling, leading to the release of cytochrome c, activation of

Abbreviations: CDP, 4'-chlorodiazepam; CSA, cyclosporine A; G-CSF, Granulocyte-colony stimulating factor; IMAC, inner membrane anion channel; I/R, ischemia–reperfusion; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species.

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caspace pathways and cell death by necrosis and/or apoptosis (Petronilli et al., 2001). Because of the deleterious effects of oxidative stress, attempts to find drugs that can attenuate this stress condition and prevent mitochondrial dysfunction have been studied extensively (Aon et al., 2003; Brady et al., 2006; Chen et al., 2008; Cortassa et al., 2004).

Granulocyte-colony stimulating factor (G-CSF) has recently been shown to have cardioprotective effects including improved cardiac function, increased myocardial blood supply, and reduced mortality after cardiac injury under several conditions, including myocardial ischemia (Cheng et al., 2008; Brunner et al., 2008; Okada et al., 2008). Despite these beneficial cardioprotective effects, the effects of G-CSF on cardiac mitochondria under oxidative stress have never been investigated. In the present study, we tested the hypothesis that G-CSF can protect mitochondrial swelling, prevent mitochondrial depolarization, and reduce ROS production under hydrogen peroxide-induced oxidative stress condition in isolated cardiac mitochondria. Isolated cardiac mitochondria were chosen as a study model since the direct effect of pharmacological interventions on the mitochondria could be directly assessed without the interference from intracellular signaling (Bognar et al., 2006; Venditti et al., 2006; Hofer et al., 2009).

2. Materials and methods

2.1. Animal preparation

All animal studies were approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University. Wistar rats (300–350 g) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. They were housed in a room maintained under constant environmental conditions (temperature 22–25 °C and a constant 12-h light/dark cycle). All animals received standard pelleted rat diet and water ad libitum.

2.2. Isolated cardiac mitochondria preparation

Rats were anesthetized using an intraperitoneal injection of thiopental (80 mg/kg). The hearts were removed and homogenized in ice-cold buffer containing (in mmol/l) sucrose 300, TES 5, and EGTA 0.2, pH 7.2 (4 °C). The tissue were finely minced and homogenized by the homogenizer. The homogenate was centrifuged at 800 g for 5 min and the supernatant was collected and centrifuged at 8800 g for 5 min. Mitochondrial pellets were resuspended in an ice-cold buffer and centrifuged one more time at 8800 g for 5 min (Larche et al., 2006). Protein concentration was determined according to Bicinchoninic Acid (BCA) assay (Walker, 1994).

2.3. Experimental protocols

Isolated cardiac mitochondria from rat hearts were used in all experiments. The first protocol investigated the protective effects of G-CSF on oxidative stress-induced mitochondrial dysfunction. Oxidative stress was induced by H₂O₂ application for 5 min in isolated cardiac mitochondria. To test the effectiveness of H₂O₂ in causing mitochondrial damage, H₂O₂ at concentrations of 0.05, 0.1, 0.5, 1.0 and 2.0 mM were used (n = 5/group), and the assessment on mitochondrial swelling, ROS production and mitochondrial membrane potential changes was done according to the experimental protocols listed in Sections 2.5, 2.6 and 2.7.

In the first protocol, cardiac mitochondria were randomly assigned into eight treatment groups: H₂O₂ (2 mM), G-CSF (50 and 200 ng/ml), H₂O₂ (2 mM) pre-treated with G-CSF (25, 50, 100 or 200 ng/ml), and vehicle-treated mitochondria as a control group (n = 8/group, see Fig. 1A). In the second protocol, the mechanism of G-CSF on cardiac mitochondria was investigated. Pharmacological interventions with

cyclosporine A (CsA) and 4'-chlorodiazepam (CDP) were used. CsA is the mPTP blocker, whereas CDP is known to inhibit the opening of IMAC. In this protocol, cardiac mitochondria were randomly assigned into 12 groups: control (vehicle), G-CSF (effective dose of G-CSF obtained from the first protocol), CsA (5 μM), CDP (100 μM), H₂O₂, H₂O₂ pre-treated with G-CSF, H₂O₂ pre-treated with CsA, H₂O₂ pre-treated with CDP, H₂O₂ pre-treated with (G-CSF + CsA), H₂O₂ pre-treated with (G-CSF + CDP), H₂O₂ pre-treated with (CsA + CDP), and H₂O₂ pre-treated with (G-CSF + CsA + CDP) (n = 8/group, see Fig. 1B). All groups were studied according to the experimental protocols listed in Sections 2.5, 2.6, and 2.7.

The involvement of the effect of G-CSF on complexes I and III in the electron transport chain was also investigated. Pharmacological interventions with 2-μM rotenone (complex I inhibitor) and 2-μM antimycin A (complex III inhibitor) were used for this study (Fontaine et al., 1998; Marcil et al., 2006). In this protocol, cardiac mitochondria were randomly assigned into eleven treatment groups: control (vehicle), rotenone-treated cardiac mitochondria for 5 min, antimycin A-treated cardiac mitochondria for 5 min, H₂O₂ pre-treated with rotenone, H₂O₂ pre-treated with antimycin A, H₂O₂ (2 mM), H₂O₂ pre-treated with G-CSF, H₂O₂-pretreated with G-CSF and rotenone, respectively, H₂O₂-pretreated with G-CSF and antimycin A, respectively, H₂O₂-pretreated with rotenone and G-CSF, respectively and H₂O₂-pretreated with antimycin A and G-CSF, respectively (n = 8/group, see Fig. 1C). Since the electron transport chain, particularly complexes I and III, is the main source of ROS production, the level of ROS in cardiac mitochondria was determined in this study protocol.

2.4. Identification of cardiac mitochondria with electron microscopy

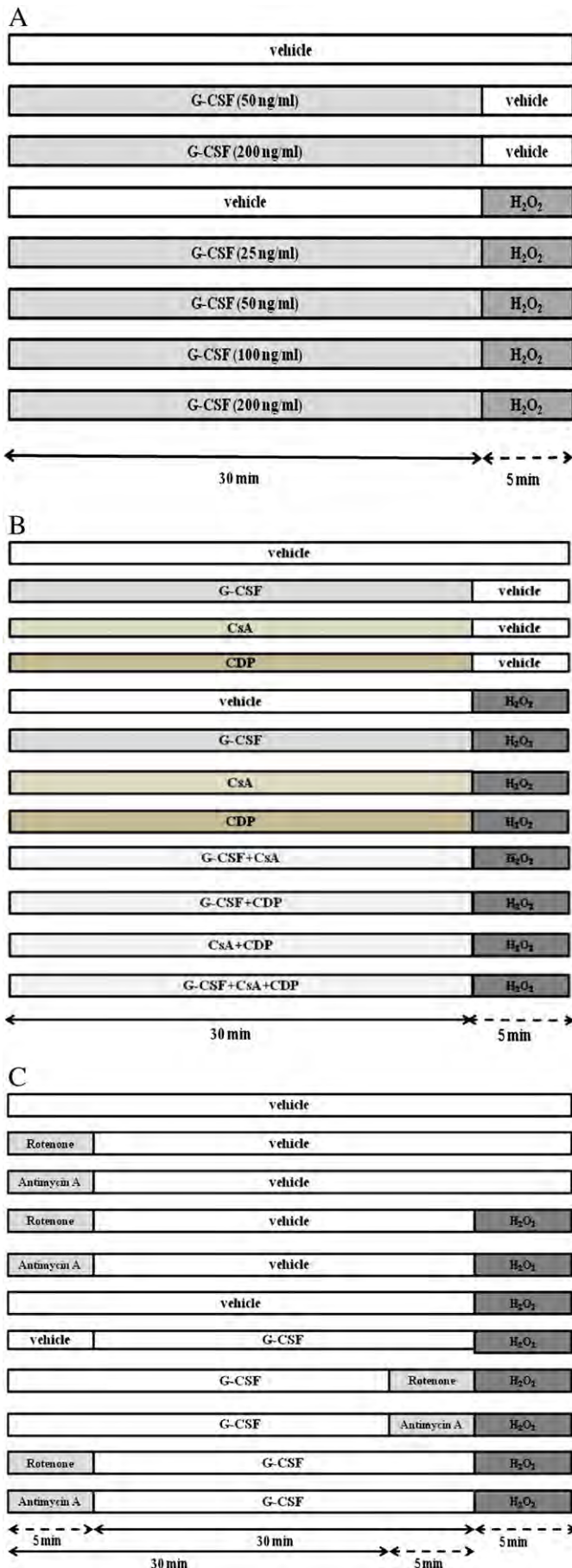
Electron microscopy was used to identify isolated mitochondria from the hearts (Chelli et al., 2001). Isolated cardiac mitochondria were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4°. After rinsing in cacodylate buffer, mitochondrial pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature, and then dehydrated in a graded series of ethanol. Mitochondria were embedded in Epon-Araldite. Ultrathin sections were cut with a diamond knife, placed on copper grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope.

2.5. Effects of pharmacological interventions on cardiac mitochondrial swelling

Cardiac mitochondria from all groups were assessed for mitochondrial swelling. In this study, swelling was assessed by measuring the change in the absorbance of the suspension at 540 nm (A₅₄₀) using a microplate reader (Ruiz-Meana et al., 2006). Mitochondria (0.4 mg/ml) were incubated in respiration buffer (containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM KH₂PO₄, pH 7.4 at 37 °C) with an addition of 10 mM pyruvate/malate. Mitochondrial swelling was indicated when the absorbance of the suspension decreased.

2.6. Effects of pharmacological interventions on ROS production in cardiac mitochondria

ROS production in mitochondria was measured using a fluorescent microplate reader with dichlorohydro-fluorescein diacetate (DCFDA) (Novaliya et al., 2003). Cardiac mitochondria (0.4 mg/ml) were incubated at 25 °C with 2 μM DCFDA for 20 min. The mitochondrial suspension was gently agitated and incubated at room temperature for measurements. DCFDA passed through membranes where it was oxidized in the presence of H₂O₂ to DCF. Fluorescence was determined at λ_{ex} 485 nm and λ_{em} 530 nm



according to the spectral characteristics of DCF. The ROS level was expressed as arbitrary units of fluorescence intensity of DCF.

2.7. Effects of pharmacological interventions on cardiac mitochondrial membrane potential

The changes in mitochondrial membrane potential from all groups of isolated mitochondria were monitored with the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Tong et al., 2005; Di Lisa et al., 1995). The interaction of JC-1 with mitochondrial membrane components can alter the fluorescence properties associated with the monomer and aggregate. JC-1 has fluorescence properties as cation. When an interaction between JC-1 and anion of O₂⁻ within mitochondrial matrix occurs, aggregate fluorescence form is seen. However, JC-1 monomer fluorescence form is seen when the JC-1 has no interaction with anion within mitochondrial matrix. The isolated cardiac mitochondria (0.4 mg/ml) were stained with JC-1 (310 nM) at 37 °C for 30 min. The intensity of fluorescence was determined using a fluorescent microplate reader. JC-1 monomer fluorescence (green) was excited at 485 nm and the emission was detected at 530 nm. JC-1 aggregate fluorescence (red) was excited at 485 nm and the emission fluorescence was recorded at 590 nm. Consequently, mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio (i.e. ΔΨm). The reduction in ΔΨm indicates mitochondrial membrane depolarization.

2.8. Data analysis

All data were presented as means ± SEM. Comparisons were made by one-way ANOVA followed by the Fisher post-hoc test. A *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of H₂O₂ on isolated cardiac mitochondria

H₂O₂ at 0.5 and 1.0 mM could only cause an increase in the ROS level, but did cause neither absorbance (i.e. no mitochondrial swelling) nor membrane potential changes, compared to the control cardiac mitochondria (Fig. 2). However, an application of 2-mM H₂O₂ significantly reduced the absorbance (i.e. mitochondrial swelling), increased ROS level and mitochondrial depolarization (Fig. 2). Since 2-mM H₂O₂ could cause the changes in all determined parameters in isolated cardiac mitochondria, this concentration of H₂O₂ was used in all protocols in this study.

3.2. Effects of G-CSF on mitochondrial swelling

Transmission electron microscope was used to identify the cardiac mitochondria (Fig. 3). The freshly isolated cardiac mitochondria are shown in Fig. 3A. Applying 2-mM H₂O₂ to cardiac mitochondria caused mitochondrial swelling with markedly unfolded cristae (Fig. 3B). When G-CSF at 50 ng/ml was applied to cardiac mitochondria 30 min prior to H₂O₂ application, the morphology of cardiac mitochondria was well preserved (Fig. 3C), indicating the effectiveness of G-CSF in preventing morphological change in cardiac mitochondria from H₂O₂ application. Mitochondrial swelling was quantitatively assessed by reduced absorbance of the mitochondrial suspension. Applying H₂O₂ to mitochondria resulted in decreased

Fig. 1. Diagrams of the study protocols: (A) Effects of G-CSF on H₂O₂-induced mitochondrial damage, (B) Effects of G-CSF, cyclosporine A (CsA), 4'-chlorodiazepam (CDP) on H₂O₂-induced mitochondrial damage, (C) Effects of G-CSF on complexes I and III.

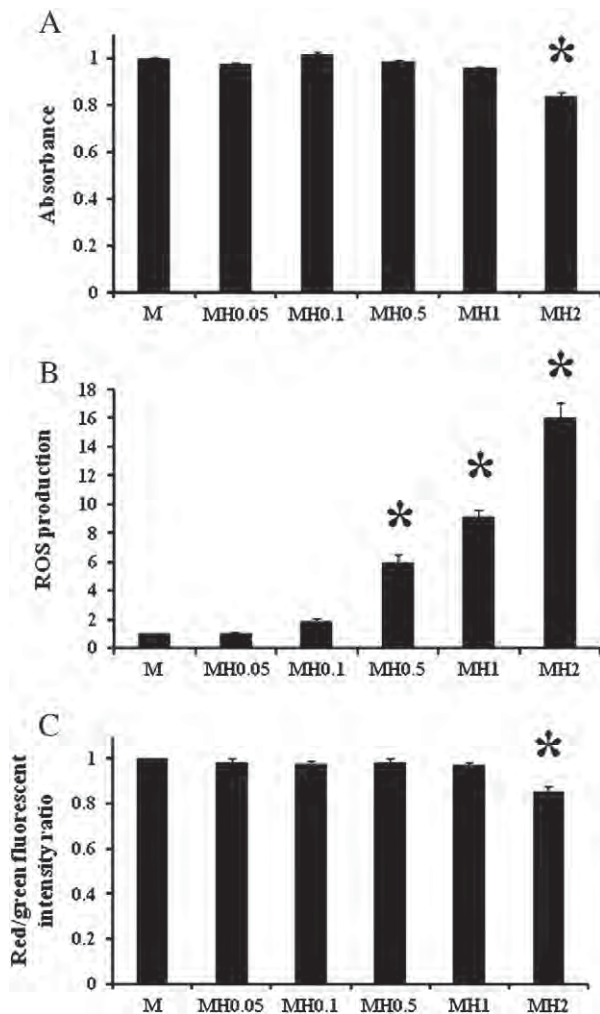


Fig. 2. Effects of H₂O₂ at various concentrations on (A) cardiac mitochondrial swelling, (B) ROS level and (C) mitochondrial membrane potential change. H₂O₂ at 0.5 and 1.0 mM caused an increased ROS level, but neither caused mitochondrial swelling nor membrane potential changes in cardiac mitochondria. However, 2-mM H₂O₂ caused mitochondrial swelling, increased ROS level, and mitochondrial membrane depolarization. M=control, MH(0.05, 0.1, 0.5, 1 and 2)=H₂O₂-treated mitochondria at 0.05, 0.1, 0.5, 1 and 2 mM, respectively. **p*<0.05 vs. M group.

absorbance (Fig. 4), suggesting that H₂O₂ can significantly induce mitochondrial swelling. G-CSF at 25 ng/ml did not prevent decreased absorbance, whereas G-CSF at 50, 100, and 200 ng/ml effectively prevented a reduction in the absorbance after H₂O₂ application (Fig. 4). From this kinetic study, a time point of 4 min after H₂O₂ application was chosen to investigate the protective effect of G-CSF. Without H₂O₂, G-CSF itself did not alter the absorbance, compared to the control group (Fig. 5). When G-CSF was applied to cardiac mitochondria prior to H₂O₂ application, G-CSF at concentrations of 50, 100, and 200 ng/ml significantly reduced mitochondrial swelling, compared to the H₂O₂ group. The absorbance measured in these mitochondria pretreated with G-CSF did not differ from that in the control (G0) group (Fig. 5).

3.3. Effects of G-CSF on ROS production

At baseline, ROS levels in cardiac mitochondria in the groups treated with G-CSF (50 and 200 ng/ml) were modestly higher than in the control group (Fig. 6). When H₂O₂ was applied to the mitochondrial suspension, the level of ROS was significantly increased, compared to the control group. However, when G-CSF was applied to cardiac mitochondria prior to H₂O₂ application, G-CSF (25, 50, 100 and 200 ng/ml) significantly reduced ROS level, compared to the H₂O₂ group. Furthermore, G-CSF at 50, 100, and 200 ng/ml decreased ROS levels more than G-CSF at 25 ng/ml. Nevertheless, the ROS levels in cardiac mitochondria pretreated with G-CSF at all concentrations were still higher than that in the control (G0) group.

3.4. Effects of G-CSF on mitochondrial membrane potential changes ($\Delta\Psi_m$)

At baseline, the $\Delta\Psi_m$ in the G-CSF (50 and 200 ng/ml) groups did not differ from the control group (Fig. 7). When H₂O₂ was added to mitochondrial suspension, mitochondrial depolarization was observed as indicated by a marked decrease in $\Delta\Psi_m$. However, when G-CSF was applied to mitochondria prior to H₂O₂ application, G-CSF (50, 100, and 200 ng/ml) significantly attenuated the changes in $\Delta\Psi_m$, compared to the H₂O₂ treated group. The $\Delta\Psi_m$ in these G-CSF pretreated cardiac mitochondria did not differ from the control (G0) group.

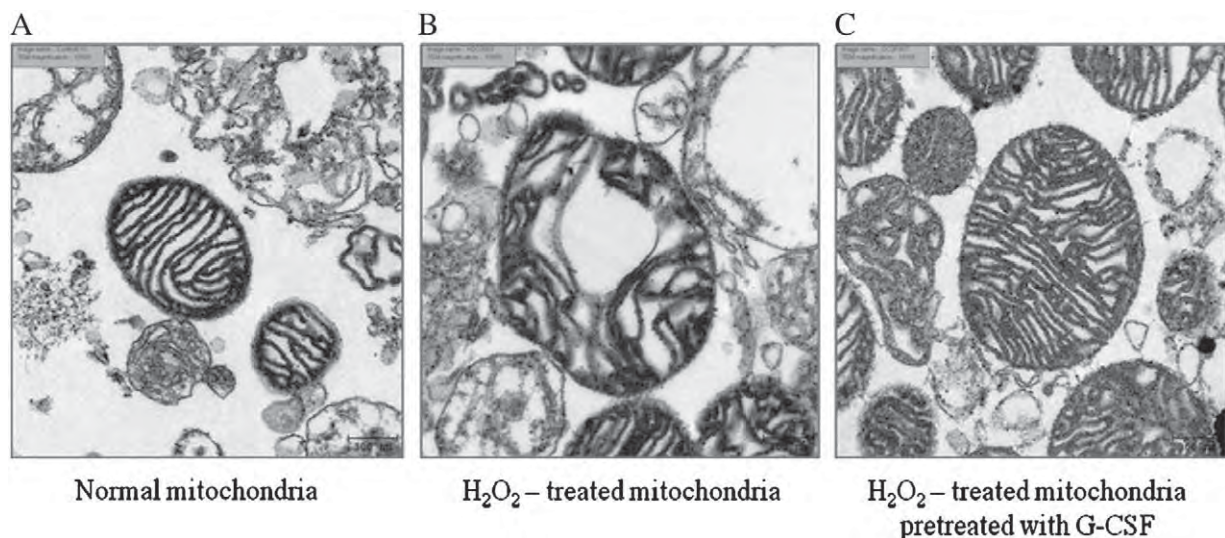


Fig. 3. Identification of the cardiac mitochondria with electron microscopy: (A) normal cardiac mitochondria, (B) cardiac mitochondria treated with 2-mM H₂O₂, (C) cardiac mitochondria pretreated with G-CSF at 50 ng/ml followed by H₂O₂ application.

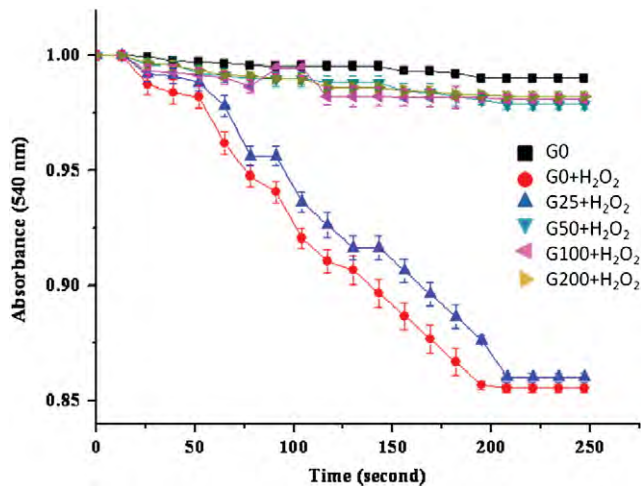


Fig. 4. Kinetic study of the effects of G-CSF on cardiac mitochondrial swelling. Mitochondrial swelling was quantitatively assessed by the reduction in the absorbance of the mitochondrial suspension. G0 = cardiac mitochondria not treated by H_2O_2 or G-CSF, (G0 + H_2O_2) = cardiac mitochondria treated with H_2O_2 , (G25, 50, 100, and 200 + H_2O_2) = mitochondria pretreated with G-CSF at 25, 50, 100, and 200 ng/ml followed by H_2O_2 application, respectively.

3.5. Effect of G-CSF, CsA, and CDP on cardiac mitochondrial swelling

At baseline, G-CSF at 50 ng/ml (i.e. G50), CsA, and CDP did not cause any changes in the absorbance of cardiac mitochondria (Fig. 8). G-CSF at 50 ng/ml was chosen since it could effectively prevent absorbance reduction after H_2O_2 application as demonstrated in the previous protocol. When H_2O_2 was applied to mitochondrial suspension, absorbance was markedly decreased compared to the control group. However, pretreating cardiac mitochondria with G-CSF or CsA or CDP prior to H_2O_2 application could attenuate the reduction in the absorbance, compared to the H_2O_2 group. No difference was found among these G-CSF, CsA, and CDP pretreated groups. Furthermore, a combination of (G-CSF + CsA) or (G-CSF + CDP) or (CsA + CDP) or (G-CSF + CsA + CDP) could effectively prevent the absorbance reduction in cardiac mitochondria caused by H_2O_2 . The measured absorbance did not differ among these groups, and did not differ from that of the control group (Fig. 8).

3.6. Effects of G-CSF, CsA and CDP on ROS production

At baseline, G-CSF at 50 ng/ml and CsA caused a slight increase in ROS production in isolated cardiac mitochondria (Fig. 9). The ROS level was significantly increased in mitochondria treated with H_2O_2 . However, in cardiac mitochondria pretreated with G-CSF or CsA or CDP prior to H_2O_2 application, the ROS level was significantly decreased, compared to the H_2O_2 group. However, the reduction of ROS level among these groups was not equal. CsA prevented ROS production with greatest efficacy, followed by CDP and G-CSF. In addition, when H_2O_2 was added to mitochondria pretreated with (G-CSF + CsA) or (G-CSF + CDP) or (CsA + CDP) or (G-CSF + CsA + CDP), the ROS level was significantly decreased, compared to the H_2O_2 group. However, the level of ROS production did not differ among these combined treatment groups. Furthermore, the reduction in ROS level in these combined treatment groups was similar to that in the (CsA + H_2O_2) group (Fig. 9).

3.7. Effects of G-CSF, CsA, and CDP on mitochondrial membrane potential changes ($\Delta\Psi_m$)

At baseline, G-CSF at 50 ng/ml and CDP alone did not cause any changes in $\Delta\Psi_m$ in cardiac mitochondria. However, CsA alone caused a slight decrease in $\Delta\Psi_m$ (mitochondrial depolarization) (Fig. 10). When H_2O_2 was added to cardiac mitochondria, $\Delta\Psi_m$ was significantly altered, compared to the control group. However, applying H_2O_2 to mitochondria pretreated with G-CSF or CsA or CDP did not alter the $\Delta\Psi_m$. When H_2O_2 was added to mitochondria pretreated with (G-CSF + CsA) or (G-CSF + CDP) or (CsA + CDP) or (G-CSF + CsA + CDP), the $\Delta\Psi_m$ was also not altered, compared to the H_2O_2 group. No difference was found for $\Delta\Psi_m$ among these combined treatment groups. Furthermore, the $\Delta\Psi_m$ in these groups did not differ from that in the control group (Fig. 10).

3.8. Effects of G-CSF on complexes I and III of the electron transport chain

At baseline, rotenone (2 μ M) and antimycin A (2 μ M) did not cause any change in the ROS level, whereas H_2O_2 caused an increase in the ROS level in cardiac mitochondria (Fig. 11). G-CSF could significantly decrease the ROS level in cardiac mitochondria when it was applied to the mitochondria before the addition of rotenone, antimycin A, and

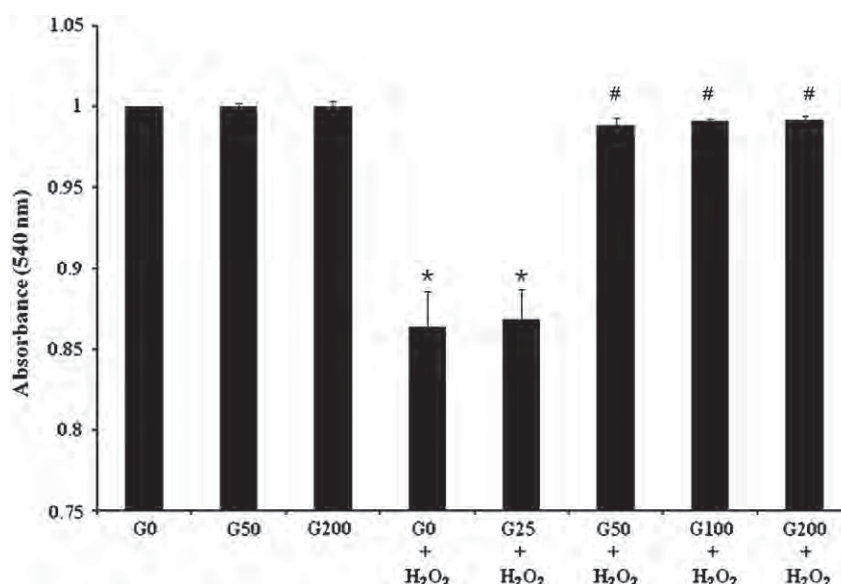


Fig. 5. Effects of G-CSF on cardiac mitochondrial swelling after 4-min H_2O_2 application. G0 = cardiac mitochondria not treated by H_2O_2 or G-CSF, G50 and G200 = cardiac mitochondria treated with G-CSF 50 and 200 ng/ml, respectively, (G0 + H_2O_2) = cardiac mitochondria treated with H_2O_2 , (G25, 50, 100, and 200 + H_2O_2) = cardiac mitochondria pretreated with G-CSF at 25, 50, 100, and 200 ng/ml followed by H_2O_2 application, respectively. * P < 0.05 vs. G0 group, # P < 0.05 vs. (G0 + H_2O_2) group.

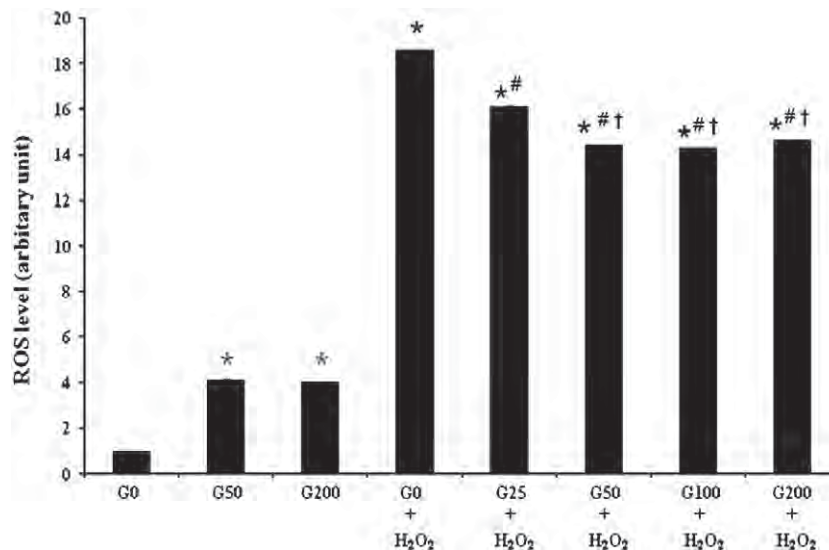


Fig. 6. Effects of G-CSF on ROS level. * $P < 0.05$ vs. G0 group, # $P < 0.05$ vs. (G0 + H₂O₂) group, † $P < 0.05$ vs. (G25 + H₂O₂) group.

H₂O₂ (i.e. ROS level in MG, MGR, and MGA groups was lower than that in the H₂O₂, MRH and MAH groups in Fig. 11). However, the effectiveness of G-CSF in reducing the ROS level was decreased when G-CSF was added to the mitochondria after rotenone and antimycin A application since the ROS levels in MRGH and MAGH were significantly higher than those in the MG, MGRH and MAGH groups.

4. Discussion

Mitochondria have been shown to play a key role in cardiac dysfunction caused by myocardial ischemia (Xiao et al., 2010). A previous study demonstrated that oxidative stress occurring in ischemic myocardium caused an increased ROS production in cardiac mitochondria (Tompkins et al., 2006). The increased ROS level in one or a few mitochondria has been shown to trigger the release of ROS from neighboring mitochondria, a mechanism known as ROS-induced ROS release (Zorov et al., 2006), resulting in a large amount of ROS accumulated in the ischemic myocardium. The increased ROS level could lead to the disruption of the electron transport chain in cardiac mitochondria, causing cardiac mitochondrial dysfunction and eventually leading to myocardial cell death (Tompkins et al., 2006).

Pharmacological interventions to attenuate mitochondrial dysfunction have been shown to have cardioprotective effects, including prevention of arrhythmia and reduction of infarct size (Matejikova et al., 2009; Moncada, 2010).

Granulocyte-colony stimulating factor (G-CSF) has recently been shown to improve cardiac function, increase myocardial blood supply, and reduce mortality after cardiac injury under several conditions including ischemic heart (Cheng et al., 2008; Brunner et al., 2008; Okada et al., 2008). However, its effect on cardiac mitochondria undergoing oxidative stress induced by H₂O₂ has never been investigated. In the present study, we found that G-CSF could prevent mitochondrial swelling, decrease ROS level, and attenuate mitochondrial membrane depolarization in cardiac mitochondria under oxidative stress condition.

In the present study, the effective dose of G-CSF (50 ng/ml) could successfully prevent mitochondrial damage. G-CSF at higher concentrations (up to 200 ng/ml) shared equally similar effects to G-CSF at 50 ng/ml. Although G-CSF could completely prevent mitochondrial swelling and $\Delta\Psi_m$ changes after oxidative stress induced by H₂O₂, it could only decrease ROS to a certain level, which was still higher than that in the control group. These findings indicate that G-CSF may act

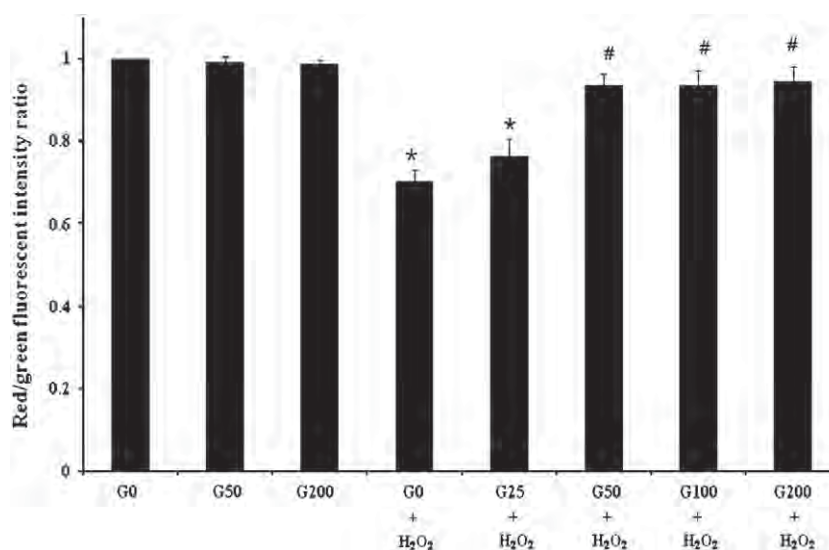


Fig. 7. Effects of G-CSF on cardiac mitochondrial membrane potential changes ($\Delta\Psi_m$). * $P < 0.05$ vs. G0 group, # $P < 0.05$ vs. (G0 + H₂O₂) group.

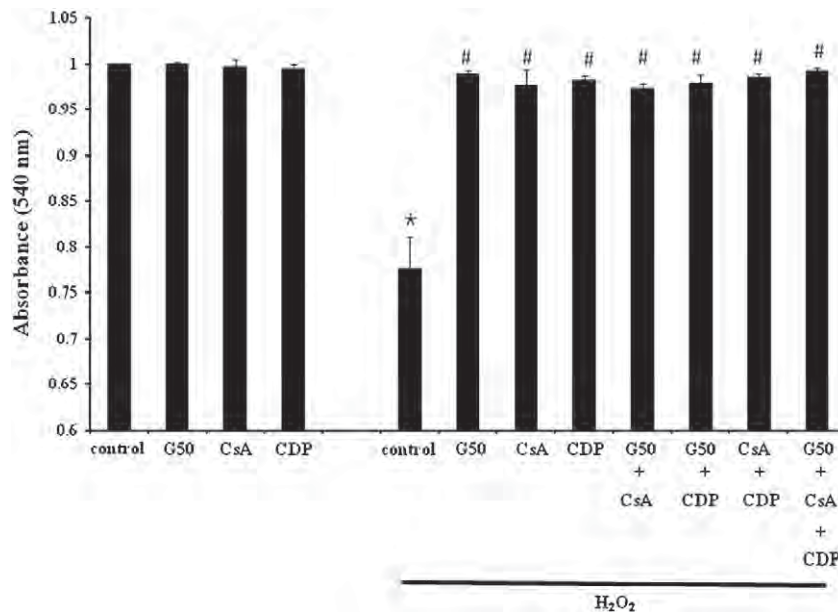


Fig. 8. Effects of G-CSF, CsA, and CDP on cardiac mitochondrial swelling. * $P < 0.05$ vs. control group, # $P < 0.05$ vs. H₂O₂ group.

more effectively in preventing mitochondrial swelling and mitochondrial depolarization, but less effective in preventing ROS production.

Mitochondrial swelling is described as an increase of mitochondrial volume. The cause of mitochondrial swelling is due to the prolonged opening of mPTP, which causes an increase of permeability to solutes with molecular masses up to 1500 Da. Since mPTP opening can be triggered by the increased ROS level (Hausenloy et al., 2003), the prolonged mPTP opening can lead to free bi-directional movement of low molecular weight molecules across the inner membrane, while proteins remain in the matrix. Consequently, colloidal osmotic pressure increases and causes mitochondrial swelling (the inner membrane cristae unfolded), the rupture of outer mitochondrial membrane, and the release of cytochrome *c* (Azzone and Azzì, 1965; Crompton, 1999). In this study, G-CSF effectively prevented mitochondrial swelling caused by H₂O₂, and this protection is as effective as the effect of the blocker of mPTP opening (CsA). These findings

suggested that G-CSF might act on mPTP, thus inhibiting the opening of this pore and leading to the prevention of mitochondrial swelling.

Mitochondria are known as a major organelle for producing ROS in cells. ROS is normally produced at sites of complexes I and III in the electron transport chain (Chen et al., 2008). Under physiological conditions, O₂⁻ is transported to the electron transport chain for oxidative phosphorylation in the mitochondria and converted into a small amount of H₂O₂ (Chen et al., 2008). However, these ROS are generally degraded by catalase and glutathione peroxidase in the mitochondria. In some pathological conditions such as oxidative stress, ROS production is markedly increased and the ROS degradation process is not sufficiently maintained, resulting in an excess of ROS including O₂⁻.

Under physiological conditions, O₂⁻ can be released across the inner mitochondrial membrane via IMAC, causing mitochondrial membrane depolarization (Brady et al., 2006). The release of ROS via

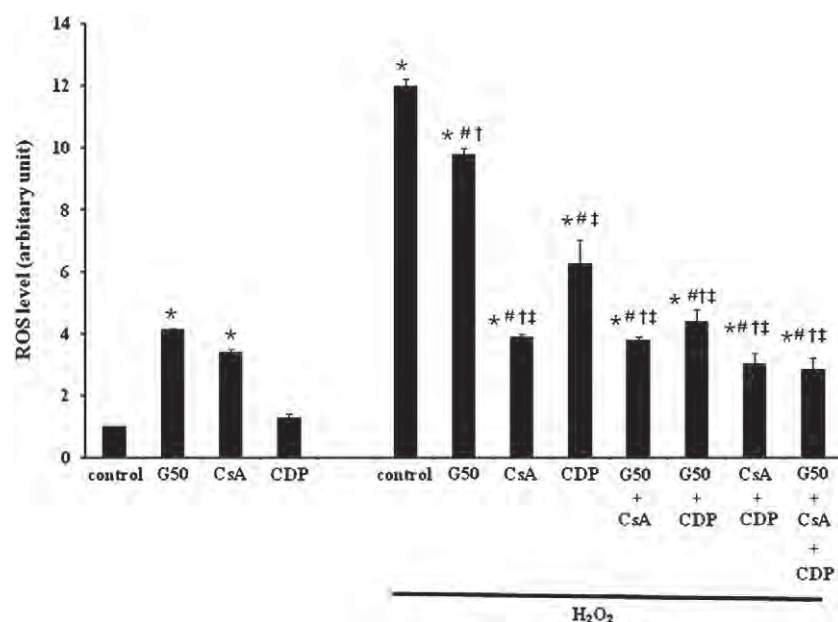


Fig. 9. Effects of G-CSF, CsA, and CDP on ROS level. * $P < 0.05$ vs. control group, # $P < 0.05$ vs. H₂O₂ group, † $P < 0.05$ vs. (CDP + H₂O₂), ‡ $P < 0.05$ vs. (G-CSF + H₂O₂).

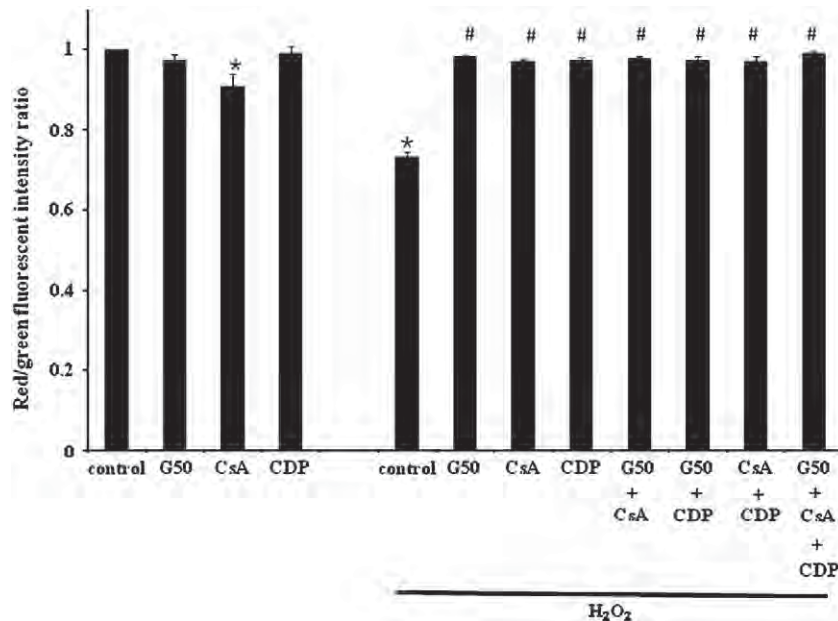


Fig. 10. Effects of G-CSF, CsA, and CDP on cardiac mitochondrial membrane potential changes ($\Delta\Psi_m$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. H_2O_2 group.

IMAC opening could activate the IMAC of its neighboring mitochondria, resulting in a great increase of ROS (Vanden Hoek et al., 1998). This process is called ROS-induced ROS release mechanism, leading to severe oxidative stress in cells (Zorov et al., 2000). IMAC opening is normally terminated by a reduction of ROS level (i.e. via decreasing ROS production and efflux from the mitochondrial matrix) and ROS scavenging by the antioxidant enzymes (Cortassa et al., 2004). Aon et al. (2003) clearly demonstrated in their excellent study that a certain amount of ROS produced from the electron transport chain is required to accumulate in the mitochondrial matrix up to a critical level in order to trigger the opening of IMAC (Aon et al., 2003). Once the ROS accumulation reaches a point of mitochondrial critical threshold, mitochondrial depolarization is observed and the mitochondrial membrane potential becomes unstable in almost the entire population of mitochondria in the mitochondrial network (Aon et al., 2003).

In the present study, our finding indicated that H_2O_2 used in this study could cause an increased ROS level in cardiac mitochondria,

which must be sufficiently high, resulting in the opening of IMAC and eventually leading to a decrease in $\Delta\Psi_m$. Through this mechanism, our findings that G-CSF, CsA, and CDP shared a similar efficacy in the prevention of the mitochondrial depolarization, despite the fact that they had different efficacy in the prevention of ROS production, indicated that these pharmacological interventions can effectively attenuate the ROS level in cardiac mitochondria to a level below the critical threshold required for triggering the IMAC opening. Our findings show that low concentrations of H_2O_2 (0.5 and 1 mM) could increase the ROS level in cardiac mitochondria but did not cause mitochondrial membrane potential changes, whereas high-concentration (2 mM) of H_2O_2 could cause much increase in ROS level and produced mitochondrial membrane depolarization, thus supporting the critical threshold hypothesis.

In the present study, the ROS level was lowest in mitochondria pretreated with CsA, followed by higher levels in the CDP and G-CSF groups. Since the efficacy of G-CSF in preventing mitochondrial

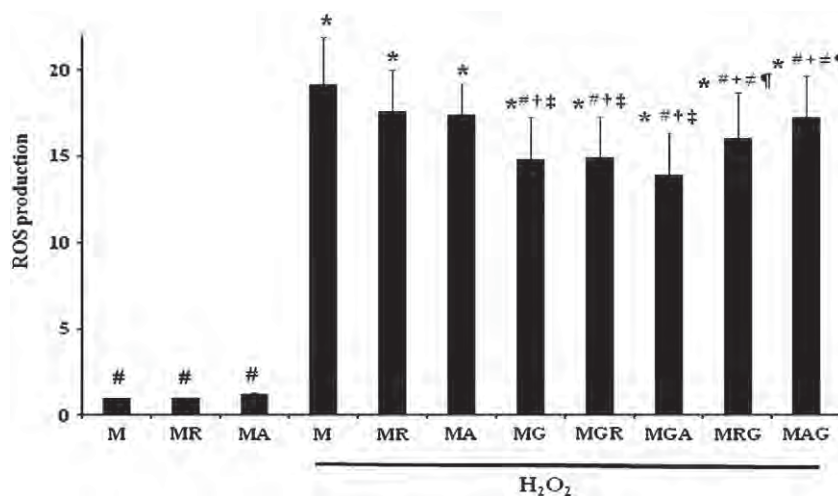


Fig. 11. Effects of G-CSF on complexes I and III. M = healthy cardiac mitochondria, MR = cardiac mitochondria treated with 2- μ M rotenone, MA = cardiac mitochondria treated with 2- μ M antimycin A, MG = cardiac mitochondria treated with G-CSF 50 ng/ml, MGR = cardiac mitochondria pretreated with G-CSF, followed by rotenone application, respectively, MGA = cardiac mitochondria pretreated with G-CSF, followed by antimycin A application, respectively, MRG = cardiac mitochondria pretreated with rotenone, followed by G-CSF application, respectively, MAG = cardiac mitochondria pretreated with antimycin A, followed by G-CSF application, respectively. * $p < 0.05$ vs. M, # $p < 0.05$ vs. (M + H_2O_2), † $p < 0.05$ vs. MR + H_2O_2 , ‡ $p < 0.05$ vs. MA + H_2O_2 , § $p < 0.05$ vs. MG + H_2O_2 , ¶ $p < 0.05$ vs. MGR + H_2O_2 , ¶ $p < 0.05$ vs. MGA + H_2O_2 .

depolarization was similar to that of CsA, our findings suggest that the decreased ROS level caused by G-CSF was sufficient to bring the ROS level below the critical threshold. As a result, the trigger of IMAC opening was inhibited, resulting in no change in $\Delta\Psi_m$ after H_2O_2 application. All these findings suggest that the accumulation of ROS in cardiac mitochondria up to a critical threshold level could be a key determinant for the cardiac mitochondrial protection.

Without H_2O_2 application, ROS levels in the G-CSF and CsA groups were modestly increased. This could be due to the fact that G-CSF itself can increase ROS production by stimulating the angiogenic factors production (Carrao et al., 2009). In cardiomyocytes, it has been shown that G-CSF can directly stimulate ROS production, which plays a pivotal role in triggering adaptations of the heart to ischemia including growth of the coronary collaterals (Carrao et al., 2009). However, the ROS level caused by G-CSF alone was not sufficient to cause the opening of the IMAC.

Our results also showed that applying CsA alone could modestly raise the ROS level in cardiac mitochondria. CsA is known to effectively block the mPTP opening, therefore the release of ROS from mitochondria can be blocked inside the matrix at some degree. Our study also showed that CsA alone can cause a modest decrease in $\Delta\Psi_m$, compared to the control group. This evidence suggests that under physiological condition, mPTP flickering (i.e. opening and closing) is essential for the exchange of metabolites between cardiac mitochondria and cytosol, thus maintaining cardiac mitochondrial membrane potential (Kroemer et al., 2007). Therefore, blocking the opening of mPTP by CsA could prevent this exchange process, resulting in a slight change in $\Delta\Psi_m$.

In the present study, CsA had the highest efficacy in preventing ROS production in cardiac mitochondria. The lower efficacy of CDP than CsA in preventing ROS production could be due to the fact that blocking IMAC only prevents the release of ROS from the mitochondrial matrix, while the remaining ROS could still be released from the intermembrane space and out of the mitochondria via mPTP. Thus, ROS-induced ROS release mechanism could still occur within the mitochondrial network, resulting in a higher level of ROS in the CDP group. Since combined treatment with G-CSF and CDP could effectively decrease ROS level to a similar degree as that in CsA, these findings suggested that G-CSF may act as a blocker of mPTP opening, but with less efficacy than CsA in preventing ROS production.

In the present study, our results indicated that the protective effect of G-CSF on mitochondria could involve complexes I and III on the electron transport chain. Complexes I and III are known as the main sources of ROS production in the mitochondria (Andruekhiv et al., 2006). In a previous study, Chen et al. reported that rotenone did not alter net ROS generation in intact mitochondria (Chen et al., 2003). However, under the pathological condition such as myocardial ischemia, administration of rotenone could decrease the production of ROS in mitochondria (Becker et al., 1999). Our results were consistent with these previous reports. In the present study, although rotenone has a tendency to decrease ROS production in cardiac mitochondria pretreated with rotenone prior to H_2O_2 application, compared to mitochondria treated with H_2O_2 , it did not reach statistical significance. Antimycin A is previously reported that it can increase ROS production (Chen et al., 2008). In the present study, antimycin A-treated cardiac mitochondria under the H_2O_2 -induced oxidative stress condition can increase ROS production compared to the control group. Similar to rotenone, antimycin A did not alter ROS level in normal cardiac mitochondria, and did not change ROS level when H_2O_2 was applied. However, the ROS level measured in the groups in which G-CSF was administered prior to the application of rotenone and antimycin A was significantly lower than that in the groups in which G-CSF was administered after rotenone and antimycin A. This finding indicated that the effect of G-CSF on mitochondria could involve complexes I and III activity in the electron transport chain. It is also important to note that although these

findings indicate that G-CSF could affect complexes I and III activity, future studies are needed to elucidate the definite mechanism of G-CSF as well as its involvement on complexes IV and V in the electron transport chain.

5. Conclusions

Under oxidative stress, G-CSF can effectively prevent mitochondrial swelling, mitochondrial membrane potential changes, and ROS production in cardiac mitochondria. Its mechanism could be due to the inhibition of mPTP opening and could involve the complexes I and III activity on the electron transport chain. These beneficial effects of G-CSF may be used to prevent cardiac mitochondrial damage under oxidative stress conditions.

Disclosures

The authors have no conflict of interest to declare.

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Granulocyte colony-stimulating factor stabilizes cardiac electrophysiology and decreases infarct size during cardiac ischaemic/reperfusion in swine

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Abstract

Aim: Effects of granulocyte colony-stimulating factor (G-CSF) on cardiac electrophysiology during ischaemic/reperfusion (I/R) period are unclear. We hypothesized that G-CSF stabilizes cardiac electrophysiology during I/R injury by prolonging the effective refractory period (ERP), increasing the ventricular fibrillation threshold (VFT) and decreasing the defibrillation threshold (DFT), and that the cardioprotection of G-CSF is via preventing cardiac mitochondrial dysfunction.

Methods: In intact-heart protocol, pigs were infused with either G-CSF or vehicle ($n = 7$ each group) without I/R induction. In I/R protocol, pigs were infused with G-CSF ($0.33 \mu\text{g kg}^{-1} \text{min}^{-1}$) or vehicle ($n = 8$ each group) for 30 min prior to a 45-min left anterior descending artery occlusion and at reperfusion. Diastolic pacing threshold (DPT), ERP, VFT and DFT were determined in all pigs before and during I/R period. Rat's isolated cardiac mitochondria were used to test the protective effect of G-CSF (100 nM) in H_2O_2 -induced mitochondrial oxidative damage.

Results: Neither G-CSF nor vehicle altered any parameter in intact-heart pigs. During ischaemic period, G-CSF significantly increased the DPT, ERP and VFT without altering the DFT. During reperfusion, G-CSF continued to increase the DPT without altering other parameters. The infarct size was significantly decreased in the G-CSF group, compared to the vehicle. G-CSF could also prevent cardiac mitochondrial swelling, decrease ROS production, and prevent mitochondrial membrane depolarization.

Conclusion: G-CSF increases the DPT, ERP and VFT and reduces the infarct size, thus stabilizing the myocardial electrophysiology, and preventing fatal arrhythmia during I/R. The protective mechanism could be via its effect in preventing cardiac mitochondrial dysfunction.

Keywords granulocyte colony-stimulating factor, ischaemic/reperfusion injury, mitochondria, oxidative stress, ventricular fibrillation.

Myocardial infarction remains the major problem in many countries throughout the world. Previous studies have demonstrated that myocardial injury during the

ischaemic-reperfusion injury could facilitate spontaneous ventricular fibrillation (VF), decrease the VF threshold (VFT) and increase the defibrillation

threshold (DFT) (Qin *et al.*, 2002, Anastasiou-Nana *et al.* 2005). The association between arrhythmic events and the infarct size during ischaemic/reperfusion (I/R) has been demonstrated in which the reduction of the infarct size could significantly ameliorate arrhythmic events from myocardial ischaemia (Sobel *et al.* 1972, Xiao *et al.* 2008).

Granulocyte colony-stimulating factor (G-CSF) is a drug known to promote the proliferation, survival and differentiation of haematopoietic cells, and has been used as an adjuvant drug for stem cell therapy in the treatment of acute and chronic myocardial infarction (Fujita *et al.* 2007, Kastrup *et al.*, 2006). Although previous studies have shown that the combination of G-CSF and stem cell therapy enhanced the regeneration of myocardium and improved reperfusion function, thus reducing the adverse events from myocardial ischaemia (Fujita *et al.*, 2007, Kastrup *et al.* 2006), recent studies have demonstrated that G-CSF can exert its own potential effects on the heart (Yagi *et al.* 2008, Abdel-Latif *et al.* 2008). It has been shown that treating acute MI patients with G-CSF resulted in improved left ventricular function and decreased VF incidence (Baldo *et al.*, 2008, Engelmann *et al.* 2008). Despite these potential benefits, some clinical reports demonstrated otherwise (Ripa *et al.* 2006, Zohnhofer *et al.* 2006, 2008). Nevertheless, the direct effect of G-CSF on the susceptibility of VF induction and defibrillation efficacy during ischaemia/reperfusion period has never been investigated.

Growing evidence indicates the important role of mitochondria in protection against arrhythmia. Cardiac mitochondrial membrane potential dissipation has been shown to be associated with cardiac arrhythmias, and drugs that could prevent depolarization of mitochondrial membrane potential has been demonstrated to prevent cardiac arrhythmia in the heart under various stress conditions (Aon *et al.* 2008, O'Rourke *et al.* 2007, Akar *et al.*, 2005, Aon *et al.*, 2009). In cardiac ischaemia/reperfusion (I/R) injury, reactive oxygen species (ROS) have been shown to be mainly responsible for cardiac and mitochondrial dysfunction (O'Rourke *et al.* 2007, Aon *et al.*, 2006, Akar *et al.*, 2005). A recent study also demonstrated that G-CSF could protect cardiac mitochondrial damage during the early phase of myocardial injury (Hiraumi *et al.* 2009).

In this study, the effects of G-CSF on cardiac electrophysiology as well as the incidence of VF during I/R period were investigated in swine. We hypothesized that G-CSF increases the VFT and decreases the DFT, leading to arrhythmia prevention and improved defibrillation efficacy. We also tested the hypothesis that G-CSF protects the heart during I/R injury by preventing cardiac mitochondrial dysfunction. Isolated rat's cardiac mitochondria were used to test this hypothesis.

H₂O₂ was used to mimic the I/R injury by causing oxidative damage in cardiac mitochondria (Yang and Cortopassi, 1998).

Methods

Thirty domestic pigs (25–30 kg) of either sex were used in this study. This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animal as adopted and promulgated by the US National Institutes of Health and has been approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. The pigs were anesthetized by a combination of atropine (0.04 mg kg⁻¹), telazol (4.4 mg kg⁻¹), and xylazine (2.2 mg kg⁻¹), and maintained by 1.5–3.0% isoflurane delivered in 100% oxygen. The vital signs including femoral arterial blood pressure (BP), heart rates (HR), lead II electrocardiogram, respiratory rate and core temperature were continuously monitored for the entire study. The PaO₂, end-tidal CO₂ and blood gases were measured and maintained under physiologic condition. Under artificial respiration, a 34-mm platinum coated titanium coil electrode catheter (Guidant Corp., St Paul, MN, USA) was inserted into the right ventricular apex and a 68-mm electrode catheter at the junction between right atrium and superior vena cava (Chattipakorn *et al.*, 2006, Kanlop *et al.* 2008, Shinlapawittayatorn *et al.*, 2006, Sungnoon *et al.* 2008). These two electrodes were used to deliver strong stimulus during VFT and DFT determination. Median sternotomy was done and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected from its surrounding tissues.

Two protocols were performed in this study; one in pigs with normal hearts without ischaemia (*n* = 14) and another in pigs with I/R hearts (*n* = 16). In each experimental model, pigs were divided equally into two groups; G-CSF and vehicle. In each pig, cardiac electrophysiological parameters, including diastolic pacing threshold (DPT), effective refractory period (ERP), VFT and DFT were determined at the beginning of the study. G-CSF (0.33 µg kg⁻¹ min⁻¹) (Takahama *et al.*, 2006) and vehicle (normal saline solution of similar amount) were administered intravenously for 30 min in G-CSF and vehicle groups, respectively. In the intact-heart model, the cardiac electrophysiological parameters were determined before and after the drug or vehicle administration. In the I/R model, the LAD was ligated at 5 cm above a distal branch of LAD to perform a complete regional occlusion. The ischaemic period was sustained for 45 min and then the LAD was reperused (Krug *et al.* 1966). During the first 20 min of occlusion, defibrillation shock would be delivered to terminate VF if VF spontaneously occurred. The time

interval for the first VF to occur after LAD occlusion was determined in each pig. Once the spontaneous VF disappeared or did not occur within 20 min, VF would be electrically induced for the VFT determination (Qin *et al.* 2002a). The DFT was also determined simultaneously with VFT determination. At the time of reperfusion, G-CSF or vehicle was infused again and all parameters were determined at 30 min after the reperfusion (Chattipakorn *et al.*, 2006, Kanlop *et al.*, 2008). Illustration of the study in the ischaemic-reperfusion protocol is shown in Figure 1.

A train of 10 S1 stimuli (square, 5-ms pulse width, 500-ms interval) was delivered from the tip of RV pacing electrode to determine the DPT. The pacing current was initially at 0.1 mA and increased in 0.1-mA steps until all stimuli exerted their ventricular responses. This pacing current was defined as the DPT. The ERP was determined by delivering an S2 stimulus after the last S1. The S1–S2 interval was initially set at 350 ms and was decremented in 10-ms steps until an S2 stimulus could not elicit a ventricular response. The last S1–S2 interval that elicited a ventricular response was defined as an ERP (Kanlop *et al.*, 2008).

VFT and DFT determination protocol: VF was induced by delivering a stimulus during the vulnerable

period (T-wave of lead II ECG). The coupling interval for VF induction was determined by delivering a train of 10 S1s for three times and the interval between the last S1 and the peak-T wave from each train was determined. The average value of these intervals was used as a coupling interval to deliver an S2 stimulus for VF induction (Kanlop *et al.*, 2008, Chattipakorn *et al.* 2000a,b,c). A biphasic S2 shock (Ventak ECD, Guidant Corp.) was delivered through shocking electrodes for VFT and DFT determination. For VFT determination, the S2 shock strength was initially at 100 V. This strength was reduced or increased in 10-V steps depending on whether VF induction was successful or failed, respectively (Kanlop *et al.*, 2008). The lowest shock strength required for inducing VF was defined as the VFT (Kanlop *et al.*, 2008). There was a minimum interval of 4 min between each VF episode to allow the hemodynamic status to return to physiological conditions (Sungnoon *et al.*, 2008). The DFT was determined using a three-reversal up/down protocol (Chattipakorn *et al.*, 2006, Shinlapawittayatorn *et al.*, 2006). In brief, the defibrillation shock was initially at 400 V and delivered after 10 s of VF. The shock strength was decreased or increased in 80-V steps depending on whether the shock succeeded or failed to terminate VF, respectively. This process was repeated with an increment or decrement of 40-V and 20-V steps. The lowest shock strength required for successful defibrillation after the third reversal was defined as the DFT (Chattipakorn *et al.* 2006, Shinlapawittayatorn *et al.* 2006).

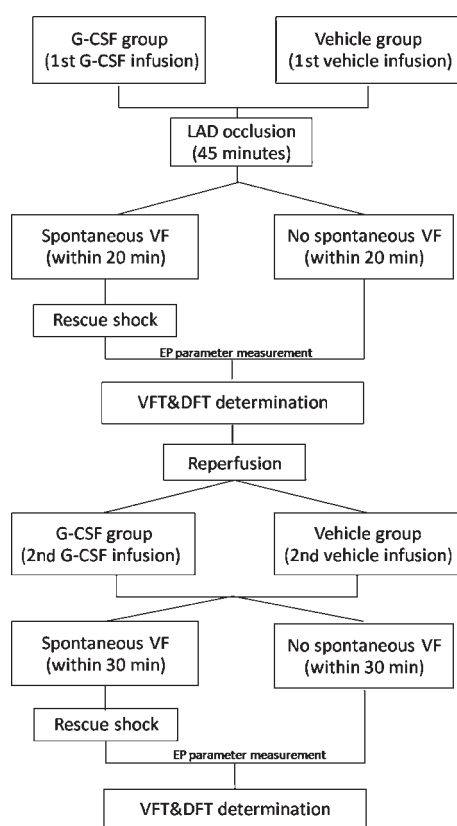


Figure 1 Illustrated diagram of the ischaemic-reperfusion protocol in swine.

G-CSF preparation

G-CSF (Neupogen®, Thousand Oaks, CA, USA) was used for intravenous infusion. A vial of Neupogen® of 1.0 mL contains 30 MU (=300 µg) of filgrastim. G-CSF 1.0 mL was diluted in 9 mL dextrose saline solution (30 µg mL⁻¹) and intravenously infused for 30 min at the rate of 0.011 mL kg⁻¹min⁻¹ (0.33 µg kg⁻¹ min⁻¹) (Takahama *et al.*, 2006).

Infarct size and area at risk (AAR) measurement

At the end of each experiment, the heart was removed and irrigated with normal saline to wash out blood from chambers and vessels. The LAD was occluded again at the same site previously done during the ischaemic period. The catheters were inserted into the right and left coronary ostia for Evan blue infusion. The area which could not be infused by Evan blue was defined as area of no blood flow during ischaemic period. The heart was frozen and cut horizontally into 5-mm thick slices, starting from the apex until 5 mm above the occluding site. Then, each slice was immersed in Triphenyltetrazolium chloride (TTC) at least 25 min

(Takahama *et al.*, 2006, Khalil *et al.* 2006), after which the area with viable tissue was seen in red. The area that was not stained with Evan blue was defined as area at risk (AAR). The area which demonstrated neither blue nor red was defined as the infarct site. The area measurements were performed with IMAGE tool software version 3.0 (University of Texas Health Sciences Center, San Antonio, TX, USA). The infarct site was calculated depending on the weight of each slice according to the formula of Riess *et al.* (2009):

$$\text{Total infarct size} = \frac{\sum[(\text{Infarct size of slice } n / \text{total area}) \times \text{weight of slice } n]}{\text{Total weight of slices}}$$

$$\text{Total AAR} = \frac{\sum[(\text{AAR of slice } n / \text{total area}) \times \text{weight of slice } n]}{\text{Total weight of slices}}$$

Cardiac mitochondria study protocol

In this protocol, cardiac mitochondria were isolated from the hearts of Wistar rats using the technique described previously (Larche *et al.* 2006), and the protein concentration was determined according to bicinchoninic acid (BCA) assay (Walker 1994). The morphology of the isolated cardiac mitochondria was confirmed using the electron microscope. In this protocol, H₂O₂ (2 mM) was used to mimic the oxidative stress condition occurring during I/R injury (Yang & Cortopassi 1998a). Cardiac mitochondria were divided into three treatment groups ($n = 5$ in each group): (1) a control group, (2) H₂O₂ treated group, (3) G-CSF (100 nM) plus H₂O₂ treated group. In group 2, cardiac mitochondria were incubated with H₂O₂ for 5 min, whereas in group 3 cardiac mitochondria were pre-treated with G-CSF (100 nM) for 30 min followed by H₂O₂ application for another 5 min. At the end-point, cardiac mitochondria in each group were determined for mitochondrial swelling, mitochondrial ROS production, and mitochondrial membrane potential changes.

Determination of cardiac mitochondrial swelling

The change in the absorbance of the mitochondrial suspension (0.4 mg mL⁻¹) at 540 nm (A₅₄₀) was determined using a microplate reader (Ruiz-Meana *et al.* 2006). The decrease in the absorbance of the mitochondrial suspension indicated mitochondrial swelling.

Determination of cardiac mitochondrial ROS production

The dye dichlorohydro-fluorescein diacetate (DCFDA) was used to determine the level of ROS production in

cardiac mitochondria (Novalija *et al.* 2003). DCFDA could pass through the mitochondrial membrane, and was oxidized by ROS in the mitochondria into DCF. Fluorescence was determined at λ_{ex} 485 nm and λ_{em} 530 nm using a fluorescence microplate reader. The ROS level was expressed as arbitrary units of fluorescence intensity of DCF.

Determination of cardiac mitochondrial membrane potential changes

The dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to determine the change in the mitochondrial membrane potential (Di Lisa *et al.* 1995, Tong *et al.* 2005). JC-1 was characterized as a cation and remained in the mitochondrial matrix as a monomer (green fluorescence) form. However, it could interact with anions in the mitochondrial matrix to form an aggregate (red fluorescence) form. JC-1 monomer fluorescence (green) was excited at 485 nm and the emission was detected at 530 nm. JC-1 aggregate fluorescence (red) was excited at 485 nm and the emission fluorescence was recorded at 590 nm. Mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio.

Statistical analysis

Values were expressed as mean $\pm$ SD. Analysis of covariance (ANCOVA) was used to assess whether the inequality of the baseline values affected the results after the treatment. Comparisons of variables before and after the drugs or vehicle administration (comparison within group) were performed individually in each group using the paired, two-tailed student's *t*-test. Comparisons among treatments of cardiac mitochondria were made by one-way ANOVA followed by the Fisher *post-hoc* test. $P < 0.05$ was considered statistically significant.

Results

In the intact-heart model, the baseline parameters such as the DPT, ERP, HR, systolic BP and diastolic BP between G-CSF and vehicle groups were not significantly different. Neither G-CSF nor saline administration altered the HR, systolic BP and diastolic BP (data not shown). The DPT was not changed after G-CSF or saline infusion. The ERP was slightly increased in the G-CSF group, however, it was not statistically significant (Table 1). For the VFT and DFT measurements, all determined parameters including the peak voltage, total energy, pulse width and impedance after G-CSF or saline infusion were not changed from their baseline values (Table 1).

Table 1 Electrophysiological parameters in the *normal-heart* pigs without ischaemia/reperfusion induction protocol

Parameters	G-CSF group (n = 7)	Vehicle group (n = 7)
Baseline		
Peak voltage (V) of VFT	47 ± 8	47 ± 8
Total energy (J) of VFT	0.2 ± 0.06	0.2 ± 0.06
Peak voltage (V) of DFT	518 ± 77	518 ± 77
Total energy (J) of DFT	21 ± 7	20 ± 7
DPT (mA)	0.3 ± 0.1	0.3 ± 0.1
ERP (ms)	284 ± 53	282 ± 53
After drug administration		
Peak voltage (V) of VFT	48 ± 6	46 ± 6
Total energy (J) of VFT	0.2 ± 0.04	0.2 ± 0.04
Peak voltage (V) of DFT	514 ± 57	502 ± 57
Total energy (J) of DFT	21 ± 5	20 ± 5
DPT (mA)	0.3 ± 0.2	0.3 ± 0.2
ERP (ms)	301 ± 36	285 ± 40

VFT, ventricular fibrillation threshold; DFT, defibrillation threshold; DPT, diastolic pacing threshold; ERP, effective refractory period.

In the I/R model, the baseline parameters such as the DPT, ERP, HR, systolic BP and diastolic BP between G-CSF and vehicle groups were not significantly different (Table 2). In the G-CSF group, the DPT and ERP were increased ($P < 0.05$) and the systolic BP was decreased ($P < 0.05$) during LAD occlusion, compared to the baseline. During reperfusion, the DPT was significantly increased from that at the baseline and ischaemic phase. The ERP which was significantly increased from the baseline during the occlusion period was returned to the baseline during the reperfusion phase (Table 2). The systolic BP was still significantly decreased ($P < 0.05$), compared to the baseline.

In the vehicle group, the DPT were increased ($P < 0.05$) and the systolic and diastolic BP were decreased ($P < 0.05$) during LAD occlusion, compared to the baseline. The ERP was not changed from the baseline during this phase. During reperfusion, the DPT was not increased from its value at ischaemic phase, but was still significantly higher than the DPT at the baseline (Table 2). Similar to the ischaemic phase, the ERP was not changed from the baseline during reperfusion.

During LAD occlusion, the incidence of VF between G-CSF (5/8) and vehicle group (4/8) were not significantly different. The time interval from the LAD occlusion to the first VF occurrence of the G-CSF (15 ± 2 min) and vehicle (19 ± 4 min) groups were not significantly different.

For the VFT measurement during the ischaemic period, both peak voltage and total energy of the VFT

Table 2 Electrophysiological and hemodynamic parameters at the baseline, ischaemia and reperfusion phases during the G-CSF or vehicle administration in the *ischaemia/reperfusion* protocol

Parameters	G-CSF group (n = 8)	Vehicle group (n = 8)
Pre-occlusion (baseline)		
DPT (mA)	0.2 ± 0.1	0.2 ± 0.1
ERP (ms)	253 ± 15	247 ± 17
HR (beats min ⁻¹)	95 ± 7	90 ± 10
Systolic pressure (mmHg)	100 ± 10	113 ± 10
Diastolic pressure (mmHg)	66 ± 10	73 ± 13
Occlusion (ischaemia)		
DPT (mA)	0.5 ± 0.2*	0.5 ± 0.2*
ERP (ms)	276 ± 18*	254 ± 15
HR (beats min ⁻¹)	92 ± 16	98 ± 14
Systolic pressure (mmHg)	85 ± 16*	96 ± 13*
Diastolic pressure (mmHg)	59 ± 14	62 ± 8*
Reperfusion		
DPT (mA)	0.7 ± 0.2*†	0.5 ± 0.1*
ERP (ms)	245 ± 31†	250 ± 20
HR (beats min ⁻¹)	96 ± 13	96 ± 8
Systolic pressure (mmHg)	66 ± 4*	73 ± 5*
Diastolic pressure (mmHg)	55 ± 9	60 ± 12*

DPT, diastolic pacing threshold; ERP, effective refractory period; HR, heart rate.

* $P < 0.05$ vs. the baseline in each group, † $P < 0.05$ vs. ischaemic phase in each group.

in the G-CSF group were significantly increased from the baseline (Fig. 2). During reperfusion, both peak voltage and total energy of the VFT in the G-CSF group were significantly decreased from the VFT during ischaemia and were not significantly different from the baseline. In the vehicle group, both peak voltage and total energy of the VFT were not significantly changed from the baseline throughout the entire study. The pulse width and impedance for the VFT were neither changed for the entire period of the study in both groups (data not shown).

For the DFT determination, all parameters including peak voltage, total energy (Fig. 3), pulse width and impedance during occlusion and reperfusion were not significantly different from the baseline parameters in both experimental groups.

The area at risk in this study was not different between the G-CSF-treated group and the vehicle-treated group ($P = 0.15$, Fig. 4a). However, the size of the infarct myocardium was significantly larger in the vehicle-treated group than in the G-CSF-treated group (Fig. 4b).

For cardiac mitochondrial study, H₂O₂ significantly caused cardiac mitochondrial swelling, increased mitochondrial ROS level, and depolarized mitochondrial

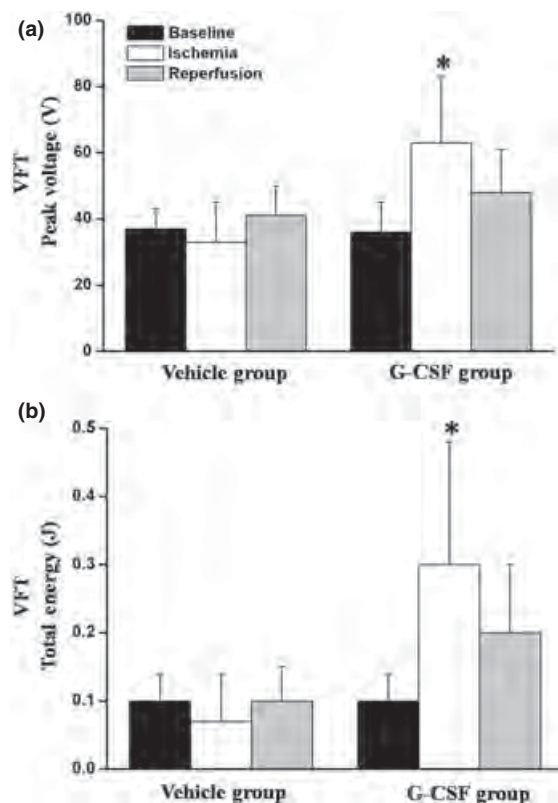


Figure 2 Effects of G-CSF and vehicle on the ventricular fibrillation threshold (VFT) at the baseline and during ischaemic and reperfusion periods. The peak voltage (a) and total energy (b) after G-CSF infusion was significantly elevated, compared to the baseline values. Vehicle did not alter the VFT. * $P < 0.05$ compared to the baseline value.

membrane potential (Fig. 5). When cardiac mitochondria were pre-treated with G-CSF, it could protect H_2O_2 -induced mitochondrial swelling, decreased mitochondrial ROS level, and prevented mitochondrial depolarization (Fig. 5).

Discussion

The major findings of this study are as follows: (1) In intact heart, G-CSF did not affect any measured electrophysiologic parameter, (2) The DPT was significantly and progressively increased from the baseline during ischaemic and reperfusion phases in the G-CSF group, (3) During the ischaemic period, the ERP and the VFT in the G-CSF group were significantly increased from the baseline, but returned to the baseline value during reperfusion, (4) The DFT during ischaemia and reperfusion was not altered from the baseline in both G-CSF and vehicle groups, (5) G-CSF significantly decreased the infarct size, and (6) G-CSF protected cardiac mitochondrial swelling, decreased ROS level, and prevented mitochondrial depolarization caused by oxidative stress.

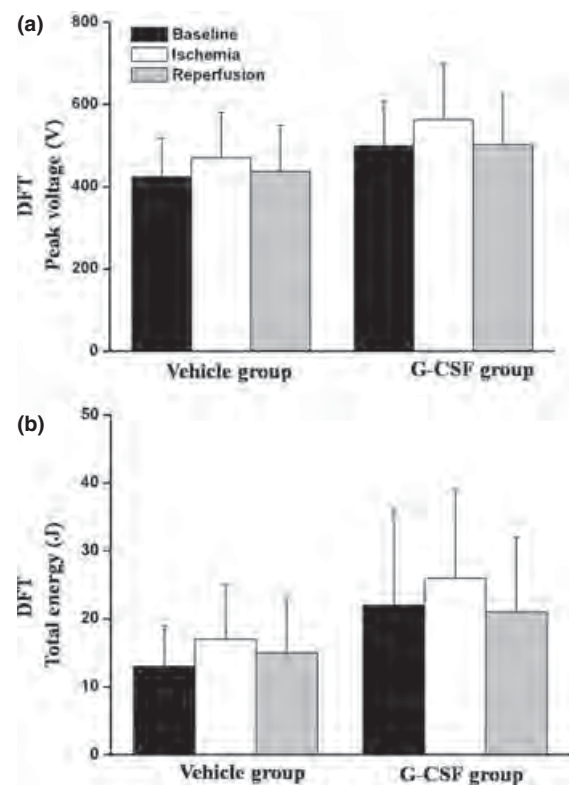


Figure 3 Effects of G-CSF and vehicle on the defibrillation threshold (DFT). The peak voltage (a) and total energy (b) after either G-CSF or vehicle infusion were not significantly altered, compared to the baseline values.

Previous clinical trials and a recent meta-analysis found that when G-CSF was used as a purpose to mobilize stem cell in patients with acute myocardial infarction, no significant differences on cardiac function or infarct size were found between G-CSF and Placebo groups (Ripa *et al.* 2006, Zohlhofer *et al.* 2006, 2008). However, the effects of acute G-CSF administration on cardiac electrophysiology during cardiac ischaemia/reperfusion have never been investigated. Our study is the first study investigated the direct effects of G-CSF on the cardiac electrophysiology and the infarct size during cardiac I/R in a swine model. The aim was to investigate the effect of G-CSF directly on the electrophysiology and infarct size of the heart under acute I/R condition, and without stem cell therapy for myocardial recovery.

Several studies that directly investigated the direct effect of G-CSF on the heart have reported that G-CSF prevents adverse effects of acute myocardial infarction such as ventricular arrhythmia and heart failure, and improves cardiac function (Baldo *et al.* 2008, Ince *et al.* 2005, Kuhlmann *et al.* 2006). The proposed cardioprotective mechanisms of G-CSF against I/R injury include its ability to limit the infarct size (Takahama

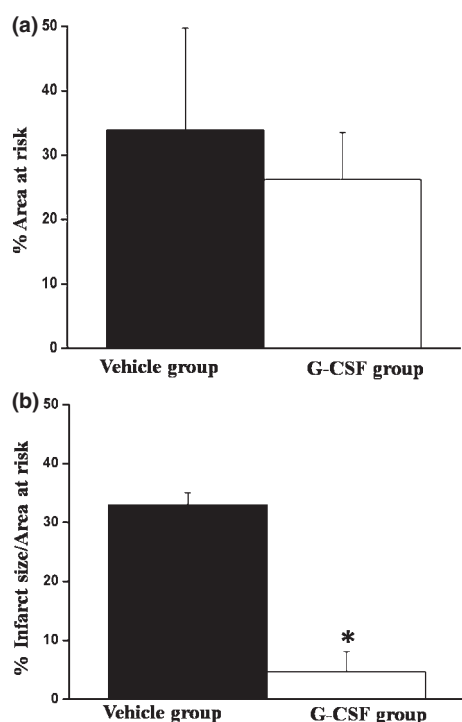


Figure 4 Effects of G-CSF and vehicle on the infarct size and area at risk (AAR). The infarct size was prominent in the vehicle group, whereas it was much smaller in the G-CSF group. The AAR was not different between the two groups. * $P < 0.05$ compared to the vehicle group.

et al., 2006). Thus, consistent with a previous report in canine (Takahama *et al.*, 2006), our results clearly indicated that in this short period of acute myocardial ischaemia, G-CSF could markedly reduce the infarct size in the swine model.

In this study, the DPT was increased after LAD occlusion in both G-CSF and saline groups. However, in the former group, the DPT was further increased even after reperfusion, whereas it was not changed in the vehicle group. This finding indicates that G-CSF could have electrophysiologically stabilized the myocardium during I/R event by raising the DPT, thus preventing the heart from arrhythmia susceptibility from this ischaemia/reperfusion injury. Furthermore, the ERP was prolonged significantly during myocardial ischaemia in the G-CSF treated groups. It is known that VF induction can be facilitated by ERP shortening, and that ERP prolongation could decrease the dispersion of refractoriness in the heart, thus impeding the VF induction (Maass *et al.* 2009). Although the incidence of VF and time interval from LAD occlusion to the first observed VF between the G-CSF and vehicle groups were not different in this study, the VFT was significantly increased during the ischaemic period in the G-CSF group. Since the VFT could effectively represent the susceptibility of the heart to ventricular arrhythmia induction, increased VFT by G-CSF suggested that ischaemic myocardium had low susceptibility to VF

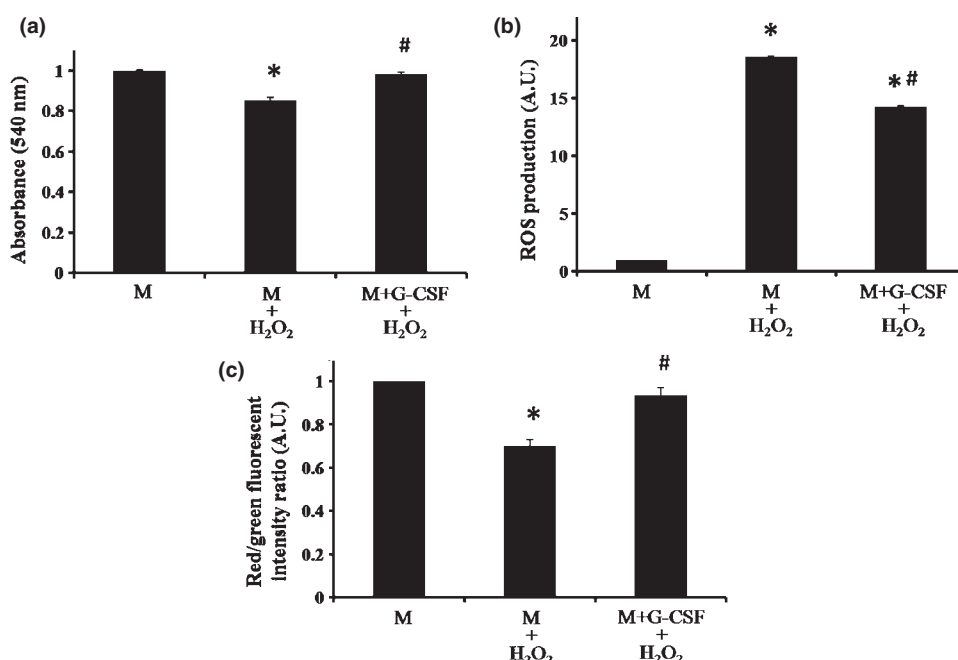


Figure 5 Effects of G-CSF on cardiac mitochondria. G-CSF could effectively prevent cardiac mitochondrial swelling (a), decrease mitochondrial ROS level (b), as well as prevent mitochondrial depolarization (c) caused by H₂O₂-induced oxidative damage. M, cardiac mitochondria; M + H₂O₂, cardiac mitochondria treated with H₂O₂; M + G-CSF + H₂O₂, cardiac mitochondria pre-treated with G-CSF, followed by H₂O₂ treatment. * $P < 0.05$ vs. M, # $P < 0.05$ vs. M + H₂O₂.

induction. Also, the VF incidence in our study is different from a previous report that demonstrated a reduction in VF incidence after G-CSF administration (Takahama *et al.* 2006). This difference could be due to the different sites of LAD occlusion since the AAR in this study was $23 \pm 0.9\%$, whereas the AAR in the previous study was $\sim 40\%$ (Takahama *et al.*, 2006).

Mitochondria are vital organelles in many organs including the heart. Previous studies demonstrated that it played a vital role in determining cardiac arrhythmia and cell death (Aon *et al.* 2008, O'Rourke *et al.* 2007, Akar *et al.* 2005, Aon *et al.* 2009). During ischaemia/reperfusion in the heart, ROS is known to be a major cause of cardiac dysfunction and lethal arrhythmia (O'Rourke *et al.* 2007, Aon *et al.* 2006, Akar *et al.*, 2005). In this study, we demonstrated that G-CSF could protect cardiac mitochondrial damage during H_2O_2 -induced oxidative stress, by protecting cardiac mitochondrial swelling, decreasing mitochondrial ROS production, and preventing cardiac mitochondrial depolarization. It is important to note that G-CSF could completely protect the cardiac mitochondria against swelling (Fig. 5a) and membrane depolarization (Fig. 5c) caused by oxidative stress in this study since no statistical differences were found between cardiac mitochondria in the control group (group 1) and cardiac mitochondria pre-treated with G-CSF followed by H_2O_2 treatment (group 3). All of these beneficial effects could result in preserving cardiac mitochondrial function during severe oxidative stress condition as seen in the heart during ischaemia/reperfusion injury. Therefore, this role of G-CSF in preserving cardiac mitochondrial function could be responsible for its cardioprotection against ischaemia/reperfusion injury as seen in a swine model in this study. Since species differences could play an important role in the effect of G-CSF, future studies including a direct investigation on the role of cardiac mitochondria in the acute I/R model are needed to verify this hypothesis. Furthermore, future investigations on the role of G-CSF administered after the occlusion are required to warrant its clinical benefits in acute myocardial infarction.

Conclusion

G-CSF increases the DPT, ERP and the VFT, thus stabilizing the myocardial electrophysiology during acute myocardial ischaemia. However, the VF incidence and the time to VF onset are not affected by G-CSF administration. G-CSF also decreases the infarct size. Since G-CSF could protect cardiac mitochondrial dysfunction caused by oxidative damage, this beneficial effect could be responsible for its cardioprotection during I/R injury.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Effects of high-fat diet on insulin receptor function in rat hippocampus and the level of neuronal corticosterone

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ABSTRACT

Aim: Chronic consumption of a high-fat (HF) diet contributes to peripheral insulin resistance and elevated plasma corticosterone. However, the effect of HF consumption on the neurofunctional insulin receptors and neuronal corticosterone level is unclear. We tested the hypothesis that HF consumption can lead to peripheral insulin resistance, elevated neuronal corticosterone, and impaired neuronal responses to insulin.

Main methods: Male Wistar rats were fed with normal diet or HF diet for 4, 8 or 12 weeks. At the end of each dietary period, plasma was collected for investigating peripheral insulin resistance parameters and corticosterone. Brains were then rapidly removed for studying the function of neuronal insulin receptors (IRs) by extracellular recording in CA1 hippocampus, neuronal IR signaling by immunoblot technique and neuronal corticosterone.

Key findings: Elevated plasma corticosterone level was initially seen in 4-week HF-fed rats. Peripheral insulin resistance developed at 8-week HF-fed rats. However, the elevated neuronal corticosterone level was found at 12-week HF consumption. The neuronal IR response demonstrated by insulin-mediated long-term depression in CA1 hippocampus was diminished in 12-week HF-fed rats. The phosphorylation levels of neuronal IR, IR substrate 1 and Akt/PKB were decreased in 12-week HF-fed rats with no change in these proteins. There was a correlation among peripheral insulin resistance, neuronal stress (elevated neuronal corticosterone), and neuronal insulin resistance in HF group.

Significance: Our findings suggest that HF consumption can lead to the elevation of corticosterone and peripheral insulin resistance, which could contribute to neuronal insulin resistance and neuronal stress.

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Introduction

Insulin has been shown to play a role in synaptic plasticity by facilitating clathrin-dependent internalization of alpha-amino-3 hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, causing long-term depression (LTD) in hippocampal CA1 neurons (Ahmadian et al., 2004; Man et al., 2000). That evidence indicates that the neurofunctional IRs in the CA1 hippocampus is insulin-mediated LTD. Moreover, the activation of insulin receptors turns on the protein kinase activity of the IR, which triggers cascades of signal transduction through its downstream substrate molecules. Several insulin receptor signaling pathways activated by IRs include insulin receptor substrate-1 (IRS-1) (Olefsky, 1990). It has been demonstrated that rats trained in a spatial learning task showed the learning-specific increase in IRS-1 in the hippocampal synaptic membrane (Morris et al., 2004).

These findings suggest that IR signaling plays a role in learning and memory by modulating activities of synaptic plasticity such as insulin-mediated LTD and by triggering signal transduction cascades such as IR, IRS-1 and serine/threonine-specific protein kinase/protein kinase B (Akt/PKB).

In hamsters with insulin resistance induced by fructose feeding, it has been shown that hippocampal synaptic plasticity, an important biological mechanism of learning and memory, was impaired (Mielke et al., 2005). Excessive fat consumption has also been shown to play an important part in the development of insulin resistance and type2 diabetes (Zierath et al., 1998). Several studies suggest that consumption of a diet rich in fat for 3 months can develop peripheral insulin resistance and impede cognitive performance (Greenwood and Winocur, 2005; Kalmijn et al., 2004). These findings suggest that the development of insulin resistance can mediate the cognitive deficit associated with HF diet. However, the effect of HF diet on the neurofunctional IRs is still unclear. In addition, the effects of time-course of HF diet consumption on the neurofunctional IRs have never been investigated. Therefore, in this study we tested the hypothesis that HF consumption for a specific period of time can cause peripheral

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insulin resistance and can lead to impaired neuronal response to insulin (or neuronal insulin resistance). We used an electrophysiological study to investigate whether the neuronal responses to insulin (insulin-mediated LTD) are altered by HF consumption at different time course in order to detect the earliest stage of the disruption of the neurofunctional IRs. We also examined the alteration of biochemical activity of insulin receptor pathways: IR, IRS-1 and Akt/PKB, in the brain following each time course of HF consumption.

Glucocorticoid participates in the control of homeostasis and stress. Previous studies demonstrated that adrenal corticosteroid level was increased in rodents with diabetes (Chan et al., 2005; Langley and York, 1990). In addition, Stranahan and colleagues demonstrated that diabetes could impair hippocampal function via glucocorticoid effects (Stranahan et al., 2008a,b). Despite these findings, the correlation among peripheral and neuronal insulin resistance, and the level of corticosterone following HF consumption has not yet been investigated. In this study, we also determined whether the neuronal insulin resistance can develop with neuronal stress after HF consumption by using the amount of neuronal corticosterone as an indicator of neuronal stress.

Materials and methods

Animals and dietary protocols

All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Male Wistar rats weighing ~200 g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were individually housed in a temperature-controlled environment with a 12:12 light–dark cycle. One week after arrival, rats were randomly assigned to one of the two dietary groups ($n = 43$ in high-fat diet group and $n = 44$ in normal diet group). The normal-diet (ND) group received a standard laboratory chow, in which 19.7% of total energy (%E) was from fat, with energy content calculated at 4.02 kcal/g (Mouse Feed Food No.082, C.P. Company, Bangkok, Thailand). The HF group consumed a diet containing fat, mostly from lard (59.3% E), with energy content calculated at 5.35 kcal/g, for 12 weeks. The animals were maintained in individual cages with unrestricted access to food and water. Body weight and food intake were recorded daily. Blood samples were collected from the tail at weeks 4, 8 and 12 after fasting for at least 5 h. Plasma was separated and stored at -80°C for subsequent biochemical analyses. At the end of each experimental period (4, 8 and 12 weeks) of both dietary regimens, animals were deeply anesthetized and decapitated. The brain was rapidly removed for brain slice preparation and one lobe of liver as well as visceral fat were removed, weighed and stored at -80°C for further biochemical analysis.

The oral glucose tolerance test (OGTT) was investigated in the 12-week HF diet and 12-week ND group. After rats being on the dietary for 12 weeks, animals were fast for 12 h before they were used in the OGTT. An OGTT consisted of 2 g/kg body weight glucose feeding by gavage. Blood was collected from a small cut at the tip of the tail immediately before and at 15, 30, 60 and 120 min after glucose feeding. The plasma was stored at -80°C until it was used for glucose analysis with a commercially available kit (Biotech, Bangkok, Thailand).

Analytical procedure for the level of glucose, triglyceride, insulin and corticosterone in plasma and neuronal corticosterone

Fasted plasma glucose and triglyceride concentrations were determined by colorimetric assay using commercially available kits (Biotech, Bangkok, Thailand). Fasted plasma insulin level was measured by Sandwich ELISA kits (LINCO Research, Missouri, USA).

Neuronal corticosterone levels from brain homogenate samples and fasted plasma corticosterone levels were measured using an enzyme immunoassay kit (Assay Designs Inc, Ann Arbor, MI, USA).

Determination of insulin resistance (HOMA index)

Insulin resistance was assessed by Homeostasis Model Assessment (HOMA) (Appleton et al., 2005; Haffner et al., 1997) as a mathematical model describing the degree of insulin resistance, calculated from fasting plasma insulin and fasting plasma glucose concentration. A higher HOMA index indicates a higher degree of insulin resistance.

Analysis of liver triglyceride concentration

Tissue homogenates were prepared for triglyceride assay by a modification of the method of Frayn and Maycock (Frayn et al., 1980). The triglyceride concentration was analyzed with a commercially available kit (Biotech, Bangkok, Thailand).

Brain slice preparation

At the end of weeks 4, 8 and 12, the animals were anesthetized with isoflurane after fasting for at least 5 h. Brain slice preparation was following our previous study (Chattipakorn and McMahon, 2002). Hippocampal slices were used for the extracellular recordings, immunoprecipitation and immunoblotting.

Extracellular recording of hippocampal slices

To investigate insulin-induced long-term depression (LTD), the hippocampal slices were transferred to a submersion recording chamber and continuously perfused at 3–4 ml/min with standard aCSF warmed to $25\text{--}28^{\circ}\text{C}$. Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collateral–commissural pathway with a bipolar tungsten electrode, while recordings were gathered from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 Mohm) filled with 2 M NaCl. Stimulus frequency was 0.033 Hz. The stimulus intensity was adjusted to yield a fEPSP of 0.8–1.0 mV in amplitude. Hippocampal slices were perfused with aCSF (as baseline condition) for 10 min and then perfused with aCSF plus 500 nM insulin (as insulin stimulation) for 10 min, after which the hippocampal slices were perfused with aCSF again (wash out) and recorded for the next 30 min.

To investigate that the reduction of insulin-mediated LTD was the result of an alteration of neuronal insulin signaling and not a non-specific alteration of synaptic transmission following neuronal insulin resistance, we examined the characterized form of synaptic plasticity that may not depend upon insulin signaling, by measuring paired pulse facilitation (PPF) and carbachol-induced LTD (mLTD). PPF is a measurement of short-term potentiation, which may occur at the presynaptic sites (Zucker and Regehr, 2002). PPF occurs following two identical stimulations, separated by 50-msec, applied to the Schaffer collateral. To investigate mLTD, the protocol was as following. The stable 10-min baseline of CA1 fEPSPs was recorded by stimulating the Schaffer collateral–commissural pathway with bipolar tungsten electrode. Stimulus frequency was 0.1 Hz and stimulus intensity was adjusted to yield fEPSPs of 0.8–1.0 mV amplitude. The cholinergic agonist, carbachol 50 μM (Calbiochem, San Diego, CA, USA), was superfused for 10 min to induce mLTD after which the carbachol was washed out and recorded for the next 30 min.

All data were filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using pClamp 9.2 software (Axon Instruments, Foster City, CA, USA). The initial slope of the fEPSPs was measured and plotted vs. time using Origin 8.0 software.

Preparation of brain homogenates for immunoprecipitation, immunoblotting and neuronal corticosterone measurement

To examine the alteration of neuronal insulin-mediated phosphorylation of the IR, the IRS-1 and the Akt/PKB following 4, 8 and 12 weeks of two dietary regimens, six brain slices per animal were placed into either aCSF or aCSF plus 500 nM insulin (Humelin R, Eli Lilly, Giessen, Germany) for 5 min. Then, three brain slices in each conditioned group were homogenized in 500 μ l of ice-cold brain slice lysis buffer [1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% Triton X-100 and supplemented with a protease inhibitor cocktail, Roche complete mini-tablets, (Roche Molecular Biochemicals, Indianapolis, IN, USA)]. Next, the homogenates were centrifuged at 9000 g for 30 min at 4 °C and the protein concentration was measured using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). These homogenates were then stored at –80 °C for further biochemical analysis of the tyrosine phosphorylation of IR, IRS-1 and Akt/PKB.

To determine the level of IR, IRS-1 and Akt/PKB protein expression as well as the level of neuronal corticosterone in the brain, another set of three brain slices in aCSF was homogenized over ice in non-ionizing lysis buffer containing: 100 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% Triton X-100, and 1% NP-40 supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals). Then, homogenates were stored at –80 °C for further western blot analysis of IR, IRS-1, Akt/PKB and biochemical analysis of neuronal corticosterone levels.

Immunoprecipitation and immunoblotting

IRS-1 protein and tyrosine phosphorylation of IR and IRS-1 were immunoprecipitated from brain homogenates with polyclonal antibodies against each protein (1 μ g antibody/500 μ g total lysate). Rabbit anti-IR and rabbit anti-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with protein A agarose beads were used to prepare each protein for immunoprecipitation as previously described (Mielke et al., 2005). Then, the proteins were separated by electrophoresis with SDS-Page on 10% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes and immunoblotting was conducted with anti IRS-1 rabbit and phosphotyrosine antibody (rabbit polyclonal, 1:600 in TBST, Santa Cruz Biotechnology) to determine the changes in IRS-1 level and insulin-mediated tyrosine phosphorylation of the IR and IRS-1, respectively.

Akt/PKB in both serine 473 and threonine 308 kinases phosphorylation were electrophoresed and immunoblotted with rabbit antibodies Akt/PKB both serine 473 and threonine 308. Examination of the levels of IR β and Akt/PKB protein was conducted with homogenates prepared from another set of three brain slices. Both proteins were resolved by the immunoprecipitation and immunoblot assay conducted with rabbit anti-IR and rabbit anti-Akt/PKB (1:1000 in TBST, Santa Cruz Biotechnology). For loading control, immunoblotting for each membrane was completed incubation with anti- β -actin (1:400; rabbit polyclonal; Sigma, Missouri, USA).

All membranes for visualizing the phosphorylation and the protein levels of IR, IRS-1 and Akt/PKB were incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (1:8000 in TBST, Bio-Rad Laboratories) and Amersham ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). Band intensities were quantified by Scion Image and the results were shown in average signal intensity (arbitrary) units.

Statistical analysis

Data were presented as means $\pm$ SE. All statistical analyses were performed using the program SPSS (version 16; SPSS, Chicago, IL, USA). The significance of the difference between the means was calculated by Student's *t*-test with $p < 0.05$ for the OGTT test and two-way ANOVA between treatment and time following with post-hoc

analysis for remaining parameters. Pearson's correlation analysis was used to determine the relationship among the plasma parameters, liver triglyceride content, visceral fat, body weight, and neuronal corticosterone in hippocampus and the percentages of insulin-mediated LTD in all animals.

Results

Peripheral insulin resistance developed in rats after 8-week HF feeding

The initial animal body weight was not different among experimental groups. Animals fed with HF for 4 weeks had an increased in body weight compared to rats fed with ND (Table 1). However, the level of glucose, triglyceride, and insulin in plasma and HOMA index did not differ between both dietary groups.

Animals in the 8-week and 12-week HF group had a significant increase in body weight, visceral fat, fasted plasma glucose, plasma insulin, liver triglyceride content and HOMA index compared to those with ND feeding ($p < 0.05$, Table 1). However, plasma triglyceride and free fatty acid level were not significantly different between both dietary groups.

In addition, we found that body weight, visceral fat and plasma glucose level were increased in a time-dependent manner in both dietary groups. However, the HOMA index was increased in a time-dependent manner only in the HF group. The glucose responses during the OGTT in the 12-week HF group was markedly increased at 15-, 30- and 60 min time points, compared to those in the ND group ($p < 0.05$). The total area under the glucose curve (AUC_g) was also significantly increased in the 12-week HF group (26.3 ± 9.3 g/dl $\times$ min vs. 21.2 ± 5.7 g/dl $\times$ min of ND, $p < 0.05$).

Rats fed with the HF diet consumed less food per day than those on the ND ($p < 0.05$). However, when the consumed food weights were converted to caloric intake, rats fed with HF consumed more calories per day than rats fed with ND ($p < 0.01$).

HF feeding for 12 weeks reduced the ability of insulin to induce LTD in hippocampal CA1 circuits

In all time courses (4-, 8- and 12-week) of ND-fed rats, we found that insulin application to hippocampal slices reduced the size of the fEPSP responses at 2–3 min after the start of insulin infusion with maximum effects appearing over the following 10–15 min, and the depression of fEPSPs was prominent and long lasted for 30–40 min (Figs. 1 and 2A). In our set-up, 500 nM insulin added to the infusion line required approximately 1 min to reach the slices.

In HF animals, the degree of insulin-mediated LTD observed from slices of 4-week and 8-week HF animals was not significantly reduced compared to the 4-week and 8-week ND animals ($n = 6$ –8 independent slices per group, $n = 6$ animals/group, Fig. 1). In the 12-week HF-fed group, the amount of insulin-mediated LTD was significantly diminished ($p < 0.05$ vs. ND group, Fig. 2A). At 30-minute post-insulin stimulation, the percentage reduction of the normalized fEPSP slope from 12-week ND was $73.60 \pm 4.18\%$ of the average slope recording during the baseline level ($n = 16$ independent slices, $n = 14$ animals/group), while the percentage reduction of fEPSPs of 12-week HF slices was $9.34 \pm 3.09\%$ of the values recorded before insulin application ($n = 17$ independent slices, $n = 13$ animals/group) (Fig. 2A). Furthermore, we found that the average degree of insulin-mediated LTD at all time courses of both dietary groups was significantly correlated with several peripheral insulin resistance parameters ($p < 0.01$; as shown in Table 2).

We found no significant difference in the pair-pulsed facilitation (PPF), which is a ratio of fEPSP slope₂/fEPSP slope₁, of slices recorded at the baseline between both dietary groups at all time courses ($p > 0.05$). To confirm whether the impairment of the insulin-mediated LTD in 12-week HF group could occur without the

Table 1
Effect of 4-, 8- and 12-week HF diet feeding on body weight, visceral fat, fasted plasma glucose, triglyceride, free fatty acid, insulin, liver triglyceride and HOMA index. *, $p < 0.05$ compared to ND group in each time course; ^a, $p < 0.05$ compared to 4-week group of the same dietary regimen; ^b, $p < 0.05$ compared to 8-week group of the same dietary regimen.

Parameters	4 weeks		8 weeks		12 weeks	
	ND	HF	ND	HF	ND	HF
Body weight (g)	354.62 ± 5.35	394.60 ± 6.82 *	427.50 ± 5.93 ^a	501.67 ± 7.31 ^{a,a}	460.18 ± 8.37 ^{a,b}	546.25 ± 8.32 ^{a,a,b}
Visceral fat (g)	16.67 ± 1.21	31.00 ± 3.97 *	17.68 ± 2.94	47.02 ± 5.43 ^{a,a}	25.19 ± 1.96 ^a	45.47 ± 2.01 ^{a,a}
Glucose (mg/dl)	130.88 ± 3.49	123.13 ± 3.10	130.03 ± 4.21	150.03 ± 5.70 ^{a,a}	149.41 ± 4.39 ^a	153.42 ± 2.95 ^a
Triglyceride (mg%)	87.44 ± 4.75	72.33 ± 7.43	72.28 ± 3.94	65.70 ± 4.24	84.89 ± 6.85	76.84 ± 5.48
Free fatty acid (mM)	0.63 ± 0.06	0.53 ± 0.06	0.66 ± 0.07	0.54 ± 0.09	0.83 ± 0.13	0.77 ± 0.08
Insulin (ng/ml)	2.42 ± 0.53	2.79 ± 0.34	2.02 ± 0.35	3.73 ± 0.73 *	2.14 ± 0.54	4.23 ± 0.59 *
Liver triglyceride (mg/g tissue)	21.15 ± 2.21	170.52 ± 19.00 *	18.51 ± 1.94	171.10 ± 27.11 *	17.50 ± 1.50	173.40 ± 6.99 *
HOMA index	17.15 ± 4.51	17.10 ± 3.72	16.35 ± 3.56	31.42 ± 7.10 ^{a,a}	20.05 ± 5.57	39.69 ± 5.69 ^{a,a}

interruption of other forms of plasticity, the experiment of carbachol-induced LTD was performed. We found that the degree of carbachol-induced LTD in CA1 hippocampus of both 12-week HF and ND groups was similar (Fig. 2B).

Depressed phosphorylation of insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and Akt/PKB levels in brain slices after 12-week HF consumption

In order to compare neuronal insulin receptor signaling within 4-, 8- and 12-week time courses between HF and ND rats, we investigated whether the protein levels of IR, IRS-1 and Akt/PKB were down-regulated in relation to the reduction of the ability of insulin-mediated LTD in CA1 hippocampus. We found that the levels of the IR, IRS-1 and Akt/PKB from 4-, 8- and 12-week HF brain slices were not significantly different from 4-, 8- and 12-week ND brains (Fig. 3, $n = 4-8$ animals/group).

Interestingly, the IRS-1 protein levels in the 12-week HF group revealed a 20% reduction from those slices observed in the 12-week ND group, however, it was not significantly different ($p = 0.1$, $n = 8$ /group, Fig. 3B). Although, there is no significant difference in the expression of IRS-1 protein in both dietary groups at each time-course, we found that the longer time of high-fat diet consumption leads to the reduction of IRS-1 expression ($p < 0.05$, Fig. 3B).

The phosphorylation status of IR, IRS-1 and Akt/PKB in the acutely prepared brain slices under basal condition following the insulin stimulation is illustrated in Fig. 4. The basal (non-insulin stimulated) phosphorylation levels of IR and IRS-1 from the 4-, 8- and 12-week ND and from the 4-, 8- and 12-week HF groups were weakly variable detected by immunoprecipitation and phosphotyrosine immunoblotting. The possible explanation of the variability of basal phosphorylation levels is that the different endogenous insulin levels in the

brainslices, could stimulate the phosphorylation of IR, IRS-1 and Akt/PKB, even though there was no exogenous insulin application onto the brainslices. However, insulin stimulation resulted in the strong observable phosphorylation of IR, IRS-1 and Akt/PKB levels in 4-, 8- and 12-week ND and 12-week HF groups (Fig. 4A–D). In 4-, 8- and 12-week ND-fed rats, the exposure to insulin stimulation resulted in an increase in IR, IRS-1 and Akt/PKB phosphorylation, compared to their own basal condition. Interestingly, at 12-week feeding, the insulin-stimulated IR phosphorylation was significantly lower in the HF group (0.96 ± 0.03) than in the ND group (1.28 ± 0.06), accounting for ~25% reduction ($p < 0.01$, $n = 11$ /group, Fig. 4A). We also found that the reduction of the insulin-stimulated IR phosphorylation in only high-fat diet consumption group was a time-dependent manner ($p < 0.05$, Fig. 4A). Similarly, the insulin-stimulated IRS-1 phosphorylation was significantly lower in the 12-week HF group (1.02 ± 0.04) than in the 12-week ND group (1.20 ± 0.03), accounting for ~15% reduction ($p < 0.01$, Fig. 4B). From the brain slices of the 12-week HF group, Akt/PKB phosphorylation of serine 473 was significantly decreased, compared to that in the ND group ($p < 0.05$, Fig. 4C), and the Akt/PKB phosphorylation of threonine 308 was slightly decreased but did not reach a statistical significance ($p = 0.05$, Fig. 4D).

Increased plasma and neuronal corticosterone levels following HF consumption

Lin and colleagues have shown that increased plasma corticosterone level could induce peripheral insulin resistance (Lin et al., 2000; Stranahan et al., 2008a,b). In the present study, we also found that the levels of plasma corticosterone in HF-fed rats were significantly higher than those in the ND rats starting from 4-week HF consumption and remained higher throughout the course of the study ($p < 0.05$, Fig. 5A). The increased level of plasma corticosterone in these HF-fed

Table 2
Correlation among peripheral insulin resistance parameters, % change of fEPSP slope in response to 500 nM insulin and neuronal corticosterone in rats that received normal diet or high-fat diet for 4, 8 and 12 weeks. *, correlation is significant at the 0.05 level; **, correlation is significant at the 0.01 level.

Parameters	% change of fEPSP slope in response to 500 nM insulin	Body weight (g)	Visceral fat (g)	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)	Liver triglyceride content (mg/g tissue)	HOMA index	Plasma corticosterone (ng/ml)	Neuronal corticosterone (ng/mg protein)
% change of fEPSP slope in response to 500 nM insulin	1	−0.808**	−0.622**	0.553**	−0.440**	−0.593	−0.634	−0.746**	−0.652**
Body weight (g)	−0.808**	1	0.775**	0.581**	0.532**	0.677**	0.684**	0.759**	0.452*
Visceral Fat (g)	−0.622**	0.775**	1	0.553**	0.470**	0.791**	0.626**	0.725**	0.497**
Plasma glucose (mg/dl)	−0.500**	0.581**	0.553**	1	0.201	0.412*	0.600**	0.571**	0.363
Plasma insulin (ng/ml)	−0.440**	0.532**	0.470**	0.201	1	0.608**	0.589**	0.414*	0.475*
Liver triglyceride content (mg/g tissue)	−0.593**	0.677**	0.791**	0.412*	0.608**	1	0.679**	0.697**	0.470*
HOMA index	−0.634**	0.684**	0.626**	0.600**	0.589**	0.679**	1	0.585**	0.547**
Plasma corticosterone (ng/ml)	−0.746**	0.759**	0.725**	0.571**	0.414**	0.697**	0.585**	1	0.387
Neuronal corticosterone (ng/mg protein)	−0.652**	0.452**	0.497**	0.363	0.475*	0.471*	0.547**	0.387	1

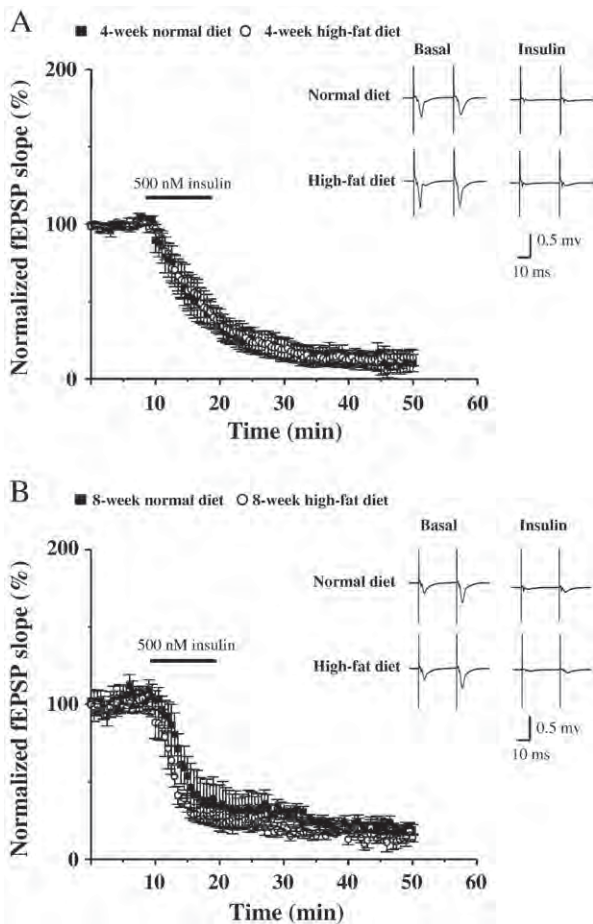


Fig. 1. Four- and eight-week HF diet feeding had no effects on the ability of insulin-mediated long-term depression (LTD) on fEPSPs in hippocampal CA1 regions. Panels A and B represent responses before and after insulin stimulation in brain slices from ND and HF groups. *Panel A:* Average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from 4-week-ND-fed ($n = 6$ –8 independent slices per 6 animals) and 4-week-HF-fed ($n = 6$ –8 independent slices per 6 animals) brain slices. *Panel B:* Average normalized fEPSPs from 8-week-ND-fed ($n = 6$ –8 independent slices per 6 animals) and 8-week-HF-fed ($n = 6$ –8 independent slices per 6 animals) brain slices. Both 4-week and 8-week HF diets had no effect on the ability of insulin to depress fEPSPs. Examples of averages of 20 consecutive traces taken from a slice treated with aCSF (basal) and with 500 nM (insulin) are shown in the insets of Panels A and B.

rats was in a time-dependent manner. Unlike plasma corticosterone, the level of neuronal corticosterone was significantly greater than that in the ND group only in the 12-week HF-fed rats (Fig. 5B, $p < 0.01$). Our results also demonstrated that the decrease in neuronal insulin receptor function was correlated well among an increase of all parameters of peripheral insulin resistance, plasma corticosterone and neuronal corticosterone (Table 2).

Discussion

The major findings in the present study are that in the HF fed rats, 1) elevated plasma corticosterone level was observed after 4-week HF feeding; 2) peripheral insulin resistance was developed after 8-week HF feeding; and 3) increased neuronal corticosterone level and the occurrence of neuronal insulin resistance were observed after 12-week HF feeding.

Earlier works found that HF diet causes insulin resistance characterized by hyperinsulinemia, hyperlipidemia, elevated corticosteroids and decreased insulin sensitivity (Hummel et al., 1966; Manco et al., 2004; Riccardi et al., 2004). In both basic and clinical studies, it has been shown that the development of insulin resistance is associated with HF consumption and is linked to cognitive deficits

(Greenwood and Winocur, 2005; Riccardi et al., 2004; Winocur and Greenwood, 2005). While considerable research has examined both the consequences and mechanisms of a diminished insulin response in various peripheral tissues, only a few studies have investigated the effects of this metabolic disruption within the CNS, particularly the metabolic disturbance following the consumption of HF diets. In addition, although much evidence suggests that neuronal insulin signaling might play a role in neuronal plasticity (Park et al., 2001; Wickelgren, 1998; Zhao and Alkon, 2001), the number of available reports in an area of HF diet consumption and neuronal insulin resistance is still limited.

Growing evidence demonstrates the influence of time-course effects on the learning and memory processes caused by HF diet. Previous studies demonstrated that rats fed with HF diet for 3 months had cognitive impairment (Greenwood and Winocur, 1996), while rats fed with HF diet for 8 months exhibited impaired learning ability and reduced hippocampal synaptic plasticity (Stranahan et al., 2008a, b). Despite these reports, the neurofunctional insulin resistance in hippocampus in different time-course of HF consumption has not

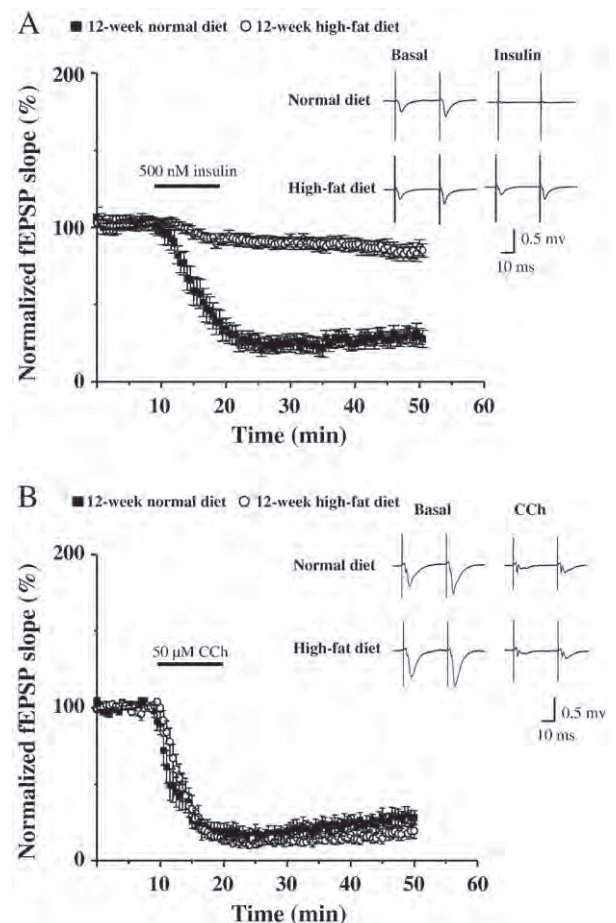


Fig. 2. *Panel A:* Twelve-week-HF diet feeding significantly diminished the ability of insulin-mediated long term depression (LTD) in CA1 hippocampus. A summary of averages of 16–17 experiments ($n = 16$ –17 independent slices, $n = 13$ –14 animals/group) shows that bath application of 500 nM insulin for 10 min produced a depression of fEPSPs in 12-week-ND brain slices and the fEPSPs did not fully recover after washout of insulin. However, 500 nM insulin-mediated LTD was significantly attenuated by 12-week-HF diets. *Panel B:* Average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from 12-week-ND-fed ($n = 17$ independent slices, $n = 14$ animals/group) and 12-week-HF-fed ($n = 16$ independent slices, $n = 13$ animals/group) brain slices. Representative traces were indicated for 12-week ND group and 12-week HF group (in upper panel). Carbachol-induced LTD (mLTD) recorded from CA1 hippocampal slices harvested from 12-week ND and 12-week HF diet, suggested no difference in mLTD occurring during the neuronal insulin resistance. CCh, carbachol.

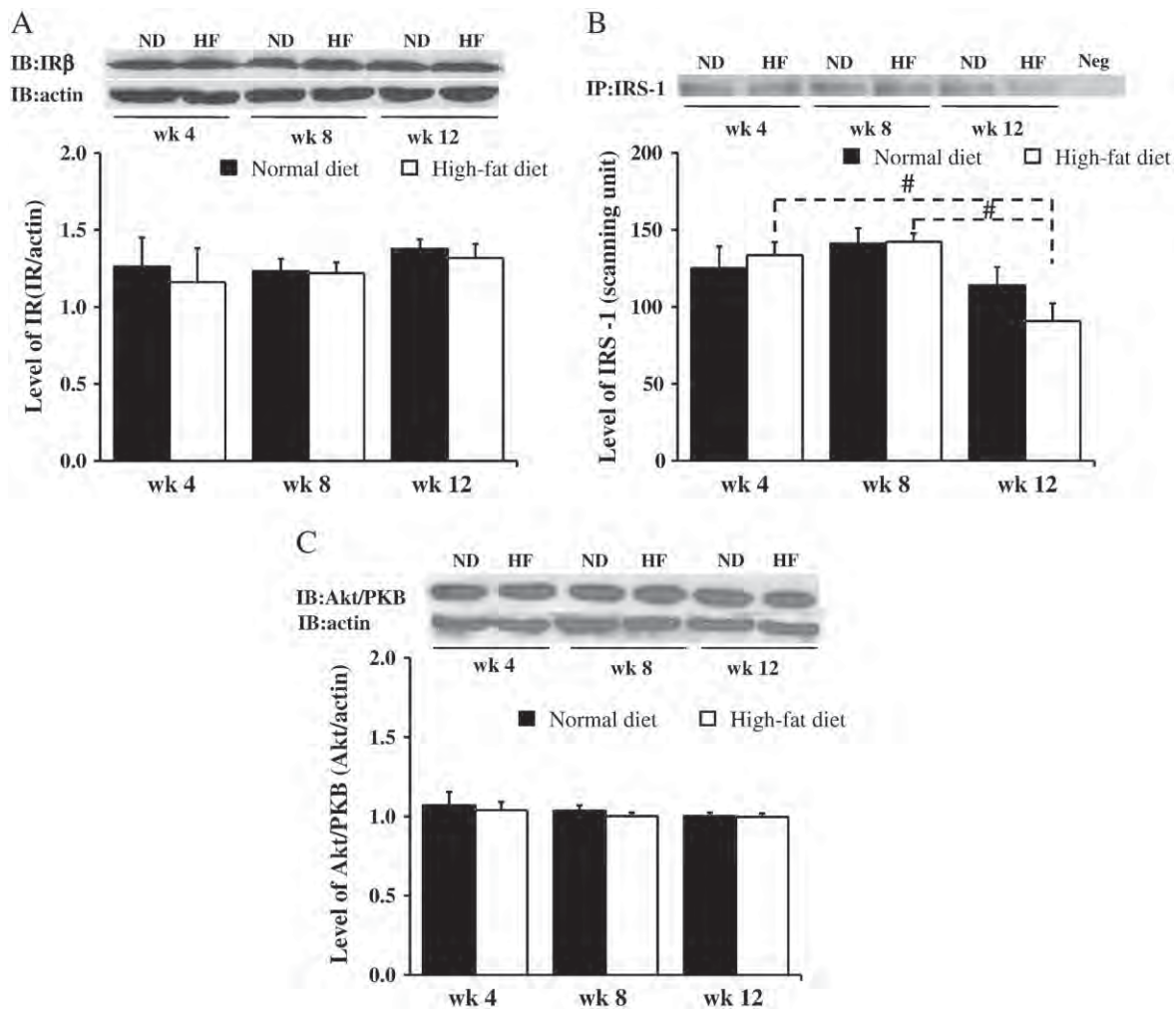


Fig. 3. The protein levels of IR, IRS-1 and Akt/PKB were not changed following 4-, 8- and 12- week HF diet feeding. *Panels A–C:* Representative immunoblots indicate that the total cellular amount of the IR (A), IRS-1 (B) and Akt/PKB (C) proteins were unchanged in the 4-, 8- and 12-week HF rat brain slices compared to the 4-, 8- and 12-week ND slices, and the densitometric quantitation of blots from both groups was not different. All immunoblotting lanes were loaded with equal amounts of protein (40 μ g/lane). #, $p < 0.05$ compared to 12-week group of the same dietary regimen. ND, normal diet fed group; HF, high-fat fed group; Negative (Neg), no proteins had been loaded.

been investigated. To determine the existence of neurofunctional insulin resistance in the brain following HF diet consumption, we determined the efficacy of the neurofunctional insulin receptor characterized as insulin-mediated LTD in CA1 hippocampus and the stimulated phosphorylation status of IR, IRS-1 and Akt/PKB in brain slices harvested from control and HF fed rats. We demonstrated a significant reduction of the insulin-mediated LTD in CA1 hippocampal slices and the insulin-mediated phosphorylation of IR, IRS-1 and Akt/PKB in 12-week HF-fed rats. Our findings suggest that the consumption of HF diet for only 12 weeks can down-regulate the neuronal insulin receptor sensitivity as well as the peripheral insulin sensitivity. A previous study on hamsters with peripheral insulin resistance resulting from a 6-week high-fructose diet has shown a reduction of IR, IRS-1 and Akt/PKB phosphorylation in the brain and insulin-mediated LTD in the hippocampus (Mielke et al., 2005). Our study demonstrates that the development of neuronal insulin resistance after HF consumption required a longer time than that after fructose consumption.

Insulin has been shown to regulate the endocytosis of AMPA receptors through calcium-dependent clathrin mediated internalization of AMPA receptors at the postsynaptic sites, which causes the depression of excitatory synaptic transmission (Ahmadian et al., 2004; Beattie et al., 2000; Huang et al., 2004; Lin et al., 2000; Man et al., 2000). In the present study, a significant reduction of insulin-mediated LTD in hippocampal slices from the 12-week HF group was

observed. The reduction of insulin-mediated LTD was well correlated with other peripheral insulin resistance such as an increase in visceral fat, weight, plasma glucose, insulin level and liver triglyceride content. These results suggest that changes in metabolic system are linked to the neuronal function. The weakening of insulin-mediated LTD indicated one of the functional consequences of impaired neuronal insulin signaling. The reduced LTD induction could be due to the significant decrease in insulin-induced phosphorylation of the insulin receptor signaling: IR, IRS-1 and Akt/PKB in brain slices of the 12-week HF group as seen in the present study (Fig. 4). Previous studies demonstrated that the phosphorylation of Akt/PKB contributed to an increase in the level of intracellular calcium (Sun et al., 2006; Viard et al., 2004; Worrall and Olefsky, 2002). Thus, the reduction in Akt/PKB phosphorylation of neuronal IR found in the present study could lead to the decreased intracellular calcium and contributes to impair calcium dependent clathrin-mediated internalization of AMPARs, resulting in the decline of insulin-induced LTD in the 12-week HF feeding rats.

Although it is possible that the diminished insulin-mediated LTD in the HF group could be due to changes in synaptic transmission in CA1 hippocampus caused by HF, the unaltered PPF hippocampal CA1 regions in both dietary treatments suggested that HF feeding did not affect presynaptic responses to electrical stimulation. Therefore, it may be concluded that 12 weeks of HF feeding only significantly affects the neuronal insulin receptor signaling function at the post-

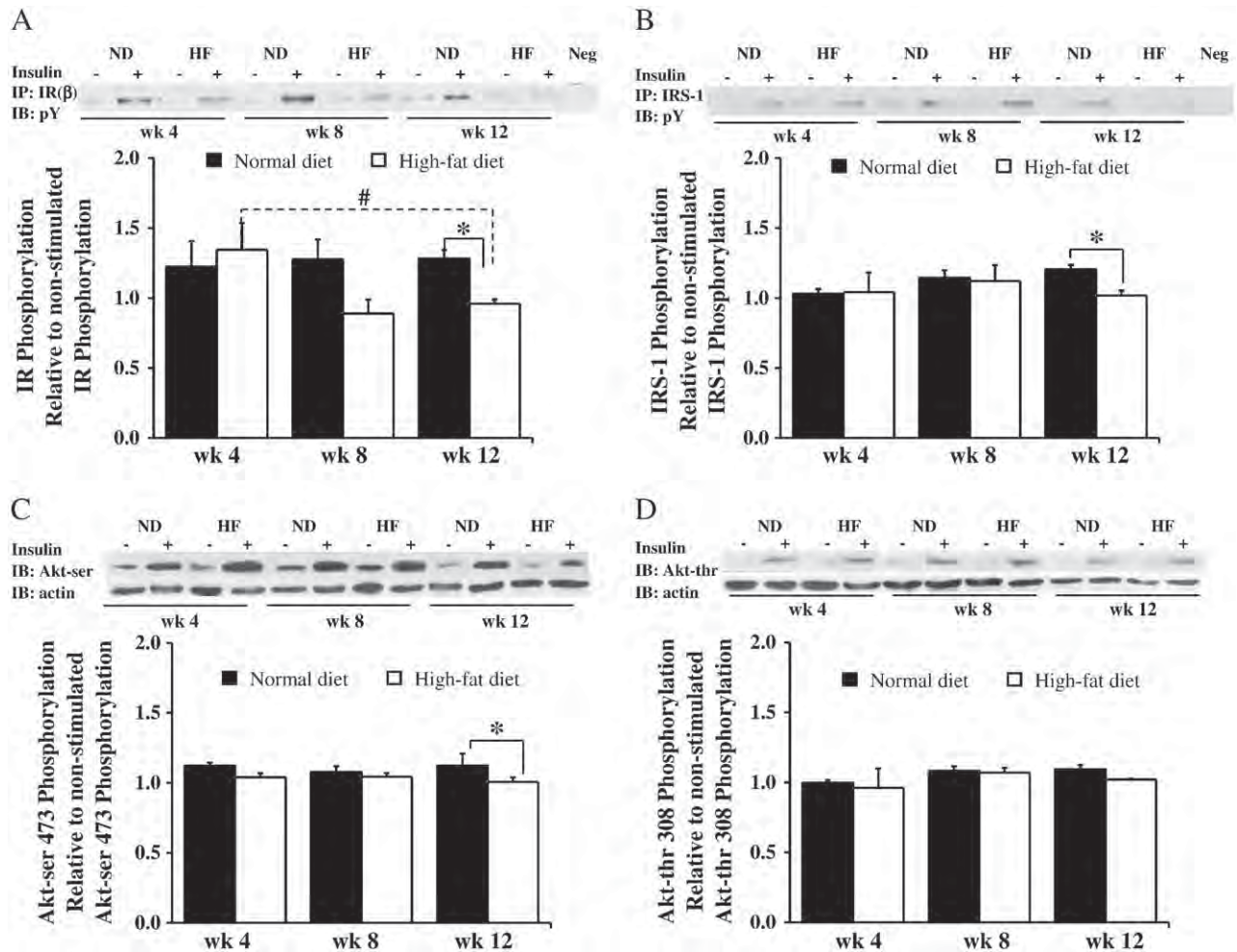


Fig. 4. Insulin-induced tyrosine phosphorylation of both neuronal insulin receptor β subunit (IR), the substrate protein IRS-1 and Akt/PKB was weakened in the 12-week HF-fed group. *Panels A–D:* Representative blots of tyrosine phosphorylation illustrated a marked decrease in the ability of insulin to stimulate IR (A), IRS-1 (B), serine 473 kinase of Akt/PKB (C) and threonine 308 kinase of Akt/PKB (D) phosphorylation in brain slices harvested from the 4-, 8- and 12-week HF-fed group compared to the 4-, 8- and 12-week ND-fed group. Densitometric quantitation of blots from insulin stimulated IR, IRS-1 and Akt/PKB was significantly greater in the 12-week ND-fed group than that in the 12-week HF-fed group. All immunoblotting lanes were loaded with equal amounts of protein (20 μ g/lane). *, $p < 0.05$ compared to ND group in each time course; #, $p < 0.05$ compared to 12-week group of the same dietary regimen; ND, normal diet fed group; HF, high-fat fed group; Negative (Neg), no proteins had been loaded; -, no insulin stimulation; +, insulin stimulation.

synaptic sites, but not at the pre-synaptic release in CA1 hippocampus. Carbachol-induced LTD or muscarinic LTD (mLTD) in CA1 hippocampus, characterized as the other form of synaptic plasticity (Scheiderer et al., 2006), is believed to be a substrate of learning and memory at the molecular level (Scheiderer et al., 2006). We demonstrated in this study that the reduction of neuronal insulin response in the 12-week HF group was unaffected in mLTD, suggesting that this form of synaptic plasticity may not require neuronal insulin signaling. Our finding was similar to previous reports, showing that the weakening of insulin-mediated LTD has no effect upon the induction and maintenance of the LTP via a high-frequency stimulation (Mielke et al., 2005, 2006), but was different from the other previous study (Stranahan et al., 2008a,b). In that study, Stranahan and colleague demonstrated that rats fed with high-fat, high glucose for 8 months reduced LTP at schaffer-CA1 synapse (Stranahan et al., 2008a,b). The differences in the results between Stranahan study and our study may depend upon the differences in dietary regimen and time-course of HF consumption.

In the present study, the phosphorylation of IR, IRS-1 and Akt/PKB in brain slices was diminished at 12 weeks, whereas the levels of IR, IRS-1 and Akt/PKB in brain slices were not altered by 4-, 8- and 12-week HF diet feeding (see Fig. 4). These changes in phosphorylation of neuronal insulin signaling confirm the impairment of insulin receptor function in the brain following 12-week HF feeding.

The unchanged levels of IR, IRS-1 and Akt/PKB proteins in the brain following HF diet consumption in the present study are consistent with those previously reported in the liver (Hahn-Obercyger et al., 2009), in the skeletal muscle (Youngren et al., 2001) and in the hippocampus (Banas et al., 2009). Furthermore, the impairment of IR, IRS-1 and Akt/PKB protein kinase activity, has been demonstrated in the skeletal muscle (Barnard et al., 1992), fat (Boyd et al., 1990) and liver tissues (Watarai et al., 1988) in 10–12 week consumption of HF diets. All of these findings indicate that HF diet could cause defective neuronal insulin receptor function, but not at the level of protein expression.

In addition to the weakening of neurofunctional insulin receptors and neuronal insulin signaling, our results demonstrated that the level of plasma corticosterone was increased prior to the development of peripheral insulin resistance in HF fed rats. It had been previously described that corticosterone influences insulin sensitivity and that enhancement of peripheral corticosterone could contribute to the development of peripheral insulin resistance (Reynolds and Walker, 2003). Furthermore, it is known that the basal level of corticosteroids physiologically protects neuronal damage, whereas the stress or high level of corticosteroids can contribute to neuronal damage (McEwen, 2007). Several studies demonstrated that chronic exposure to high level of corticosteroids can produce neuronal stress and neuronal loss in CA3 pyramidal neurons of rodents (Sapolsky et al., 1985) and

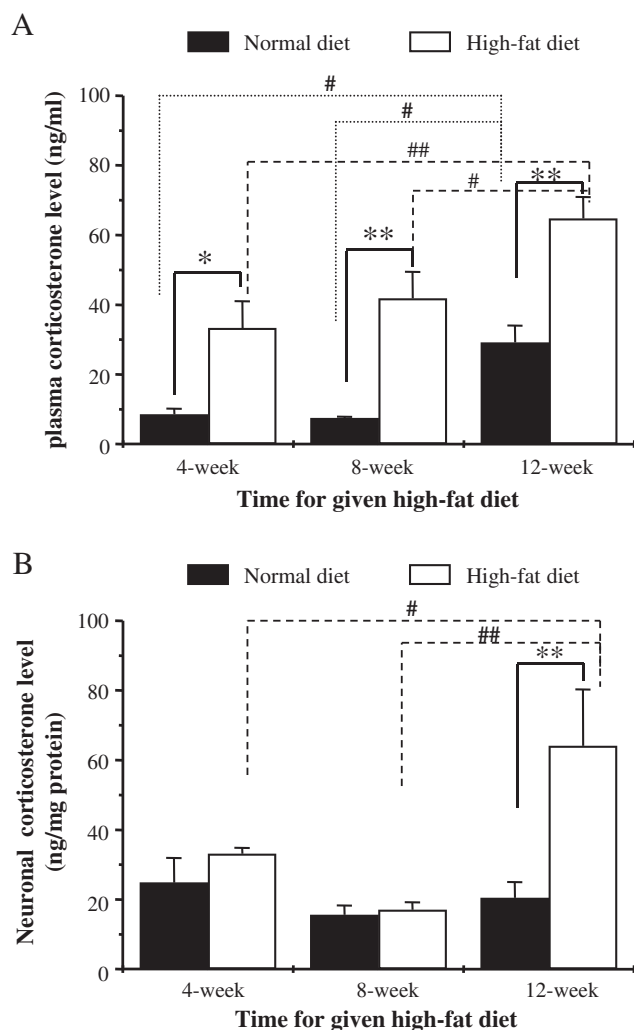


Fig. 5. Increased plasma and neuronal corticosterone levels following HF diet feeding. *Panel A:* Plasma corticosterone level following 4-, 8- and 12-week feeding of both ND and HF diet. *Panel B:* Neuronal corticosterone level following 4-, 8- and 12-week feeding of both ND and HF diet. *, $p < 0.05$ compared to ND group in each time course; **, $p < 0.01$ compared to ND group in each time course; #, $p < 0.05$ compared to 12-week group of the same dietary regimen; ##, $p < 0.01$ compared to 12-week group of the same dietary regimen.

primates (Sapolsky et al., 1990; Uno et al., 1989). In addition, a previous study demonstrated that corticosterone could induce insulin resistance in the skeletal muscles (Zhao et al., 2009). Thus, the elevation of neuronal corticosterone level found in our study could also promote neuronal insulin resistance. Our findings suggest that obesity following HF diet consumption could lead to neuronal insulin resistance and neuronal stress as shown by an increased release of neuronal corticosterone (Stranahan et al., 2008a,b).

Study limitation

The limitation of this study is that the morphological data on the integrity of dendritic spines and terminals in the CA1 region of the hippocampus were not investigated. However, the present study demonstrated that the amount of insulin receptor was not different between the high-fat fed and normal diet fed rats, suggesting that the reduction of insulin-induced LTD in high-fat fed rats was due to the decreased function of insulin signaling, as supported by the decreased phosphorylated IR, IRS and Akt/PKB levels in the high-fat fed rats.

Conclusion

The present study demonstrates that a significant modification of important neuronal insulin receptor signaling can be induced by a fat-enriched diet. Fed for 12 weeks, the HF diet clearly induces neuronal insulin resistance, which is identified as a significant reduction in the ability of insulin to induce LTD, and a reduction in the stimulated phosphotyrosine activity of IR, IRS-1 and Akt/PKB in brain slices. Twelve-week HF feeding not only causes neuronal insulin resistance, but also leads to neuronal stress, as indicated by increased neuronal corticosterone level. Since the defective insulin receptor signaling has been shown to associate with the pathogenesis of Alzheimer's disease (Watson and Craft, 2004), cognitive impairment (Greenwood and Winocur, 1990, 1996, 2005; Winocur and Greenwood, 2005) and the presence of cognitive impairment in patients with type II diabetes (Gispén and Biessels, 2000), the neuronal insulin resistance developing after 12-week HF consumption could be responsible for the impairment of cognition through the glucocorticoid-mediated effect in this animal model.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Review

Roles of Mitochondrial Benzodiazepine Receptor in the Heart

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*Cardiac Electrophysiology Unit, Department of Physiology, Cardiac Electrophysiology Research and Training Center, Faculty of Medicine and Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand***ABSTRACT**

Mitochondrial benzodiazepine receptor (mBzR) is a type of peripheral benzodiazepine receptor that is located in the outer membrane of mitochondria. It is an 18-kDa protein that can form a multimeric complex with voltage-dependent anion channel (32 kDa) and adenine nucleotide translocator (30 kDa). mBzR is found in various species and abundantly distributed in peripheral tissues, including the cardiovascular system. The mitochondria are well known as the site of energy production, and the heart is the organ that highly requires this energy supply. In the past decades, it has been shown that mBzR plays a critical role in regulating mitochondrial and heart functions. A growing body of evidence demonstrates that mBzR is associated with regulation of mitochondrial respiration, mitochondrial membrane potential, apoptosis, and reactive oxygen species production. Moreover, mBzR has been suggested to play a role in alteration of physiological effects in the heart such as contractility and heart rate. mBzR is involved in the pathologic condition such as ischemia/reperfusion injury, responses to stress, and changes in electrophysiological properties and arrhythmogenesis. In this review, evidence of the roles of mBzR in the heart under both physiological and pathologic conditions is presented. Clinical studies regarding the use of pharmacologic intervention involving mBzR in the heart are also discussed as a possible target for the treatment of electrical and mechanical dysfunction in the heart.

RÉSUMÉ

Le récepteur mitochondrial des benzodiazépines (mBzR) est un type de récepteur périphérique des benzodiazépines situé dans la membrane externe de la mitochondrie. C'est une protéine 18 kDa qui peut former un complexe multimérique avec le canal anionique voltage-dépendant (32 kDa) et le translocateur des nucléotides adényliques (30 kDa). Le mBzR se retrouve dans différentes espèces et est abondamment distribué dans les tissus périphériques, incluant le système cardiovasculaire. Les mitochondries sont bien connues comme étant le site de production d'énergie, et le cœur est l'organe qui requiert le plus grand approvisionnement énergétique. Au cours des dernières décennies, il a été démontré que le mBzR joue un rôle critique dans la régulation mitochondriale et les fonctions du cœur. Un nombre croissant de preuves démontrent que le mBzR est associé à la régulation de la respiration mitochondriale, au potentiel de la membrane mitochondriale, à l'apoptose et à la production d'espèces réactives de l'oxygène. De plus, le mBzR semble jouer un rôle dans les modifications physiologiques du cœur comme la contractilité et la fréquence cardiaque. Le mBzR est impliqué dans des pathologies tels les lésions d'ischémie-reperfusion, les réponses au stress, et les changements dans les propriétés électrophysiologiques et l'arythmogénèse. Dans cette revue, les rôles du mBzR sur le cœur sont mis en évidence tant dans des conditions physiologiques que pathologiques. Des études cliniques au sujet de l'utilisation d'interventions pharmacologiques impliquant le mBzR pour le cœur sont aussi discutées comme un objectif possible dans le traitement des dysfonctions électriques et mécaniques du cœur.

Peripheral benzodiazepine receptor (PBR) is a receptor found in peripheral tissues such as adrenal, kidney, and brain in various species and is found in abundance in cardiovascular system.^{1–3} Initially discovered in rat peripheral tissues by Braestrup and Squires during 1970s⁴ as benzodiazepine binding site, it is distinct from the central benzodiazepine receptors, which are localized solely to neuronal cells in the central ner-

vous system. The majority of PBRs are located in the mitochondrial outer membrane and are normally recognized as mitochondrial benzodiazepine receptors (mBzRs).^{5,6} However, several studies demonstrated that PBRs are also located outside the mitochondria, such as in the nuclear fractions⁷ and plasma membrane⁸ of various cell types). PBR is an 18-kDa protein consisting of 169 amino acids⁹ that can form a protein complex with voltage-dependent anion channel (VDAC; 32 kDa) at the outer membrane and adenine nucleotide translocator (ANT; 30 kDa) at the inner membrane.⁶ Recently, PBR has been renamed “translocator protein 18 kDa” (TSPO) by a group of scientists to represent a specific structure and molecular function of PBR.¹⁰ The name indicates the location of proteins:

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See page 262.e11 for disclosure information.

mitochondrial translocator protein (mitoTSPO) and nuclear translocator protein (nucTSPO). To date, "PBR" is also widely accepted among the scientific community.

A growing body of evidence indicates that mBzR could play vital regulatory and protective roles for cellular and organ functions in various organs.¹¹ These roles include cardioprotective effects in the heart, steroidogenesis in the adrenal glands, astrocyte and microglia activation in the brain, and heme biosynthesis in the liver.¹²⁻¹⁵ Under cardiac ischemia/reperfusion (I/R) condition, it has been reported that mBzR plays an important role in the regulation of cardiac cell under this pathologic condition. The abnormality at the level of organelle such as mitochondria in this condition can tremendously affect cardiac electrophysiology as well as an excitation-contraction coupling process in cardiomyocytes.

In this review, the roles of mBzR in the heart under physiological conditions and during I/R injury are mainly discussed, with a focus on its effects on cardioprotection, electrophysiological alterations, reactive oxygen species (ROS) production, and the undesired effect caused by mBzR stimulation in the heart during I/R injury. The disputed findings regarding the effects of mBzR ligands on cardiac electrophysiology under a variety of stresses as well as several clinical studies available at this time are also presented with the hope that they will encourage further studies to help elucidate the mechanism of mBzR in the heart and to warrant its clinical usefulness as a potential therapeutic target for the treatment of ischemic heart disease in the near future.

Cardiac Mitochondrial Benzodiazepine Receptor

The mitoTSPO, or mBzR, is located on the mitochondrial outer membrane of a cardiac cell and has a structure similar to that found in the other peripheral tissues. mBzR is a 18-kDa protein that can form a multimeric complex with VDAC and ANT. mBzR itself has minimal function in the interaction with some ligands, but the forming of mBzR and other proteins has been shown to drive drug-binding properties and functions. In the heart, mBzR has been shown to be involved in many critical cellular controls, including apoptotic cell death, mitochondrial membrane potential ($\Delta\Psi_m$) changes, mitochondrial permeability, mitochondrial respiration, and intracellular calcium overload and responses to stress.¹² Endogenous ligands such as protophophyrin IX, diazepam binding inhibitor, triakontatetrapeptide, and phospholipase A₂ have been shown to affect mBzR in these processes.¹² In the past decades, a number of exogenous ligands have been synthesized and were specific to mBzR. These include PK11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide], 4'-chlorodiazepam (4'-Cl-DZP) or Ro5-4864 [7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one], SSR180575 (7-chloro-*N,N*,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4*H*-pyridazino[4,5-*b*]indole-1-acetamide), FGIN-1-27 [*N,N*-di-*n*-hexyl 2-(4-fluorophenyl)indole-3-acetamide], pyrrolo[2,1-*d*]^[1,5] benzodiazepine derivative, and AHN086 [1-(2-isothiocyanatoethyl)-7-chloro-1,3-dihydro-5-(4-chloro-phenyl)-2*H*-1 and 4-benzodiazepine-2-one HCl]. These ligands have been extensively investigated for their cellular effects on mBzR in the heart.^{12,16,17}

In thyroidectomized Holtzman adult male rats, it has been shown that the mBzR binding sites with exogenous ligand (4'-Cl-DZP) from cardiac ventricular homogenate were decreased,

whereas its affinity was increased.¹⁸ However, in the heart of male mice after copulation, cardiac mBzR binding sites were increased and its affinity was not altered.¹⁹ Stress has been shown to influence mBzR binding affinity in the heart. In cardiac membrane of rats, 2 and 4 hours after an 80-minute session of inescapable tail shock-induced experiment, stress has been shown to reduce mBzR levels.²⁰ In mouse ventricles, the density of mBzR was upregulated after an acute stress but was downregulated after a repeated stressor.^{21,22} In a study using stress induced by 6 and 12 hours of noise exposure, mBzR binding sites were significantly decreased in the right atrial "crude membranes" and "mitochondrial fractions."²³ This change was related to the duration of noise stress without any change in affinity. However, in this stress-induced condition, mBzR reduction could be prevented by the administration of mBzR ligands PK11195, 4'-Cl-DZP, and diazepam.²³

In summary, mBzR levels could be increased after acute stress, whereas they were downregulated after prolonged stimulation. However, under myocardial I/R injury in rats, cardiac mBzR binding sites and affinity were not altered.²⁴ The proposed mechanisms of mBzR density and affinity alterations in the heart have been due mainly to hormonal and stress conditions.²⁵ However, the existence and the role of mBzR in the cardiac innervations remain unknown and require further investigation. The conditioning effects on mBzR levels and affinity are summarized in Table 1.

Physiological Effects of Mitochondrial Benzodiazepine Receptor Activation in the Heart

A number of studies have demonstrated that mBzR has strong physiological effects on the heart.^{12,26-36} Specific synthetic mBzR ligands such as benzodiazepines have been shown to affect cardiac chronotropy and inotropy.²⁷⁻³⁶ A possible interaction between mBzR and voltage-gated Ca²⁺ channel has been reported and has been proposed to be responsible for the alteration in cardiac action potential duration (APD) and contractility.²⁷ However, a definite mechanism of mBzR activation on the physiological effects in the heart remains controversial because discrepancies in findings still largely exist.

Negative inotropic effect was observed with the use of mBzR ligands (ie, 4'-Cl-DZP) in various models including papillary muscle from right and left ventricles of guinea pigs, isolated perfused rats and rabbit hearts, and isolated canine right atrium.²⁷⁻³² Despite these findings, Weissman et al.³⁵ and Leeuwijn et al.³³ reported otherwise. The former study demonstrated that 4'-Cl-DZP augmented the increase in inotropic response that is initially induced by BAY K8644, a Ca²⁺ channel activator, by 2-fold, whereas it did not have an inotropic effect by itself.³⁵ The latter study found that 4'-Cl-DZP had a dose-dependent positive inotropic effect and increased coronary blood flow in the isolated perfused rat hearts.³³ These responses were abolished in the presence of PK11195, the mBzR ligand.³³ However, Grupp et al. demonstrated that 4'-Cl-DZP had no effects on inotropic response but induced a dose-dependent increase of coronary flow rate in the isolated perfused rat heart.³⁴ These inconsistent findings, either positive, negative, or unchanged effects on inotropic response of mBzR ligands, have not been elucidated. It could be due to the differences in 4'-Cl-DZP doses, experimental models, and species used in those studies. This effect is summarized in Table 2.

Table 1. Conditioning effects on mBzR levels and affinity

Conditions	Models	mBzR ligands	mBzR levels	mBzR affinity	Reference
Thyroidectomized	Cardiac ventricular homogenate of Holtzman adult male rats	[³ H]Ro5-4864	↓	↑	Kragie and Smiehorowski ¹⁸
Copulation	Male mice hearts	[³ H]Ro5-4864	↑	↔	Saano et al. ¹⁹
Inescapable tail shock	Rat cardiac membrane	[³ H]Ro5-4864	↓	—	Drugan et al. ²⁰
Inescapable tail shock	Mouse cardiac ventricles	[³ H]Ro5-4864	↑/↓	↔	Drugan et al. ²¹
Acute maximum extra shock	Mouse cardiac ventricles	[³ H]Ro5-4864	↑	↔	Basile et al. ²²
In vivo 6-, 12-hr noise stress	Atrial crude membrane and cardiac mitochondrial fractions	[³ H]PK11195	↓	↔	Salveti et al. ²³
Ischemia/reperfusion	Mitochondria of Wistar rat hearts	[³ H]PK11195	↔	↔	Obame et al. ²⁴
Fearfulness	Maudsley reactive rat (a high level of fearfulness) hearts	[³ H]Ro5-4864	↓	—	Drugan et al. ²⁵

↓, Decrease; ↑, increase; ↔, no change; ↑/↓, increase then decrease; —, not studied.

Although PK11195 and 4'-Cl-DZP seem to act differently, they display the same affinity as that of mBzR.³⁷ PK11195 does not change either inotropic effect or coronary flow rate by itself, but it antagonized the negative inotropic response in papillary muscle and abolished positive inotropic response caused by 4'-Cl-DZP in isolated perfused rat heart.^{28,33} These responses indicated that the effects of PK11195 may be dependent on the actions of 4'-Cl-DZP. However, Edoute et al.³⁰ demonstrated in an isolated perfused rat heart model that PK11195 was a more potent depressant in heart functions than 4'-Cl-DZP in decreasing aortic flow, dP/dt_{max}, end-systolic pressure, and stroke work. Furthermore, the differences in the findings between the two mBzR ligands 4'-Cl-DZP and PK11195 could be attributed to the nature of the ligand-receptor interactions, which are enthalpy and entropy driven, the basis of thermodynamic parameters for the binding equilibrium.³⁷ 4'-Cl-DZP has been classified as agonist and PK11195 has been classified as antagonist. However, this classification

was ambiguous,^{24,30,34,38,39} as in some physiological conditions, these two ligands have been shown to have similar effects. Furthermore, in the past few years, the mBzR ligands that caused adverse effects in the heart have been identified as agonist, whereas mBzR ligands that were shown to have protective effects have been identified as antagonist.^{39,40}

Although mBzR ligands have been shown to have no effect on heart rate at low concentrations,^{30,34,35} several studies have demonstrated that negative chronotropic could be found at high concentrations (Table 2).^{31,32,36} In canine isolated right atrium, 4'-Cl-DZP injected into sinus node decreased both atrial rate and contractile force at the concentration of 100-1000 μg.³¹ Using positron emission tomography technique in canine living heart, Charbonneau et al.³⁶ demonstrated that the injection of 4'-Cl-DZP induced a significant drop in heart rate. Recently, Brown et al.³² reported that in intact isolated perfused rabbit heart, high concentration of 4'-Cl-DZP (ie, >24 μM) led to a negative chronotropic effect.

Table 2. Physiological effects of mBzR ligands in the heart

Ligands	Properties	Concentration	Models	Species	Physiological effects		References
					Contractility	Heart rate	
4'-Cl-DZP	mBzR antagonist	3 nM-3 μM	Papillary muscle	Guinea pig	↓	—	Mestre et al., ^{27, 28}
		≥3 μM	Papillary muscle	Guinea pig	↓	—	Holck and Osterrieder ²⁹
		≤10 μM	Isolated perfused heart	Sprague-Dawley rat	↔	↔	Grupp et al. ³⁴
		10 μM	Isolated perfused heart	Sprague-Dawley rat	↓	↔	Edoute et al. ³⁰
		10 μM	Isolated atrium	Guinea pig	↔	↔	Weissman et al. ³⁵
		24 μM	Isolated perfused heart	Rabbit	↓	↓	Brown et al. ³²
		20-400 μM	Isolated perfused heart	Wistar rat	↑	—	Leeuwin et al. ³³
		100-1000 μg/kg	Isolated right atrium	Canine	↓	↓	Saegusa et al. ³¹
		≥200 μg/kg	In vivo heart	Canine	—	↓	Charbonneau et al. ³⁶
		—	—	—	—	—	Mestre et al. ^{27,28}
PK11195	mBzR agonist	<3 μM	Papillary muscle	Guinea pig	↔	—	Grupp et al. ³⁴
		1 nM-10 μM	Isolated perfused heart	Sprague-Dawley rat	↔	↔	Edoute et al. ³⁰
		0.1 μM	Isolated perfused heart	Sprague-Dawley rat	↓	↔	Leeuwin et al. ³³
		50 μM	Isolated perfused heart	Wistar rat	↔	—	Charbonneau et al. ³⁶
		Excess	In vivo heart	Canine	—	↔	Mestre et al. ²⁸
PK11195 + 4'-Cl-DZP		PK (30 nM-3 μM)	Papillary muscle	Guinea pig	↑	—	Leeuwin et al. ³³
		4' (3 nM-3 μM)	—	—	—	—	—
		PK (1 μM-50 μM), 4' (20-400 μM)	Isolated perfused heart	Wistar rat	↓	—	—

↓, Decrease; ↑, increase; ↔, no change; ↑/↓, increase then decrease; —, not studied.

Furthermore, there are pieces of evidence to show that mBzR ligands affect ion currents in the isolated cardiomyocytes, as detailed in the following paragraph. Therefore, the decrease in heart rate may be due to a reduction in net inward current during phase 4 of the pacemaker cell, leading to a hyperpolarization of the resting membrane potential.⁴¹

Because peripheral-type benzodiazepine binding sites are pharmacologic receptors coupled to calcium channels, it has been proposed that the binding of ligands to mBzR may play a role in transportation of calcium in and out of the cell.²⁷ This hypothesis is supported by studies in isolated guinea pig and rabbit ventricular myocytes.^{29,32} In a study by Holck and Osterrieder,²⁹ 4'-Cl-DZP at concentration $>3 \mu\text{M}$ reduced inward Ca^{2+} current and rectified K^{+} current in isolated guinea pig cardiac myocytes. Brown et al. also demonstrated that 4'-Cl-DZP ($24 \mu\text{M}$) decreased Ca^{2+} inward current during phase 2 of action potential in rabbit cardiomyocytes.³² In cardiomyocytes, pyrrolo[2,1-d]^[1,5]benzodiazepine derivatives, an mBzR ligand, decreased Ca^{2+} current.¹⁷ The decrease in Ca^{2+} current then diminished the trigger for Ca^{2+} release from the sarcoplasmic reticulum, which explains the negative inotropic effect of the compound. However, in Leeuwijn et al.'s³³ finding, the positive inotropic effect of 4'-Cl-DZP was not clearly explained.

Table 2 summarizes the physiologic effects of mBzR activation reported to date. A high concentration of 4'-Cl-DZP ($\geq 10 \mu\text{M}$) was found to induce negative inotropic effect in isolated perfused rat and rabbit hearts,^{30,32} except for one study.³³ In contrast, a low concentration of $<10 \mu\text{M}$ did not affect the contractility in either isolated perfused rat heart or isolated atrium models.^{34,35} Despite these reports, inconsistent results derived from studies using the same model and the same species still exist.²⁷⁻²⁹ Mestre et al. demonstrated that 4'-Cl-DZP of $<3 \mu\text{M}$ reduced contractility in papillary muscle,^{27,28} whereas Holck and Osterrieder reported that it did not alter the contractility, the inward calcium current nor the inwardly rectifying potassium current in isolated cardiac ventricular myocytes.²⁷⁻²⁹ It is important to note that the negative chronotropic effect of mBzR ligand 4'-Cl-DZP has been shown only in the moderate-sized to large animal models (ie, canine^{31,36} and rabbit³²), whereas no chronotropic effect was found in rats^{30,34} and guinea pig.³⁵

In summary, mBzR plays an important physiological role in the heart. Findings from previous studies strongly indicate that the physiological roles of mBzR in the heart differ in different species, suggesting that its effects in the human heart could be similar or different from those found in basic studies and will need further validation in future studies.

Mitochondrial Benzodiazepine Receptor and Ischemia/Reperfusion Injury

During myocardial I/R, mitochondria are known to be involved in apoptotic or necrotic cell death. In the apoptosis pathway, I/R injury causes mitochondrial swelling and release of cytochrome c to the cytoplasm.⁴² The release of cytochrome c and induction of apoptotic cell death have been shown to involve in the opening of mitochondrial permeability transition pore (mPTP).⁴³ mPTP is a multiprotein complex that can form large nonselective pores in the mitochondrial inner membrane. Opening of this pore allows free passage of any mole-

cules up to 1.5 kDa and also disrupts the permeability barrier of the inner membrane. The major components of the mPTP involve VDAC, ANT, and cyclophilin D, whereas mBzR, hexokinase, creatine kinase, and Bcl2 have been shown to play regulatory roles with this complex.⁴⁴ Since a multimeric complex of mBzRs has structurally linked to mPTP, it is believed that both of them could play a role together in cardioprotective effects.⁹

During I/R, the energy production is decreased due to the reduced oxidative phosphorylation rate at the inner mitochondrial membrane. This event also rapidly induces ROS production.^{45,46} ROS that is generated from the electron transport chain and released to cytosol from any mitochondria can destabilize its neighboring mitochondrial membrane by inducing the release of ROS from these neighboring mitochondria. This mechanism is known as ROS-induced ROS release.⁴⁷ These events lead to $\Delta\Psi_{\text{m}}$ and metabolic mitochondrial oscillation. The loss of $\Delta\Psi_{\text{m}}$ causes a rapid mitochondrial dysfunction and leads to apoptotic and necrotic cell death.⁴⁸

mBzR-mediated Cardioprotection Against Ischemia/Reperfusion Injury

In I/R injury, the mBzR ligands 4'-Cl-DZP, SSR180575, and PK11195 have been shown to improve cardiac functions and reduce the incidence of arrhythmias.^{24,32,39,49,50} As shown in Table 3, in both in vivo and ex vivo ischemia models, pretreatment with 4'-Cl-DZP before ischemia induction or at the onset of reperfusion improved left ventricular developed pressure (LVDP) and reduced apoptosis, infarct size, and postischemic arrhythmias.^{24,32,39,49} Leducq et al. found that the new, potent, and selective mBzR ligand SSR180575^{51,52} was able to restore the left ventricular function after it was impaired during reperfusion in low-flow and regional ischemia in rat and rabbit models, respectively. The restoration behaved in a dose-dependent manner, without changing the baseline values.⁴⁹ Interestingly, although PK11195 has been shown previously to antagonize the effects of 4'-Cl-DZP in intact isolated perfused heart,³³ it has been demonstrated to improve LVDP and $\text{dP}/\text{dt}_{\text{max}}$ and reduce infarct size under a pathologic condition (ie, in isolated global ischemic rat heart).²⁴ These cardioprotective effects of PK11195 were similar to those with the administration of 4'-Cl-DZP alone, with co-treatment with 4'-Cl-DZP, and with ischemic preconditioning.²⁴ Moreover, in an ischemic phase induced by coronary artery ligation in dog, the administration of PK11195 significantly increased the heart rate and decreased the mean arterial blood pressure.⁵⁰

The loss of mitochondrial integrity that resulted from calcium overload has been shown to induce mPTP opening, leading to massive swelling of mitochondria, membrane rupture, release of cytochrome c from mitochondria, and activation of proapoptotic Bcl-2 family member proteins such as Bax. These effects eventually cause cell death.^{53,54} In an in vivo rat model of occlusion/reperfusion, the levels of cytosolic cytochrome c and caspase-9 were significantly increased in a time-dependent manner during reperfusion. In addition, the level of the reactive 10- to 15-kDa form of Bax was increased in mitochondrial extract with its sudden drop in cytosolic levels during reperfusion.⁴² The activation of Bax that is translocated into the mitochondria triggers mitochondrial membrane permeability, regulates the release of cytochrome c, and supports the apop-

Table 3. Effects of mBzR ligands on ischemia/reperfusion heart

Ligands	Properties	Models	Type of Ischemia	Effects of I/R					Reference
				Infarct size	Apoptosis	Improved LVDP	dp/dt _{max}	Arrhythmias	
4'-Cl-DZP	mBzR antagonist	Isolated rabbit hearts	Global ischemia	—	—	Yes	—	—	Brown et al. ³²
		Isolated Wistar rat hearts	Global ischemia	—	—	Yes	↑	—	Obame et al. ²⁴
		Wistar rats	LAD occlusion	—	—	—	—	—	Obame et al. ²⁴
		Isolated guinea pig hearts	Global ischemia	—	—	—	—	—	Akar et al. ³⁹
SSR180575	mBzR antagonist	Isolated Sprague-Dawley rat hearts	Low-flow I/R	—	—	Yes	—	—	Laducq et al. ⁴⁹
		Isolated Sprague-Dawley rat hearts	Low-flow I/R	—	—	—	—	—	Li et al. ⁴⁰
		Isolated Sprague-Dawley rat hearts	Low-flow I/R	—	—	Yes	—	—	Laducq et al. ⁴⁹
		Rabbit	LAD occlusion	—	—	Yes	↑	—	Laducq et al. ⁴⁹
PK11195	mBzR agonist	Wistar rat	LAD occlusion	—	—	—	—	—	Laducq et al. ⁴⁹
		Dog	LAD occlusion	—	—	—	—	—	Steenbergen et al. ⁵⁰
PK11195 + 4'-Cl-DZP FGIN-1-27	mBzR agonist	Isolated Wistar rat hearts	Global ischemia	—	—	Yes	↑	—	Obame et al. ²⁴
		Isolated Wistar rat hearts	Global ischemia	—	—	Yes	↑	—	Obame et al. ²⁴
		Isolated guinea pig hearts	Global ischemia	—	—	—	—	—	Akar et al. ³⁹
		Isolated Sprague-Dawley rat hearts	Low flow I/R	—	—	—	—	—	Li et al. ⁴⁰

I/R, Ischemia/reperfusion; LAD, left anterior descending coronary artery; LVDP, left ventricular developed pressure; ↓, decrease; ↑, increase; —, not studied.

tosome formation and apoptotic process during cardiac reperfusion. Recently, 4'-Cl-DZP has been shown to limit apoptosis by reducing the release of apoptosis inducing factor and cytochrome c from intermembrane space of mitochondria. These resulted in the prevention of the redistribution of the Bcl-2 family proteins and reduction of the 10-kDa form of Bax during I/R period.²⁴ In addition, 4'-Cl-DZP administered before ischemia or during reperfusion was able to reduce infarct size in isolated myocardium and the effect was similar to that of ischemic preconditioning.²⁴ Hence, it is possible that the protective effects against I/R injury induced by the mBzR ligands and preconditioning could share common signaling pathways.²⁴

It has been shown that the rise in end-diastolic pressure, particularly during the ischemic phase, involves the combination of calcium overload and the depletion of ATP.⁵⁵ Stabilizing effects of 4'-Cl-DZP on mitochondrial function have been shown to maintain energy supply by improving oxidative phosphorylation and maintaining mitochondrial yields during I/R in rats.²⁴ This protection against I/R was associated with an increase in respiratory control ratio related to the increase in the state 3 respiratory rate.^{24,56} Furthermore, the administration of cyclosporin A (CsA), the mPTP blocker, did not change the respiratory rate, indicating that the mitochondrial inner membrane was not affected, thus confirming that mPTP opening was not involved in this process.²⁴ The effects of mBzR ligands on I/R injury are summarized in Table 3. It is clear that the definite mechanism regarding the role of mBzR and mPTP on I/R injury process is still debated. Studies in mitochondria isolated from I/R rat hearts showed that the administration of CsA blocked Ca²⁺-induced mitochondrial swelling, whereas 4'-Cl-DZP had no effect.²⁴ However, previous studies demonstrated that mBzR ligands such as 4'-Cl-DZP, PK11195, and ANH086 by themselves could induce mPTP opening in a dose-dependent manner and could induce mitochondrial Ca²⁺ release in rat cardiac mitochondrial fraction, and that these effects were abolished by CsA or other mPTP inhibitors.^{24,53,57,58} Further studies are needed to resolve these discrepancies.

In summary, mBzR antagonists such as 4'-Cl-DZP demonstrated its protection against I/R injury, whereas mBzR agonists demonstrated opposite results. These effects include improved cardiac function, reduced infarct size, and arrhythmia prevention. The proposed mechanism of this protection of mBzR antagonist against I/R injury is thought to be partly due to its antiapoptotic effects, as well as its ability to stabilize mitochondrial function.

mBzR and Cardiac Electrophysiological Alterations

Cardiac arrhythmias are known to frequently occur during myocardial I/R process.³⁹ Growing evidence indicated that mBzR plays an important role in this phenomenon.^{32,39,40,50} In isolated hearts with global ischemia, administration of 4'-Cl-DZP either before ischemia induction or prior to reperfusion can reduce the incidence of postischemic arrhythmias.^{32,39} Moreover, it has been shown that PK11195, the specific mBzR ligand, protected dogs from early and delayed ventricular arrhythmias after 20 minutes of ischemia by left anterior descending coronary artery (LAD) occlusion and significantly prevented ventricular fibrillation after reperfusion.⁵⁰

Under physiological condition, 4'-Cl-DZP has been shown to decrease APD in a dose-dependent manner in guinea pig papillary muscle^{27,28} and in isolated cardiomyocytes.^{29,32} However, in intact perfused rat heart, 4'-Cl-DZP had no effect on sarcolemma ion channel and did not alter the action potential.³⁹ Under pathologic conditions (ie, ischemia or flash-induced stress), 4'-Cl-DZP rapidly prevented APD shortening, facilitated normal action potential morphology, and prevented postischemic tachyarrhythmias.³⁹ Furthermore, when the bolus dose was administered at the onset of reperfusion, 4'-Cl-DZP could stabilize the action potential and completely prevented arrhythmias.³⁹ These effects of mBzR ligands on the incidence of arrhythmias and cardiac electrophysiology are summarized in Tables 3 and 4.

Although mBzR has a functional link with Ca^{2+} channels, its effects can be seen beyond the Ca^{2+} channels. It has been shown that the cardioprotective properties of Ca^{2+} channel blockers were effective only when given as preischemic treatment; they were not effective when administered at the onset of reperfusion.^{59,60} However, the administration of mBzR antagonist 4'-Cl-DZP as a pretreatment or a bolus at the onset of reperfusion could successfully reduce the incidence of both arrhythmia and mechanical dysfunction.^{32,39} Previous studies demonstrated that mBzR was associated with the activation of 2 important channels at mitochondrial membrane—these were mPTP, which was shown to be opened during reperfusion but not during ischemia,⁶¹ and mitochondrial inner membrane anion channel (IMAC), which might be activated during ischemia and early reperfusion.³⁹ Furthermore, these 2 channels have been implicated in $\Delta\Psi_m$ depolarization. Huser et al.⁶² showed that $\Delta\Psi_m$ fluctuation in individual cardiac mitochondria was caused by repetitive opening and closing of the mPTP, oxidative stress, and/or cellular Ca^{2+} overload. These events initially triggered pore openings at shorter duration followed by prolonged openings, resulting in the dissipation of $\Delta\Psi_m$ as efflux of large solutes coincide with swelling of mitochondrial and loss of $\Delta\Psi_m$.⁶² O'Rourke⁶³ demonstrated that IMAC was involved in $\Delta\Psi_m$ oscillation induced by substrate deprivation and suggested that IMAC may be activated under metabolic stress in intact cardiomyocytes. Still, the functional link between mBzR and mPTP or IMAC is unclear. In a patch-clamp study of isolated mitoplasts, 4'-Cl-DZP has been shown to inhibit mPTP and IMAC opening at nanomolar concentration.⁶⁴ Nevertheless, previous studies have suggested that mPTP was not involved in the arrhythmogenesis of I/R myocardium. It has been shown that the mPTP blocker CsA could well preserve rat cardiac mitochondria from Ca^{2+} -induced mPTP opening^{23,53} but did not prevent the loss of $\Delta\Psi_m$ and did not completely reduce the incidence of postischemic arrhythmias.^{32,39,65} Furthermore, it has been suggested that IMAC activation during ischemia and early reperfusion could be a precursor to the activation of mPTP.^{39,66}

In summary, inactivation of mBzR can prevent mitochondrial dysfunction and cardiac electrophysiology alterations under stresses such as I/R injury by stabilized $\Delta\Psi_m$, preserved cardiac action potential in both duration and morphology, and prevented postischemic arrhythmias. The proposed mechanism of these actions has been related to IMAC and mPTP inactivation, leading to the prevention of cellular deterioration from I/R injury.

mBzR and ROS in the Heart

It is known that mitochondria, a vital source of energetic pool in the cell during oxidative phosphorylation, are the main source of ROS production.⁶⁷ In cardiac cell, the population of mitochondria in cytoplasm is >50% of the cell volume. A small amount (1%-5%) of electrons that flow through the electron transport chain in the mitochondria and leak into the cytoplasm and mitochondrial matrix contributes to ROS production.^{66,68} ROS has been shown to play an important role as the determinant of cell survival in ischemia.^{69,71} It has been shown that ROS release during ischemic preconditioning can act as signaling molecules that may activate protein kinase C and mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}). This results in the protection of cardiomyocytes against injury.^{69,70,72-74} Despite that, an increase in ROS production in postischemic hearts shows different effects. Under physiological conditions, there is a balance between ROS production and the intracellular ROS scavenging capacity.⁷¹ However, such a balance is upset under pathologic conditions. It has been shown that oxidative stress is induced by an increase in ROS production or a depletion of ROS scavengers or the depletion of cytosolic reduced glutathione (GSH).⁷⁵ These ROS may lead to mitochondrial dysfunction, apoptotic cell death, and persistent contractile dysfunction.^{45,66,67,76}

In models of metabolic stress induction in the form of substrate deprivation^{77,78} or localized ROS generations by laser flash-induced stress,^{66,79} ROS was found to trigger cellwide oscillations and collapse of $\Delta\Psi_m$ in the entire mitochondrial network of isolated cardiomyocytes. Aon et al.⁶⁶ demonstrated that the effects of ROS generation triggered by the flash on cellwide mitochondrial oscillation were not abolished by either acute addition or the preincubation of 1 μM CsA, the inhibitor of mPTP. These suggest that mPTP was not involved in this process. Previous studies demonstrated that ROS can trigger the opening of IMAC.^{39,66} Aon et al.⁶⁶ found that PK11195, which is one of several classes of amphipathic inhibitors of IMAC, could prevent the cellwide synchronized mitochondrial oscillations and ROS bursts after a flash. Although matrix ROS was found to increase markedly in the flash region of the cell during mitochondrial depolarization, these ROS did not dissipate to other regions, confirming that PK11195 could prevent ROS-induced ROS release in these cells.⁶⁶ Consistently, 4'-Cl-DZP has been shown to suppress the entire cell oscillation of $\Delta\Psi_m$, eliminate APD oscillation in isolated cardiomyocytes,³⁹ and improve mitochondrial phosphorylation rate.^{24,49}

In summary, a high level of ROS following reperfusion can greatly destabilize the system and produce changes in the cardiac action potential, which may contribute to the genesis of arrhythmia. Because these effects were abolished by mBzR antagonists, it was proposed that mBzR activates IMAC, the precursor of mPTP opening, to release ROS and leads to neighboring mitochondrial instability, known as ROS-induced ROS release mechanism (Fig. 1). This process is the primary cause of mitochondrial oscillation in the whole cell and cardiac physiology alterations. Nevertheless, the modulations of mBzR on mitochondrial ion channels and other proteins are still unclear and need further investigation.

Table 4. Electrophysiological effects of mBzR in the heart

Ligands	Properties	Concentration	Models	Species	Electrophysiological effects				Reference
					APD	[Ca ²⁺] _i	Oscillation of $\Delta\Psi_m$ depolarization	ROS production	
4'-Cl-DZP	mBzR antagonist	0.03-3.0 μ M 3 μ M <3 μ M $\geq$ 3 μ M 24 μ M 32 μ M	Papillary muscle	Guinea pig	↓	—	—	—	Mestre et al. ²⁸
			Papillary muscle	Guinea pig	↓	—	—	—	Mestre et al. ²⁷
			Isolated cardiomyocytes	Guinea pig	↔	μ	—	—	Holck and Osterrieder ²⁹
			Isolated cardiomyocytes	Guinea pig	↓	↓	—	—	Holck and Osterrieder ²⁹
			Isolated cardiomyocytes	Rabbit	↓	↓	—	—	Brown et al. ³²
			Isolated cardiomyocytes; flash-induced	Guinea pig	↑	—	No	—	Akar et al. ³⁹
PK11195	mBzR agonist	32-100 μ M 32-100 μ M 64 μ M	Intact perfused heart	Guinea pig	↔	—	—	—	Akar et al. ³⁹
			Global ischemia	Guinea pig	↑	—	—	—	Akar et al. ³⁹
			Isolated cardiomyocytes; flash-induced	Guinea pig	—	—	No	—	Aon et al. ⁶⁶
			Papillary muscle	Guinea pig	↔	—	—	—	Mestre et al. ²⁸
			Papillary muscle	Guinea pig	↔	—	—	—	Mestre et al. ²⁷
			Isolated cardiac mitochondrial flash-induced	Sprague-Dawley rat Guinea pig	—	—	Yes No	— ↓	Li et al. ⁵⁷ Aon et al. ⁶⁶
PK11195 + 4'-Cl-DZP FGIN-1-27	mBzR agonist	PK (30-300 nM), PK (3 nM-3 μ M), 4.6 μ M 4.6 μ M 92 μ M	Papillary muscle	Guinea pig	↑	—	—	—	Mestre et al. ²⁸
			Global ischemia	Guinea pig	↓	—	—	—	Akar et al. ³⁹
			Isolated cardiomyocytes	Sprague-Dawley rat	—	↓	—	—	Li et al. ⁴⁰
			Isolated cardiomyocytes; flash-induced	Guinea pig	—	—	No	—	Aon et al. ⁶⁶

↓, Decrease; ↑, increase; ↔, no change; —, not studied.

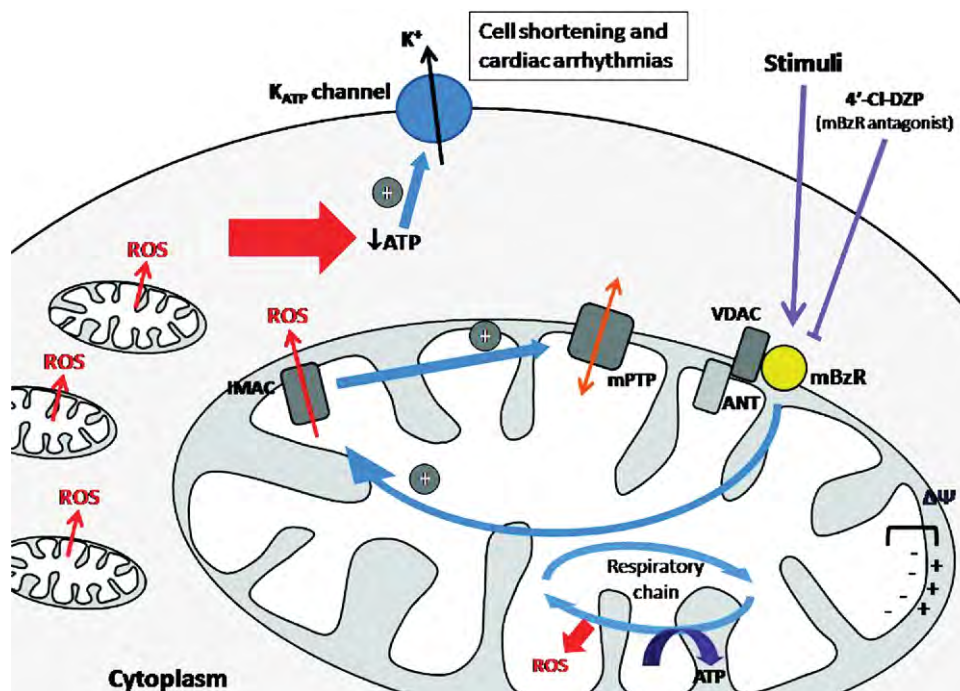


Figure 1. Hypothetical scheme of mitochondrial benzodiazepine receptor (mBzR) and other mitochondrial ion channels on the effect of reactive oxygen species (ROS)-induced ROS release mechanism and the cardiac physiology alterations. Under stress condition, ROS is increased from respiratory chain, and activation of mBzR leads to the opening of mitochondrial inner membrane anion channel (IMAC) and mitochondrial permeability transition pore (mPTP). These processes result in the oscillation or collapse of mitochondrial membrane potential ($\Delta\Psi_m$) and ROS-induced ROS release mechanism. The loss of mitochondria membrane integrity leads to the decrease of energy supply and activates sarcolemma adenosine triphosphate (ATP)-sensitive potassium channel (K_{ATP}) channel, resulting in the change in cardiac action potential, and finally leads to fatal arrhythmias. mBzR antagonists such as 4'-Cl-DZP that could inhibit $\Delta\Psi_m$ depolarization and prevent postischemic electrical dysfunction are used for protective effect against arrhythmia. ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel.

Adverse Effect of mBzR Stimulation in I/R Heart

With the use of mBzR agonists, important findings have been reported regarding the adverse effects of mBzR stimulation in the heart that underwent I/R injury. FGIN-1-27 is the mBzR agonist that bound with high affinity to mBzR but not to the central benzodiazepine receptors.⁸⁰ It has been demonstrated that adding FGIN-1-27 to the perfusate worsened the effect of I/R on the heart, because an increased incidence of arrhythmias was observed.³⁹ Furthermore, hearts pretreated with FGIN-1-27 have been shown to accelerate the APD shortening; reduce action potential amplitude (APA), upstroke velocity (dF/dt), and time for the onset of inexcitability; and eventually lead to arrhythmias.³⁹ These findings have led to a novel proposed mechanism of arrhythmogenesis in the myocardium.³⁹ At the mitochondria level, mBzR has been shown to be involved in the oscillations of $\Delta\Psi_m$ in which 4'-Cl-DZP abolished the flash-induced $\Delta\Psi_m$ oscillation, whereas FGIN-1-27 caused permanent $\Delta\Psi_m$ depolarization.⁶⁶ It has been proposed that the mechanism of arrhythmogenesis involves the loss of $\Delta\Psi_m$, which induces mitochondrial network interaction, uncoupling of metabolism, and whole-cell electrical and mechanical dysfunction and results in the reentry of cardiac arrhythmias in postischemic heart. This mechanism, proposed by Akar et al. and called "metabolic sink/block,"³⁹ is a block of electrical propagation that is distinct from other existing conduction blocks (ie, gap junction block, anatomic block, and dynamic functional block). Here, metabolic sink/block is dependent on the formation of areas undergoing regional or tem-

poral changes in $\Delta\Psi_m$. The loss of $\Delta\Psi_m$ induced by mBzR agonist may lead to postischemic arrhythmias.³⁹ Furthermore, to investigate the correlation between $\Delta\Psi_m$ and APD, Akar et al.³⁹ demonstrated that glibenclamide, the ATP-sensitive potassium channel (K_{ATP}) inhibitor, blunted the effects of FGIN-1-27, which initially reduced APA and dF/dt in an isolated ischemic rat heart study. This indicates that K_{ATP} activation was responsible for the ischemia-induced electrophysiological changes in the hearts.

In summary, mitochondrial stress such as that under ischemia can lead to mitochondrial depolarization, failure of oxidative phosphorylation, and depletion in energetic pool, resulting in an activation of sarcolemma K_{ATP} channels and changes in the duration of cardiac action potential in individual cells or myocardium, and leads to fatal arrhythmias. The mBzR antagonist 4'-Cl-DZP could blunt these effects through the inhibition of $\Delta\Psi_m$ depolarization, which has been shown to be more effective in preventing postischemic electrical dysfunction by preserving energy depletion and inhibition of sarcolemmal K_{ATP} channels, whereas FGIN-1-27, an mBzR stimulator, shows adverse effects (see Fig. 1).

Clinical Studies of Mitochondrial Benzodiazepine Receptor in the Heart

Characterization and localization of mBzR in human using noninvasive method had been reported in several studies. In a report by Charbonneau et al.,³⁶ ¹¹C-¹¹PK11195, the highly spe-

cific activity and short-lived cyclotron isotope, was injected into 7 normal male subjects, and positron emission tomography was used to locate the distribution of the isotope. ^{11}C -PK11195 was found to be concentrated in the myocardium. In an in vivo evaluation of biodistribution, displacement, and metabolism, [^{123}I]iodo-PK11195 has been shown to rapidly decay into 2 polar metabolites on high-performance liquid chromatography, in plasma samples of healthy volunteers.^{81,82} These results were similar to those in mice in which 2 polar metabolites were found in the blood.⁸¹ Unlike in the blood, only original radioactive compounds were found in the hearts of healthy humans.⁸¹ These findings have led to the hypothesis that either no metabolism of this isotope occurs in the heart or metabolites of [^{123}I]iodo-PK11195 that were found in the blood do not enter the heart.⁸¹

In patients with coronary heart disease who were treated intravenously with PK11195 at the concentration of 20 mg, no hemodynamic changes or anti-ischemic effects were observed.⁸³ These findings were different from those reported in animal models, suggesting that the effects of this ligand could vary among different species. Although the effects of other mBzR ligands such as 4'-Cl-DZP have been shown to have cardioprotective effects in various animal models in I/R injury, none has been investigated in humans. This is partly due to the fact that most studies were performed in in vitro or small animal models, without the supporting data from any preclinical studies. It is known that the physiological roles of mBzR could vary from species to species; therefore, future studies performed in human-like animal models are needed prior to clinical studies to validate findings from previous studies. If the results warrant its cardioprotective effects, clinical studies can be the next step to investigate the effects of mBzR ligands, which have been proved to have cardioprotective benefits in those animal models to warrant the possibility of their clinical usefulness.

Conclusion

A growing body of evidence demonstrates the ability of mBzR ligands to improve cardiac functions via mitochondrial functions, despite some discrepancies in results. The improvements were achieved by mBzR ligands maintaining energy supply, reducing calcium overload, preventing the mitochondrial ROS-induced ROS release, and restoring normal electrical activity in the heart. Given the important clinical implications of these mBzR ligands, they may represent novel therapeutic strategies for preventing electrical and mechanical dysfunctions at the mitochondrial level in the heart in the near future.

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Disclosures

The authors have no conflicts of interest to disclose.

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Roles of the nitric oxide signaling pathway in cardiac ischemic preconditioning against myocardial ischemia-reperfusion injury

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Summary

Nitric oxide (NO), a vasoactive gas that can freely diffuse into the cell, has many physiological effects in various cell types. Since 1986, numerous studies of ischemic preconditioning against ischemia-reperfusion (I/R) injury have been undertaken and the roles of the NO signaling pathway in cardioprotection have been explored. Many studies have confirmed the effect of NO and that its relative signaling pathway is important for preconditioning of the cardioprotective effect. The NO signaling against I/R injury targeted on the mitochondria is believed to be the end-target for cardioprotection. If the NO signaling pathway is disrupted or inhibited, cardioprotection by preconditioning disappears. During preconditioning, signaling is initiated from the sarcolemmal membrane, and then spread into the cytoplasm via many series of enzymes, including nitric oxide synthase (NOS), the NO-producing enzyme, soluble guanylyl cyclase (sGC), and protein kinase G (PKG). Finally, the signal is transmitted into the mitochondria, where the cardioprotective effect occurs. It is now well established that mitochondria act to protect the heart against I/R injury via the opening of the mitochondrial ATP-sensitive K⁺ channel and the inhibition of mitochondrial permeability transition (MPT). This knowledge may be useful in developing novel strategies for clinical cardioprotection from I/R injury.

key words: nitric oxide • ischemic preconditioning • ischemia/reperfusion injury • heart

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BACKGROUND

Acute myocardial infarction (MI) is one of the major causes of death worldwide [1]. Acute MI occurs when the large epicardial coronary artery is occluded by a thrombotic blood clot or lipid accumulation. When the occlusion lasts over 20–40 minutes, ischemic tissues become irreversibly damaged, resulting in infarction [2]. The precise time to the development of infarction varies between species. For example, faster development is observed in small rodents with a high heart rate and low collateral blood flow, and slower development is found in larger animals [3]. The progressive and irreversible damage incurred during myocardial ischemia can only be stopped by an immediate reperfusion. Despite this fact, severe and irreversible myocardium damage during the ischemic phase could be caused by reperfusion itself, referred to as reperfusion injury [4]. Irreversible reperfusion injury is defined as an injury caused by reperfusion after the ischemic episode, and results in the death and loss of cells that had only been reversibly injured and primed for death during the preceding ischemic episode [2].

During ischemia, the cardiomyocytes become depleted of oxygen and energy. Higher CO_2 and lactate production in the cell is associated with greater acidosis. The increased acidosis that is produced by anaerobic metabolism increases the influx of Na^+ via the Na^+/H^+ exchanger and the depletion of ATP for Na^+/K^+ ATPase activity, which in turn inhibits the eradication of Na^+ from the cell. This results in the accumulation of Na^+ in cytoplasm [5]. The large amount of Na^+ in cytoplasm drives the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, leading to the overload of Ca^{2+} . Finally, the elevated cytosolic Ca^{2+} levels contribute to the opening of a mitochondrial permeability transition pore (mPTP) and result in the death of cells [6]. Large infarct size and failure of infarct tissues to perform a physiological function may lead to heart failure. Protecting the heart from these harmful consequences of coronary occlusion has been the goal of ongoing research by a number of investigators for many decades [7–12]. In recent years, cardiac ischemic preconditioning (i.e., the brief sequences of coronary occlusion and reperfusion before prolonged occlusion) has been extensively studied and found to be cardioprotective against ischemia/reperfusion (I/R) injury. Its various roles in cardioprotection involve many factors, including the nitric oxide signaling pathways [13]. In this review, factors involving the cardioprotective process of ischemic preconditioning, particularly nitric oxide (NO), are presented, the mechanisms of this process are explained, and clinical implications for future therapeutic approaches involving the role of nitric oxide (NO) are discussed.

ISCHEMIC PRECONDITIONING

Ischemic preconditioning (IPC) is the induction of a brief episode of ischemia and reperfusion in myocardium to markedly reduce tissue damage induced by prolonged ischemia [7]. Ischemic preconditioning as a potent cardioprotective method against I/R injury was first reported by Murry et al. in 1986 [8]. In their study using dogs, four sequential episodes of 5-minute occlusion each followed by 5-minute reperfusion were induced in the left circumflex coronary artery, preceding a sustained 40-minute prolonged occlusion period. Another group of dogs were left on a prolonged coronary ligation without IPC. After 4 days of reperfusion, the infarction generated

in the former group was reduced by 75% compared to that of the latter group. In their study there was no significant difference in the collateral blood flows of both groups. These results suggested that the tolerance of cardiac tissue to I/R injury is not related to the change of regional blood flow. This remarkable cardioprotection process has also been demonstrated in various species of experimental animals, including rats, mice, rabbits, chicken, and swine [9–13].

Although many investigations have documented preconditioning as an intervention capable of protecting the heart against I/R injury, the cardioprotective effect of IPC is relatively short-lived and the underlying mechanism is not completely understood. More recently it was found that the fundamental processes and the end-target of cardioprotection of IPC mechanisms are related to the prevention of mitochondrial permeability transition (MPT) [1]. Any drug that blocks MPT, such as cyclosporine A, is able to mimic the IPC effect [6]. The MPT and intramitochondrial signaling against I/R injury will be described later in this review.

Even after IPC was first described by Murry et al. in 1986, its mechanisms remained largely unknown for several years. The only clue at the time from studies in various species was that IPC was not involved in the coronary blood flow through the damaged area [14]. However, in 1991 Liu et al. discovered that stimulation of cardiac G_i -coupled adenosine receptor type 1 (A_1) was necessary for the preconditioning effect [14]. The study also showed that IPC's protection could be attenuated by an adenosine receptor antagonist, whereas the infusion of adenosine, or the A_1 -specific agonist N6-1-(phenyl-2R-isopropyl) adenosine (R-PIA), could reduce the infarct size. Therefore, cardioprotection of IPC was achieved as ischemic myocardium rapidly degraded ATP to adenosine, which then accumulated in this area [7].

Bradykinin and opioid receptors are also involved in the IPC process [15–17]. During the preconditioning ischemia, bradykinin and endogenous opioid are released from the heart, together with the production of adenosine as a result of metabolic breakdown of ATP [7]. These 3 ligands activate their respective G-protein coupled receptors (GPCRs), which work on the other pathways to protect the heart from ischemic conditions [7]. Nevertheless, the protection of IPC could not be completely blocked if 1 of these 3 receptors is inhibited. On the other hand, it was suggested that combining 2 or more of these receptors produces an incremental cardioprotective effect against I/R injury of IPC [18]. Further research has shown that increasing the number of preconditioning cycles leads to greater resistance of the heart to I/R injury [19]. It was proposed that the additional brief I/R cycles produced more of the trigger substances, and more activities of these receptors could be observed [19]. In addition to these substances and receptors, nitric oxide (NO) has been shown to play an important role in the cardioprotective process of IPC during myocardial ischemia, which will be described later in this review.

NITRIC OXIDE IN THE HEART

NO is a signaling molecule that affects the cardiovascular system as a vasodilator [20]. In hypertensive patients the level of NO metabolites (nitrite and nitrate) are found to be attenuated from their normal levels and inversely correlated

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with blood pressure [21]. In the heart, NO is known to be an important regulator of cardiac contractility in physiological condition [22]. In cardiomyocytes all 3 isozymes of NO synthase (NOS) are expressed – neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3) [22]. Despite the fact that NO is a highly diffusible signaling molecule that can diffuse freely across the membranes, many studies have shown that the regulation of nNOS and eNOS are localized due to their compartmentalization. nNOS is located in the sarcoplasmic reticulum (SR), whereas eNOS is located in the caveolae of the sarcolemma [22]. Each type of NOS performs a different cardiac contraction modulation. While nNOS signaling potentiates the response of β -adrenergic stimulation [23], eNOS signaling depresses the functional regulation of β -adrenergic stimulation [24]. Although nNOS and eNOS are membrane-bound enzymes and regulate cardiomyocytes under physiological condition, iNOS is a soluble enzyme and produces less expression in physiological condition [24]. Many factors appear to be involved in the regulation of iNOS induction in response to cytokines or cardiac stress [25].

NOS produces NO from the conversion of L-arginine into citrulline, using a large number of cofactors, including nicotinamide adenine dinucleotide phosphate (NADPH) and flavin mononucleotide (FMN) [25]. Then, the activation of soluble guanylyl cyclase (sGC) by NO leads to the formation of cGMP from the nucleotide GTP [25]. cGMP-dependent protein kinase (PKG) is further activated, initiating numerous physiological regulations in cardiomyocytes. As a result, L-type Ca^{2+} channel (LTCC) activity is depressed by NO via cGMP-dependent pathways [26–28]. Moreover, a phosphorylation of α_{1C} subunit at position Ser⁵³³ of LTCC can be developed by the activated PKG, causing an inhibition of L-type Ca^{2+} current ($I_{\text{Ca,L}}$) [26]. The rate of Ca^{2+} reuptake into SR, by the increased phosphorylation of phospholamban, can be increased by NO signaling [29]. NO that is released from nNOS when it binds with the superoxide anion, can form peroxynitrite, which exerts this specific effect on phospholamban [29]. Apart from the studies of the effects of NO and cGMP on the Ca^{2+} channel, other cardiac ion channels have also been shown to be regulated by this pathway. The ATP-sensitive K^+ channel is activated by NO donors, and this involves both activation of PKG and the cGMP-independent effect [30,31]. The hyperpolarization-activated pacemaker current (I_p) is also activated by NO and the cGMP-dependent pathway in isolated guinea pig sinoatrial node cells and in human right atrial appendage cells [32,33].

ROLE OF NITRIC OXIDE SIGNALING PATHWAY IN THE CYTOSOLIC SIGNALING OF IPC

NO and its signaling pathway have been shown to be important in cardioprotection against I/R injury [1]. Numerous studies have shown the cardioprotective effect of both endogenous and exogenous NO on IPC (Table 1). In 1995 it was shown for the first time that NO has a cardioprotective effect against I/R injury, but another report in the same year showed the opposite result [34,35]. Williams et al. reported that endogenous NO plays a critical role in the reduction of infarct size after a 30-minute period of ischemia and a 120-minute period of reperfusion in rabbit hearts in an *in vivo* model [34]. Using L-nitro-arginine (L-NA), a

non-specific NOS inhibitor, to treat one group before coronary occlusion and another group during reperfusion (with the same dose administered in each group), increased infarct areas in both groups were observed, compared to a vehicle-treated group. In addition, there was no significant difference in infarct sizes between the L-NA-treated groups before and after coronary occlusion [34]. Despite this result, Woolfson et al. reported that when the isolated rabbit heart was perfused with N (G)-nitro-L-arginine methyl ester (L-NAME), the non-specific NOS inhibitor, at 10 min before the coronary occlusion and continued for 15 min of reperfusion period in 45 min of ischemia and 180 min of reperfusion episode, the infarct size was decreased compared to the untreated group [35]. These inconsistent findings could be due to use of different study models as well as differences in the duration of the treatment with NOS inhibitors and/or the duration of I/R episodes. Moreover, NO is claimed to be a pro-apoptotic factor when it reacts with superoxide anion to form peroxynitrite [36]. This result may be the cause of the myocardial injury in I/R condition found by Woolfson et al. [35], who also found that the NOS inhibitor could reduce infarct size in non-preconditioned hearts after I/R injury [35]. Nevertheless, the mechanisms of NO in the heart are still unclear and need to be investigated further.

The cardioprotective effect of NO on I/R injury was also supported by Zhao et al. in 1997 [37], who suggested that monophosphoryl lipid A (MLA) has an IPC-mimetic effect due to an increase in iNOS activity after coronary occlusion and reperfusion in rabbit hearts [37], as well as discovering the important role of iNOS in the cardioprotective effect against I/R injury when aminoguanidine (AMG), the specific-iNOS inhibitor, was used. In groups treated with AMG alone or with MLA, the infarct size was significantly increased compared to the MLA-only treated group and was not different from the vehicle-treated group [37]. These results suggested that when iNOS is inhibited, the cardioprotective effect of MLA disappears. In a pacing-induced preconditioning model of the rat heart, N^G-nitro-L-arginine (L-NNA), a non-specific NOS inhibitor, increased the release of lactate dehydrogenase, a marker of necrotic cell death, from the coronary-occluded ischemic area [38]. Yang et al. found that L-NAME could abolish the cardioprotective effect of adenosine receptor agonist 5'-(N-ethylcarboxamido) adenosine (NECA) or bradykinin in the I/R model in rabbit hearts [39]. Prendes et al. demonstrated in 2007 that IPC increased cardiac contractility after global ischemia and reperfusion in isolated rat hearts [40]; however, the effect of IPC disappeared when treated with L-NAME [40]. All of these studies demonstrated the cardioprotective effect of NO against I/R injury.

The role of exogenous NO in cardioprotection during ischemia was demonstrated by Nakano et al. [41], who found that S-nitroso-N-acetylpenicillamine (SNAP), an NO donor that serves as the resource of exogenous NO, was able to mimic preconditioning by decreasing infarct size in rabbit hearts after a long period of I/R without IPC, compared to those without SNAP infusion. The study also reported that protein kinase C (PKC) and free radicals or reactive oxygen species (ROS) were important for cardioprotection of the preconditioning-mimetic effect of exogenous NO. When chelerythrine, an inhibitor of PKC, or N-(2-mercapto-propionyl)-glycine (MPG), a ROS scavenger, were combined with SNAP,

Table 1. Effects of sources of NO in cardioprotection against ischemia-reperfusion injury.

Source of NO	Model of I/R	Animal Models	Effect of NO	References
Endogenous	Isolated heart (coronary ligation) Isolated heart (global ischemia)	Rabbit	Reduce infarct size (in both models of I/R injury)	[34]
Endogenous	Cultured embryonic ventricular myocyte	Chick	Decrease cell death	[58]
Endogenous	Isolated heart (coronary ligation)	Rat	Decrease cell death by decreasing of lactate dehydrogenase released	[38]
Endogenous	Isolated heart (global ischemia)	Rat	Improve cardiac function but do not change in cell viability	[40]
Endogenous	<i>In vivo</i> (coronary ligation)	Rabbit	Reduce infarct size	[62]
iNOS	<i>In vivo</i> (coronary ligation)	Rabbit	Reduce infarct size	[37]
iNOS	Isolated heart (global ischemia)	Mouse	Reduce infarct size	[44]
iNOS	<i>In vivo</i> (coronary ligation)	Mouse	Reduce infarct size	[82]
iNOS, eNOS	Isolated heart (global ischemia) Isolated cardiomyocyte	Rat	Reduce infarct size Decrease cell death	[61]
eNOS, iNOS and exogenous	Isolated heart (global ischemia)	Mouse	Reduce infarct size	[43]
eNOS	Isolated heart (global ischemia)	Mouse	No response	[48]
eNOS	Isolated heart (global ischemia)	Mouse	Reduce infarct size	[50]
Exogenous	Isolated heart (coronary ligation)	Rat	Reduce infarct size	[59]
Exogenous	Heart slice	Rat	Decrease necrosis and apoptosis	[60]
Exogenous	Cultured neonatal cardiomyocyte	Rat	Decrease cell death	[85]
Exogenous	Isolated heart (coronary ligation)	Rabbit	Reduce infarct size	[41]
Exogenous	Isolated heart (coronary ligation)	Rat	Reduce infarct size	[42]

the cardioprotective effect of SNAP was eliminated, as shown by the enlargement of infarct size as compared to the SNAP-only treated group [41].

The cardioprotective effects of NO have also been supported by other studies [42,43]. Kanno et al. in 2000 showed that iNOS was overexpressed in eNOS knockout mice after 30 minutes of ischemia followed by 60 minutes of reperfusion; however, nitrite formation and infarct area were not different from that of wild type mice [43]. The role of iNOS was also reported by Zhao et al. in 2000 [44], who found that an increase in iNOS expression could be induced by treating with 2-chloro-N⁶-cyclopentyladenosine (CCPA), an adenosine A₁ receptor agonist, while the effect disappeared when treated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an adenosine A₁ receptor antagonist [44]. CCPA was also found to reduce infarct size after I/R, while DPCPX or S-methylisothiourea (SMT), a specific iNOS inhibitor, cancelled this effect [44]. Results of a study by Wang et al. in 2002 supported the dominant effect of iNOS in preconditioning [45]. It was discovered that IPC, with a sequence of 6 cycles of 4-minute coronary occlusion and 4-minute reperfusion, increased the expressions of iNOS mRNA and protein at 3 hours after the last cycle of IPC [45]; however, the expression of eNOS remained unchanged [45]. Although iNOS has been shown to protect the heart against myocardial

injury after I/R, in 2008 Heinzel et al. found that the treatment of iNOS inhibitor aminoguanidine (AG) in the sustained moderate regional ischemia by a reduction in coronary artery pressure to ~45 mmHg for 6 h in miniswine, AG caused an increase in the cell shortening compared to a non-treated group combination with L-arginine in an isolated cardiomyocyte model [46]. This result suggests the role of iNOS in the attenuation of cardiac function after hypoperfused ischemia [46]. The role of iNOS in the late phase of IPC was also observed. Guo et al. found that iNOS knockout mice had larger infarct size than wild-type mice after being subjected to 30-min coronary occlusion and 24-h reperfusion in the presence of IPC (6 episodes of 4-min occlusion and 4-min reperfusion cycles) [47]. Moreover, at 24 h after I/R, wild-type mice with IPC had smaller infarct size than both non-IPC wild-type and iNOS knockout mice at 24 h after IPC [47]. This cardioprotective effect of iNOS was supported by the increased level of iNOS expression in the IPC group compared to the non-IPC group at 24 h after IPC [47].

The significance of eNOS on cardioprotection against I/R injury has also been investigated. Bell et al. reported that the infarct size in eNOS knockout mice was not different from that of wild-type mice after IPC [48]. This finding is consistent with a 2008 study by Guo et al., which reported that both eNOS-knockout mice and wild-type mice have a

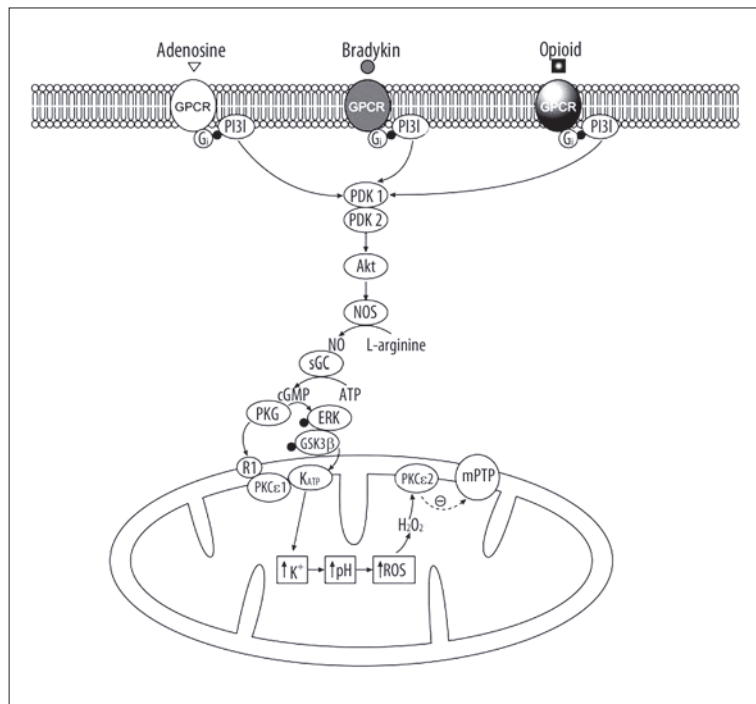


Figure 1. The schematic diagram represents the ischemic preconditioning pathway in cardiomyocytes. IPC induces cardiomyocytes to release adenosine, bradykinin and endogenous opioid which occupy their specific G-protein coupled receptors. After that, the signal will pass into the cytosol via the activation of the enzyme series including PI3K, PDKs, Akt and NOS. The latter enzyme produces NO which acts as signaling molecule to activate sGC and resulted in the cGMP formation. Then, cGMP activates PKG which has the separated mechanism on the mitochondria, the direct and indirect mechanism. The direct mechanism of PKG is on the R1 protein on outer mitochondrial membrane which then activates the opening of mitoK_{ATP} channel on the inner mitochondrial membrane via the phosphorylation of PKCε1. The indirect mechanism of PKG is the phosphorylation of ERK and GSK3β which then act on mitoK_{ATP} channel. However, the mechanism of how GSK3β activates the opening of mitoK_{ATP} channel is still unclear. After the mitoK_{ATP} channel opening, K⁺ then enters the mitochondrial matrix and H⁺ is ejected out of the matrix to balance the positive charge. When H⁺ level decreases, the electron transport chain is interrupted and leads to the formation of superoxide anion and then H₂O₂. Finally, H₂O₂ will act as signaling molecule to activate PKCε2 which then inhibits the opening of mitochondrial permeable transition pore (mPTP). This inhibition of mPTP opening helps to protect mitochondrial damage during I/R injury. (Modified with permission from [63]).

similar infarct size after I/R injury in the non-preconditioned state [49]. In eNOS knockout mice, these findings suggested that the role of eNOS in preconditioning is less prominent than that of iNOS. Although the infarct size was reduced in the overexpressed eNOS compared to wild type mice, du Toit et al. found that there was no difference in the infarct size between IPC and non-IPC in this overexpressed eNOS mouse model [50]. It has been proposed that the heart with eNOS overexpression may be already maximally protected against I/R injury by elevated endogenous NO levels produced by eNOS in this model [50]. Nevertheless, it is not known whether exogenous NO would be beneficial in overexpressed eNOS mice, and further studies are needed to investigate this possibility.

In biological systems, NO has also been found to be produced from enzyme-independent generation [51]. Under acidic conditions, such as intracellular acidosis from ischemia, NO can be generated in the tissues from the reduction of nitrite, NO₂⁻ [52]. Zweier et al. found that although all NOS isoforms in the heart were totally blocked by L-NAME, the NO formation was partially inhibited in the hearts after 30 min of ischemia [53]. They also reported that in the I/R condition of 30-min ischemia followed by 45-min reperfusion, the time course of the recovery of rate pressure product in L-NAME-treated hearts were higher than in the untreated group; however, when L-NAME was combined with nitrite, the time course of the recovery of rate pressure product decreased [53]. This finding suggested that nitrite can reverse the inhibitory effect of NOS inhibitor, indicating the enzyme-independent generation of NO as a possible mechanism protecting against myocardial I/R injury.

The cardioprotective mechanisms of NO beyond the cGMP/PKG-dependent pathway have also been reported. Since NO can modify the structure of protein through the mechanism called S-nitrosylation, some recent studies have

also demonstrated the cardioprotective effect of NO via this mechanism [54,55]. S-nitrosylation occurs when NO, from NO sources such as NOS, acts through the post-translational modification of cysteine thiol on the targeted proteins that colocalized with it to form S-nitrosothiol (SNO), resulting in a change in their protein functions [56,57]. A study by Sun et al. in 2007 reported that when treated with S-nitrosoglutathione (GSNO) (i.e., the exogenous source of NO for S-nitrosylation), the infarct area was smaller than in the untreated group after 20 min of no-flow ischemia and 20 min of reperfusion in isolated mouse hearts [54]. Lin et al. in 2009, using female ovariectomized mice, demonstrated that 17β-estradiol (E2) and the estrogen receptor-β-selective agonist 2,2-bis(4-hydroxyphenyl)-propionitrile (DPN) decreased the infarct size in the heart after being subjected to 20 min of ischemia followed by 30 min of reperfusion [55]. Proteomic analysis also demonstrated that an increase in S-nitrosylation of a number of proteins could be found in the DPN- and E2-treated hearts, as well as in the preconditioning group [55]. This finding suggests that the increased SNO protein from DPN and E2 treatment after

I/R could lead to cardioprotection against myocardial injury [55]. Therefore, the S-nitrosylation by NO could be one of the major targets for cardioprotection against I/R injury.

NITRIC OXIDE AND INTRAMITOCHONDRIAL SIGNALING OF IPC

A growing body of evidence demonstrates the important relationship among NO, ROS, and ischemic preconditioning [41,58–62]. In 2003 Lebuffe et al. found that H_2O_2 and NO were important for preconditioning-like cardioprotection [58]. Furthermore, when the mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel was inhibited by 5-hydroxydecanoate (5-HD) in the IPC model, the cardioprotective effect of IPC disappeared [58], suggesting the importance of the mitoK_{ATP} channel in cardioprotection against I/R injury.

Recent research on the mechanisms of IPC suggests that during a brief episode of ischemia, 3 ligands are released from the cardiomyocytes, and long sequences of activities are found here [7]. These ligands (bradykinin, endogenous opioid, and adenosine), occupying their respective G-protein coupled receptors (GPCRs), result in activations of phosphatidylinositol 3-kinase (PI₃K) and series of phospholipid-dependent kinase (PDK) (Figure 1). PDK causes phosphorylation and activation of Akt, where the latter induces further phosphorylation onto NOS, causing NO to be generated. After that, sGC, activated by NO, transforms GTP into cGMP, in which PKG is finally activated. In the last step of cytosolic signaling, PKG then reacts on mitochondria, resulting in the opening of the mitoK_{ATP} channel (Figure 1) [63]. The opening of the mitoK_{ATP} channel leads to the inhibition of the mitochondrial permeability transition pore (mPTP), resulting in the protection of mitochondria from damage during ischemia. Thus, a cGMP-dependent mechanism has been proposed as the main pathway to activate the mitoK_{ATP} channel via phosphorylation by PKG [60,62].

In mitochondria, the mPTP has also been shown to play a critical role in I/R injury. The mPTP is the megachannel that is the formation of 3 specific components, including the voltage-dependent anion channel (VDAC), the adenosine nucleotide transporter (ANT), and cyclophilin D [64]. Currently, the molecular structure of this pore is still under debate [64]. A high concentration of calcium, including calcium overload due to myocardial ischemia, has been shown to trigger the opening of mPTP [65]. Previous studies demonstrated that cyclosporine A, the mPTP inhibitor, could protect the heart from myocardial I/R injury [66,67]. In 2002, Hausenloy et al. proposed that the inhibition of mPTP opening could be the end-target of IPC [68]. Therefore, if mPTP opening is inhibited or interrupted by the process of mitoK_{ATP} channel opening, mitochondrial damage from I/R injury could be prevented, resulting in cardioprotection against I/R injury.

Exogenous NO by SNAP can increase ROS generation in isolated rat cardiomyocytes [59]. However, when SNAP was co-incubated with 5-HD, the ROS production decreased. On the other hand, when treated with diazoxide, the mitoK_{ATP} channel opener, without the treatment of SNAP, the ROS production was instead increased [59]. These findings suggested a relationship between NO, mitoK_{ATP} channel, and ROS production, in which ROS production via the opening of mitoK_{ATP} channel is activated by NO.

Growing evidence supports the proposed hypothesis that the NO-cGMP-PKG pathway causes the mitoK_{ATP} channel opening [58,60,62,69,70]. In this pathway, PKG was proposed as the last step in the signaling process before the involvement of mitochondria (Figure 1). This hypothesis was established when it was shown that exogenous PKG plus cGMP, when added to the isolated mitochondria, causes the increase of mitochondrial matrices due to the opening of the mitoK_{ATP} channel [71]. Furthermore, this effect could be reversed by several substances such as KT5823 (the specific PKG inhibitor), 5-HD or glibenclamide (the mitoK_{ATP} channel inhibitors), and ϵV_{1-2} (the protein kinase C ϵ isoform [PKC ϵ]-specific inhibitor) [71].

The mitoK_{ATP} channel is located in the mitochondrial inner membrane (MIM); however, PKG is a cytosolic enzyme that is unable to cross the mitochondrial outer membrane (MOM). Therefore, how PKG interacts with the mitoK_{ATP} channel remains unknown. It has been proposed that phosphorylation of serine or threonine by an unknown protein called R1, located on MOM, is a necessary step taken by PKG (Figure 1) [63]. The PKG-dependent mitoK_{ATP} channel opening seems to require the intact MOM, and is reversed by Ser/Thr phosphatase PP2A [63]. The phosphorylation of this unknown protein R1 causes the signal to be transmitted to PKC ϵ on the MIM, leading to phosphorylation and opening of the mitoK_{ATP} channel (Figure 1).

The confirmation of the presence of R1 was demonstrated by Costa and Garlid in 2008 [72], who used mitochondria and mitoplast (mitochondria without MOM) to test the effects of many specific activators and inhibitors on intramitochondrial signaling [72]. By using phorbol 12-myristate-13-acetate (PMA), a PKC ϵ activator that can cross the MOM, they found that it caused mitochondria matrix swelling due to the opening of the mitoK_{ATP} channel in both mitochondria and mitoplast. However, when treated with isolated PKG, the swelling was observed in mitochondria but not in mitoplast [72]. Both the effects of PMA and PKG, nonetheless, could be removed when treated with the PKC ϵ phosphatase PP2A [72]. This clearly shows that the specific protein R1 located in MOM has the ability to interact with PKG, as well as the ability to open the mitoK_{ATP} channel. PKG itself does not directly open the mitoK_{ATP} channel, and does not cause the mitochondrial matrix swelling in the mitoplast [72].

Another mechanism by which PKG mediates the opening of the mitoK_{ATP} channel was demonstrated by Das et al. in 2008 and 2009 [73,74]. The 2008 study demonstrated that the expression of ERK and GSK3 β protein in the ischemia-reoxygenation condition was increased in mouse cardiomyocytes treated with sildenafil citrate, the phosphodiesterase type 5 (PDE-5) inhibitor [73]. They also demonstrated that when treated with the combination of PD98059, an ERK inhibitor, and sildenafil citrate, the level of necrosis and apoptosis increased compared to sildenafil citrate treated alone in ischemia-reoxygenation cardiomyocytes [73]. In 2009, their subsequent study demonstrated that after treatment with sildenafil citrate for 24 h, the phosphorylation of ERK and GSK3 β increased in the intact mouse heart [74]; however, when treated with PD98059, the phosphorylation of ERK and GSK3 β decreased and were not different from the control. These findings suggested that GSK3 β is downstream

RA

of ERK [74], and led the researchers to propose a mechanism downstream from PKG, in which ERK and GSK3 β protect the heart against I/R injury via the opening of the mitoK_{ATP} channel (Figure 1).

GSK3 β , a multifunctional Ser/Thr kinase, is one of the main target signaling cascades in cardioprotection against I/R injury [75]. This protein was originally found to be involved in the disruption of glycogen synthesis via the inhibition of glycogen synthase, and was named glycogen synthase kinase (GSK) [75,76]. The role of GSK3 β phosphorylation in the inhibition of mPTP opening in the heart was first reported by Juhaszova et al. in 2004 [77], showing that the threshold for mPTP opening was increased when GSK3 β was knock-down via RNAi in isolated neonatal rat cardiomyocytes [77]. The GSK3 β inhibitors were also reported to have a cardioprotective effect against I/R injury [78]. A 2002 study by Tong et al. demonstrated that pretreatment with lithium as well as SB216763, the GSK3 β inhibitors, could decrease the infarct size after 20-min global ischemia and 30-min reperfusion in isolated rat hearts [78]. A 2006 *in vivo* study in rats by Nishihara et al. also supports the hypothesis that the GSK3 β inhibitor SB216763 can limit infarct size after 20-min LAD occlusion and 40-min reperfusion in a dose-dependent manner [79]. Moreover, a study by Gross et al. in 2004 found that SB216763 and another GSK3 β inhibitor, SB415286, when administered for 10 min before ischemia or 5 min before reperfusion, could decrease infarct size, and that infarct size was not different between pre- and post-treatment in 30-min ischemia and 2-hr reperfusion in rats [80]. In 2008, Obame et al. demonstrated that the cardioprotective effect of the GSK3 β inhibitor against I/R injury was related to the inhibition of mPTP opening [81].

In mitochondria, the opening of the mitoK_{ATP} channel leads to an increase in K⁺ influx, causing swelling and alkalization of the mitochondrial matrix. The increased K⁺ influx then replaces H⁺ in the electron transport chain, and as a result, superoxide anion, the first ROS that appears in the mitochondria, is produced [63]. The superoxide anion then reacts with H₂O to form H₂O₂, which directly activates PKC ϵ on the MIM. Two isoforms of PKC ϵ are found in MIM – PKC ϵ 1 and PKC ϵ 2 [63]. PKC ϵ 1, located close to the mitoK_{ATP} channel, causes the phosphorylation and opening of the mitoK_{ATP} channel. The other isoform, PKC ϵ 2, is located close to mPTP, and its activation causes the inhibition of mPTP opening [63]. A growing body of evidence currently supports this cardioprotective effect against I/R injury by the ROS production in mitochondria from the opening of the mitoK_{ATP} channel [72,82,83]. The end-target effect of mPTP inhibition allows cells to survive due to an inhibition of the released apoptotic signal from mitochondria by the MPT process (the summarized diagram of the pathway is shown in Figure 1) [1,84]. Moreover, the irreversible mPTP opening can lead to the reduction of ATP production by abolishing mitochondrial membrane potential, which finally results in necrotic cell death from energy depletion [69]. Despite the inhibition of MPT by the opening of mitoK_{ATP} channels, a 2008 study by Rickover et al. demonstrated that exogenous NO from SNP decreased intracellular Ca²⁺ overload in cardiomyocytes after ischemia-reoxygenation [85]. Since the intracellular Ca²⁺ overload can directly activate mPTP opening, the reduction of intracellular Ca²⁺ could attenuate the activation of mPTP in I/R condition [86].

RESEARCH TRANSLATION OF NITRIC OXIDE AGAINST I/R INJURY

NO-mediated signaling is intimately involved in the pathway that triggers protection against I/R injury. The preconditioning pathway initiates on the sarcolemmal membrane of cardiomyocytes, and carries messages to intracellular enzyme cascading, and, finally, to mitochondria. During the IPC process, myocardial resistance to hypoxic conditions can occur as a result of a brief ischemia before the reperfusion episode. Unfortunately, IPC is not feasible in patients presenting at the hospital with an acute MI. The goal of effective treatment of acute MI in humans, therefore, is to induce an IPC-like effect that can help myocardium to survive the prolonged ischemic process, similar to that in preconditioning.

Enhancing the NO signaling pathway could become a widely accepted method for mimicking the preconditioning effect, as this signaling plays an important role in both physiological and pathological conditions. Drugs such as NO donor or PDE-5 inhibitor are widely used to interrupt the NO signaling pathway. Many studies have found that sildenafil citrate, the PDE-5 inhibitor used for treatment of erectile dysfunction, has abilities to attenuate ischemic cardiomyopathy and reduce cell death from hypoxic conditions [87–90]. Ockaili et al. demonstrated that sildenafil citrate was able to decrease infarct size after coronary artery occlusion in rabbits, and that the effect was blocked by inhibiting mitoK_{ATP} channel (5-HD) [87]. Another study by this team showed that the expression of iNOS and eNOS mRNA and protein in isolated mouse hearts could be increased by sildenafil citrate [88]. The level of eNOS mRNA increased transiently and peaked at 45 minutes after the sample was treated with the drug [88]. The rate of iNOS mRNA expression was slower, but the peak was higher than that of eNOS. Moreover, a significant increase in iNOS and eNOS proteins was detected 24 hours after treatment with sildenafil citrate. The cardioprotective effect against I/R injury induced by sildenafil citrate was eliminated by 1400W, the specific iNOS inhibitor [88]. Das et al. demonstrated that, in an isolated ventricular myocyte, necrosis and apoptosis were reduced, whereas the expression of iNOS and eNOS was elevated, when sildenafil citrate was applied after an ischemia-reoxygenation [89].

CONCLUSIONS

Since sildenafil citrate and other PDE-5 inhibitors have been approved by the U.S. Food and Drug Administration (FDA) as a vasoactive drug for the treatment of erectile dysfunction, the drugs may be useful for cardioprotection against I/R injury. Moreover, other pharmacological agents that are related to the NO signaling pathway, including NO donors such as nitroglycerin or drugs that have been shown to have positive effects on NO signaling in β -blockers such as nebivolol, could also impart the NO-related cardioprotection against ischemic cell death [91,92]. According to our understanding of the IPC mechanism, the drugs that regulate some parts of its cascade, including adenosine, bradykinin, opioid agonists, or PI3K-Akt activators, could be applied in clinical practice as possible cardioprotective agents against I/R injury [93]. However, clinical trials of these agents against I/R injury have not yet been conducted. Future demonstration

of the cardioprotective effect of pharmacological IPC in patients should be explored, and this could have an enormous impact on development of drugs for clinical application of pharmacological preconditioning.

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Effects of cilostazol in the heart

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Cilostazol is a selective phosphodiesterase 3 (PDE3) inhibitor approved by the Food and Drug Administration for treatment of intermittent claudication. It has also been used in bradyarrhythmic patients to increase heart rates. Recently, cilostazol has been shown to prevent ventricular fibrillation in patients with Brugada syndrome. Cilostazol is hypothesized to suppress transient outward potassium (I_{to}) current and increase inward calcium current, thus, maintaining the dome (phase 2) of action potential, decreasing transmural dispersion of repolarization and preventing ventricular fibrillation. Although many PDE3 inhibitors have been shown to increase cardiac arrhythmia in heart failure, cilostazol has presented effects that are different from other PDE3 inhibitors, especially adenosine uptake inhibition. Owing to this effect, cilostazol could be an effective cardioprotective drug, with its beneficial effects in preventing arrhythmia. In this review, the cardiac electrophysiological effects of cilostazol are presented and its possible cardioprotective effects, particularly in

preventing ventricular fibrillation, are discussed, with emphasis on the need to further verify its clinical benefits. *J Cardiovasc Med* 12:88–95 © 2011 Italian Federation of Cardiology.

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Introduction

Cilostazol (PLETAL or PLETAAL) is a quinolinone derivative and a selective phosphodiesterase 3 (PDE3) inhibitor. In humans, the PDE3 isoenzyme is mainly found in platelets, in vascular smooth muscle, in the liver, in the smooth muscle of the airway, in T lymphocytes, in adipose tissue and in cardiac tissue [1]. Because of its potent vasodilation effect and antiplatelet aggregation, cilostazol has been approved by the US Food and Drug Administration (FDA) for claudication treatment to relieve leg pain symptoms, allowing greater tolerance in patients with chronic arterial occlusion [2,3]. Although cilostazol has been used mainly in intermittent claudication (chronic arterial occlusion), it has been shown to be useful in preventing embolic stroke in arrhythmic patients [4], and could decrease the wall thickness of coronary arteries in type 2 diabetic patients [5]. In addition, it has been found that cilostazol could affect the automaticity of the heart and also increase heart rate, leading to an improvement of bradyarrhythmias [4], including bradycardic atrial fibrillation, atrioventricular block and sick sinus syndrome (SSS) [6–11]. Recently, a case report of Brugada syndrome patients showed that the oral administration of cilostazol at therapeutic dose could prevent the frequency of ventricular fibrillation in these patients [12]. The report suggested that, in addition to its vasodilating effect, cilostazol could potentially have antiarrhythmic effects.

In recent decades, PDE3 inhibitors such as milrinone and vesnarinone have been shown to be arrhythmogenic and to increase mortality rate in heart failure patients [13,14]. The proposed mechanism for this undesirable effect was the drug's effects in increasing the cyclic AMP (cAMP) level [13,14]. Thus, according to the FDA, all PDE3 inhibitors, including cilostazol, are contraindicated in chronic heart failure patients of any severity because of the recognition that they increased the mortality rates in this group of patients [15]. Despite the fact that cilostazol is a PDE3 inhibitor, it is important to note that its roles in the heart are different from those of other PDE3 inhibitors. Cilostazol has been demonstrated to not only inhibit PDE3 but also to inhibit adenosine uptake, resulting in a reduction of cyclic adenosine monophosphate (cAMP) level [16–19]. Therefore, unlike other PDE3 inhibitors, these actions of cilostazol may be beneficial in cardio-protection [16–19].

Although the vasodilation and antiplatelet aggregation effects of cilostazol have been extensively investigated and reviewed [20–23], it is still unclear whether cilostazol would have beneficial or adverse effects on the heart. As cilostazol is currently of interest to many investigators and because it could have different effects on the heart compared with other PDE3 inhibitors, this review will present the roles of this PDE3 inhibitor and its arrhythmogenic effect on the heart. The electrophysiological

and cardioprotective effects of cilostazol, on the basis of its cellular mechanism, which is different from that of other PDE3 inhibitory drugs, will be discussed. The mechanisms of cilostazol on arrhythmogenic prevention, particularly its role on ventricular fibrillation prevention in Brugada syndrome, will also be presented. With increasing knowledge and understanding of its mechanisms, cilostazol may be clinically useful for arrhythmia prevention in the future. Finally, the ultimate goal of this review is to provide the pathophysiological basis for future investigations to comprehend the rationale of the study design that verifies the effects of cilostazol in a heart failure model.

Phosphodiesterase 3 inhibitor and the heart

PDE is the enzyme that hydrolyses cAMP and cyclic guanosine monophosphate (cGMP) to their inactive state, thus regulating levels of intracellular cAMP and cGMP. PDE has been classified into 11 isoenzymes, depending on the differences of the affinity for cAMP, cGMP and the physiochemical and pharmacological properties of PDE [1,24]. Generally, once adenylate cyclase and guanylate cyclase pathways are generated by the induction of extracellular signals, ATP and GTP are then changed to cAMP and cGMP, respectively, depending on their corresponding pathways. cAMP and cGMP are the second messengers that mediate biological responses. The active forms of both cAMP and cGMP are converted to their inactive forms by PDE [1,24].

PDE3 belongs to the PDE family that has high affinity for both cAMP and cGMP [1]. As the binding of cGMP at the active site of PDE3 can competitively inhibit cAMP hydrolysis, this enzyme is also defined as cGMP-inhibited PDE [1]. However, the amount of cAMP that is hydrolyzed by PDE3 is 10 times greater than that of cGMP [1]. The increased level of intracellular cAMP could activate cAMP-dependent protein kinase A (PK-A), leading to phosphorylation of important proteins or receptors in the heart, which involve a diversity of cellular functions (Fig. 1). In a sinoatrial node, cAMP enables a signal transduction involving the activated PK-A, the L-type Ca^{2+} channel (I_{CaL}), and a component of a delay rectifier potassium channel (I_{Ks}). Therefore, drugs that block PDE3 pathways would cause a positive chronotropic effect [6,8,9,11].

PDE3 is primarily associated with the regulation of contractile function in the heart. When the activity of PK-A in the myocardium is raised by the increased intracellular cAMP from the PDE3 inhibitor, phosphorylation of I_{CaL} channels in sarcolemmal membrane and ryanodine receptor (RyR) occurs. The Ca^{2+} influx through I_{CaL} channels trigger a release of Ca^{2+} from sarcoplasmic reticulum via RyR. The binding of this increased cytoplasmic Ca^{2+} concentration to troponin C activates actin–myosin cross-bridge formation, thus, eli-

citing the contractile function [25]. In many studies, the benefits of inotropic action of PDE3 inhibitors such as amrinone, enoximone and milrinone in chronic heart failure patients have been demonstrated, for example, the reduction of left ventricular end-diastolic pressure and the increase in ejection fraction and stroke volume [26–31]. It has been observed that the use of PDE3 inhibitors actually produce desirable effects in the short term, however, deleterious effects arise over time [26–28].

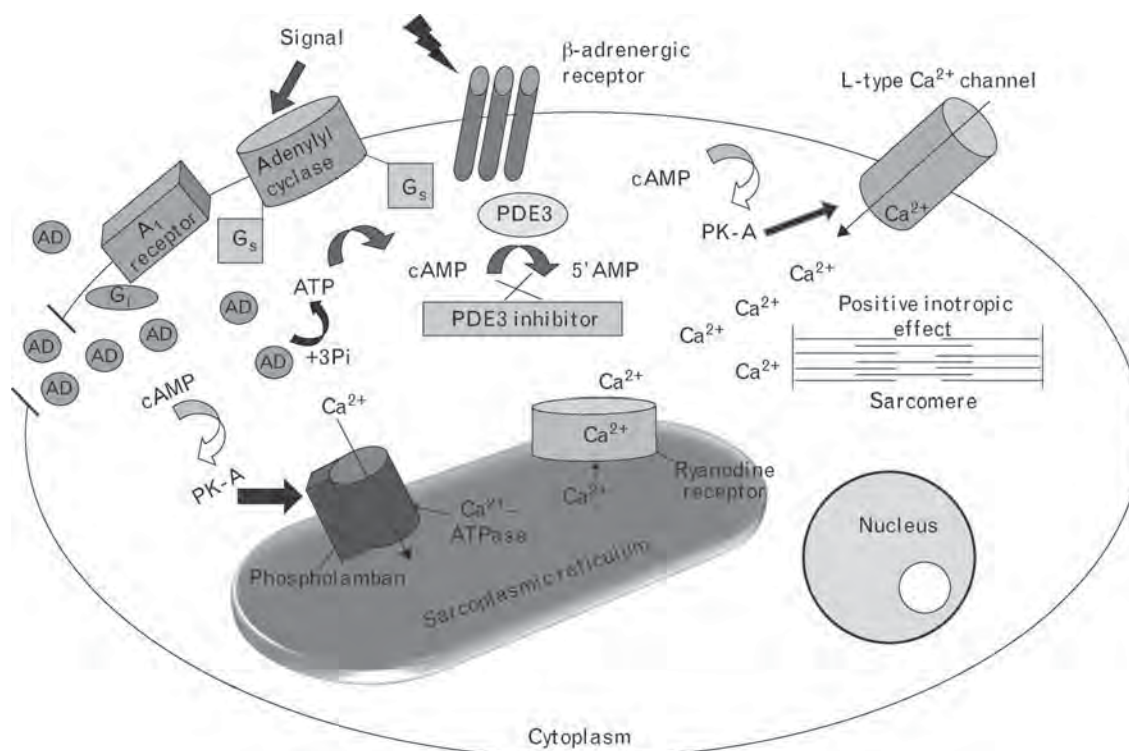
Undesirable effects of phosphodiesterase 3 inhibitors in the heart

The adverse effects of PDE3 inhibitors on the heart have been demonstrated, especially the acceleration of the progression of heart failure and the induction of ventricular tachyarrhythmia [13,14,32,33]. The remarkable clinical studies that demonstrated the significant increase in cardiovascular mortality were the Prospective Randomized Milrinone Survival Evaluation (PROMISE) [14] and the Vesnarinone trial (VEST) [13] studies, which reported increased cardiovascular mortality due to PDE3 inhibitor administration. In the latter study, 120 mg of vesnarinone was suddenly withdrawn due to its severe adverse effect. These reports were consistent with the data of the randomized controlled trial that investigated the effects of PDE3 inhibitors versus placebo in chronic heart failure patients [32].

As the deleterious effects of PDE3 inhibitors could be due to the exertion of cAMP, it is interesting to explore the signal pathway that causes changes in the level of cAMP. The β -adrenergic agonist, which shows undesirable effects, including mortality rates and progressive heart failure, also behaves in a manner similar to the PDE3 inhibitors [34,35]. It has been shown that the β -adrenergic antagonist can decrease intracellular cAMP content in cardiac myocytes by decreasing the transmembrane signal, hence, improving the symptoms and the survival rate in heart failure. The studies of PDE3 inhibitors adjuvant to the β -adrenergic antagonist have shown an improvement of the ejection fraction and functional status in patients with dilated cardiomyopathy and chronic heart failure [36–39].

Previous studies have demonstrated that the level of cAMP was lower in failing hearts than in normal hearts, which could be an adaptive body response to protect myocardial cells from further injury [32]. Several studies demonstrated that cAMP could be the substrate that caused adverse effects on the failing heart [32,34,35]. The reduction of cAMP in heart failure may be caused by the downregulation of β -adrenoreceptors, enhanced expression of G_i -protein or increased β -adrenoreceptor kinase [40]. In heart failure, there are disturbances of Ca^{2+} modulation in which abnormal expression and function of a sarco/endoplasmic reticulum ATPase (SERCA) plays an important role [41]. Schmidt *et al.*

Fig. 1



Effect of phosphodiesterase 3 (PDE3) inhibitors in cardiomyocytes. The pathway can be directly activated by signals through adenylyl cyclase or the β -adrenergic receptor. The myocardium uptakes adenosines (ADs) to synthesize ATP. Then, the adenylyl cyclase enzyme converts ATP to cyclic AMP (cAMP). The increasing of cAMP can lead to many physiological responses via the activation of cAMP-dependent protein kinase A (PK-A). This PK-A can phosphorylate L-type Ca^{2+} channels and ryanodine receptors to stimulate Ca^{2+} -induced Ca^{2+} -release mechanism, leading to cardiac contraction. During the cardiac relaxation period, the phosphorylation of phospholamban by PK-A can elicit Ca^{2+} reuptake through Ca^{2+} -ATPase on the sarcoplasmic reticulum. Under physiological condition, cAMP is hydrolyzed to an inactive product (5'AMP) by PDE3 enzyme. Therefore, inhibition of PDE3 (i.e. by PDE3 inhibitors) can cause an increased cAMP level, thus enhancing the cAMP action, especially the positive inotropic effect. In heart failure, this is an undesired effect caused by PDE3 inhibitors.

[42] demonstrated that an impaired SERCA function in human end-stage heart failure patients could be due to the reduction of cAMP-dependent phosphorylation of the phospholamban. These situations altered intracellular Ca^{2+} homeostasis by reducing systolic peak Ca^{2+} , increasing diastolic Ca^{2+} and sustaining diastolic Ca^{2+} sequestration, thus producing electrophysiological abnormality in the heart [42].

As a result of intracellular cAMP and Ca^{2+} dynamic disturbances, PDE3 inhibitors can cause ventricular arrhythmia by several mechanisms. First, the increased intracellular cAMP, which triggers early and delayed after-depolarizations (DADs), could provoke a slow conduction and alter refractoriness in depolarized tissues [43]. Second, an abnormal Ca^{2+} homeostasis due to the increased intracellular cAMP, that is, an abnormal increase in RyR2 opening probability during diastole, which is induced by chronic PK-A-mediated hyperphosphorylation in failing hearts [43,44]. This condition can promote the occurrence of DAD via transient activation of a Ca^{2+} -dependent

inward current [43,44]. Third, the higher myocardial oxygen consumption due to the inotropic effect of these PDE3 inhibitors can increase the frequency of arrhythmias [33,45]. One should be aware of these adverse effects of PDE3 inhibitors if they are to be used as inotropic drugs, particularly in heart failure patients [13,33,34,46–48].

Cilostazol and its dual inhibitory effect in the heart

Cilostazol is a quinolinone derivative that selectively inhibits PDE3. Because of its potent vasodilating and anti-thrombotic effects, cilostazol has been used for effective treatment of intermittent claudication, a manifestation of chronic arterial occlusive disease. As cilostazol is a PDE3 inhibitor, it falls into the same category as the other PDE3 inhibitors whose use the FDA prohibits with heart failure patients [2]. Currently, there is no study that directly investigates the effect of cilostazol in heart failure.

Growing evidence has demonstrated that cilostazol could have many potential benefits on the heart and that the

prohibition of its use should be reinvestigated and reconsidered [4,6,8–11,16,21,23,46,49,50]. This is due to the findings that although cilostazol is a PDE3 inhibitor, its effects on the heart could be different from other PDE3 inhibitors (Fig. 2) [16,17]. One such piece of evidence is the finding that the positive inotropic effect caused by cilostazol was much less than that caused by milrinone [16,19,21]. It has been shown that cilostazol has a dual inhibitory effect, in which it not only selectively inhibits PDE3 but also inhibits adenosine uptake into cardiac myocytes [17]. The latter mechanism is only found in cilostazol and not in other PDE3 inhibitors [17].

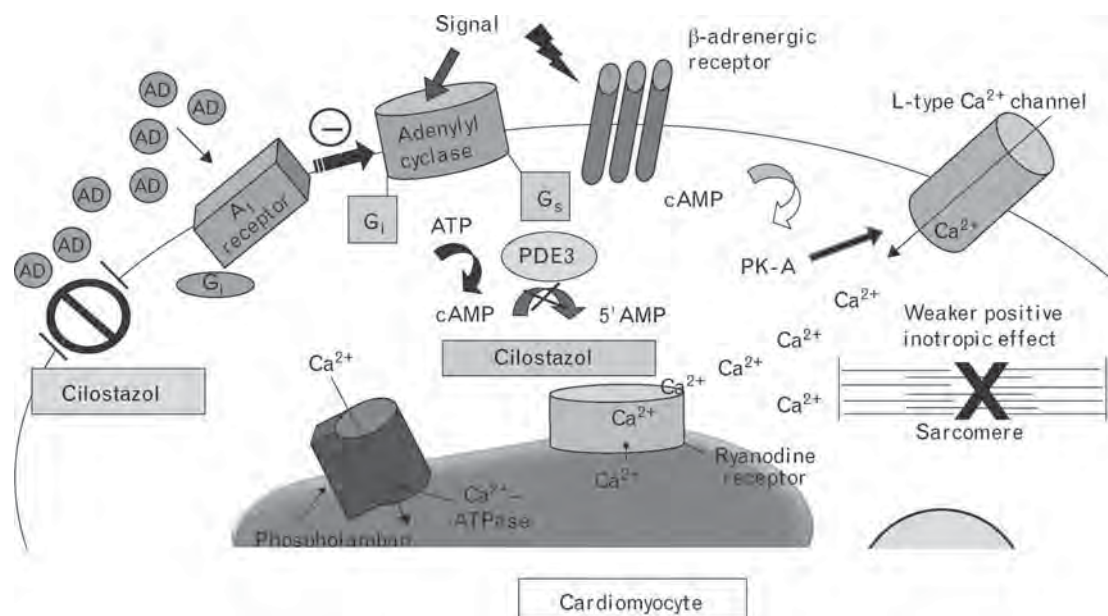
In the myocardium, adenosine acts on the A_1 receptor which then activates G_i -protein. As a result, the adenylate cyclase pathway is inhibited by adenosine through the adenosine A_1 -receptor, thus, attenuating the positive inotropy. In addition, the inhibitory effect of cilostazol on adenosine uptake could lead to low intracellular adenosine level, resulting in decreased cAMP level [17,19]. Thus, this effect could attenuate the inotropic effect, compared with other PDE3 inhibitors (Fig. 2). However, this effect is different in pacemaker cells, in which the ATP and adenine derivatives potentially enhance the negative chronotropic effect [51]. Therefore, cilostazol could cause a positive chronotropic effect due to its inhibitory effect on adenosine uptake. Furthermore,

if adenosine uptake into cardiomyocytes is inhibited, adenosine in the interstitial space and blood circulation will be increased, resulting in an activation of adenosine A_2 -receptors in platelets and vascular smooth muscle and causing vasodilation and antiplatelet aggregation [16,19,21]. Therefore, unlike other PDE3 inhibitors that have been shown to cause an increase in mortality rate in heart failure patients and induced tachyarrhythmia via cAMP [19,32,33,52], cilostazol may provide the beneficial effects of PDE3 inhibitors, without needing to be over-concerned about its adverse effects in heart failure patients, due to its mechanism as described above. It is important to note that current evidence regarding cilostazol and tachyarrhythmia is still limited and unclear. Nevertheless, recent evidence has indicated that cilostazol could lower, at least not increase, major cardiovascular events in patients with coronary artery or peripheral artery disease [53–56].

Cilostazol and bradyarrhythmia

For symptomatic bradycardia, the implantation of a cardiac pacemaker is the first-line regimen of treatment [57,58]. Nevertheless, the overuse of hospitalization and long bed rest is still a cost burden. Therefore, alternative therapies have been proposed, especially in terms of medication use. Recently, theophylline, a

Fig. 2



Effects of cilostazol in cardiomyocytes. Unlike other phosphodiesterase 3 (PDE3) inhibitors, cilostazol has a dual inhibitory effect. In addition to inhibition of PDE3, cilostazol also inhibits cellular adenosine uptake. As the intracellular adenosines (ADs) are reduced, the cAMPs would be decreased. Although adenylate cyclase can still function normally when the PDE3 is inhibited, the cyclic AMP (cAMP) level will not be sufficiently increased in this case. Furthermore, cAMP signaling can be attenuated due to the accumulation of adenosines outside the cell, resulting in the attenuation of the adenylate cyclase activity pathway through the G_i -protein of the A_1 -receptor. This dual inhibitory effect of cilostazol may be responsible for diminished inotropic effect, compared with other PDE3 inhibitors. PK-A, cAMP-dependent protein kinase A.

nonselective PDE inhibitor, had been shown to have clinical efficacy in bradyarrhythmia treatment [4]. However, it had produced adverse reactions in which it increased the number and degree of ventricular arrhythmias. Previous studies also demonstrated that amrinone, a PDE3 inhibitor, decreased the effective refractory period of the right atrium and functional refractory period of the atrioventricular node [4,33,59,60]. In studies using milrinone, there was a trend toward a lower corrected sinus-node recovery time and maximal 1:1 atrioventricular conduction [33]. These findings suggested that PDE3 inhibitors could have positive chronotropic and dromotropic effects.

Atarashi *et al.* [4] demonstrated that cilostazol increased heart rates and improved the symptoms in patients with bradyarrhythmia, bradycardic atrial fibrillation, SSS and Wenckebach-type atrioventricular block. In patients with third-degree atrioventricular block, cilostazol also increased heart rates and additionally increased ventricular escape rate, without abolishment of atrioventricular block or change in the frequency of premature ventricular beats [6]. Furthermore, cilostazol has been shown to decrease atrial natriuretic peptide and brain natriuretic peptide concentration [6,7], the hormones that are present in atrioventricular asynchrony and heart failure patients [61].

Investigations of the roles of cilostazol on heart rate variability (HRV) have shown that cilostazol significantly improved the slow heart rate, the frequency domain in SSS and bradycardic atrial fibrillation patients [9,11]. Although the mechanism underlying the chronotropic effect of PDE3 inhibitors has been clarified, the direct mechanism of cilostazol is still unclear, as there is no cardiac electrophysiological study regarding the effect of cilostazol. Nevertheless, the positive chronotropic effect of cilostazol has been proposed to be due to several mechanisms. Cilostazol causes an increased coronary blood flow and blood supply to the sinus node or the atrioventricular node in patients who have bradyarrhythmia arising from the ischemic process [4]. Cilostazol also antagonizes ATP, which is a factor causing sinus bradyarrhythmia and atrioventricular delays [4]. It is known that ATP and adenosine produce a negative chronotropic effect by causing a hyperpolarization of maximum diastolic potential. The hyperpolarization occurs when adenosine or ATP induces a time-dependent inwardly rectifying outward K^+ current [61]. It has also been proposed that adenosine inhibits noradrenaline release [51,61]. Furthermore, cilostazol can inhibit the adenosine uptake, resulting in a strong positive chronotropic effect (Fig. 2). It has been shown that the increase of cAMP in nodal cells caused by cilostazol could also cause adrenergic stimulation [4]. In addition, it is known that cilostazol causes systemic vasodilatation, thus producing a reflex adrenergic stimulation [4]. Although the positive dromotropic effect of PDE3

inhibitors is still unclear, it is possible that the negative dromotropic effect of adenosine is antagonized by cilostazol [62]. Despite the fact that cilostazol has a positive chronotropy and some evidence has suggested its possible undesired tachyarrhythmic effect [8,63–65], recent reports and meta-analysis have indicated that it could either lower the incidence of cardiac death and major cardiac events or, at least, that it did not increase major cardiovascular events or mortality in patients treated for coronary artery disease and peripheral vascular disease [53–56].

Cilostazol and ventricular tachyarrhythmia

Brugada syndrome is another cardiac electrophysiological abnormality caused by an abnormal *SCN5A* gene [66]. It causes a decreased sodium current and gives prominent transient outward potassium current (I_{to}) in the epicardium rather than in the endocardium, wherein this imbalance of current induces a prominent notch in phase 1 of the action potential. Consequently, the loss of the dome of action potential in the ventricular epicardium, but not endocardium, produces ST elevation and develops transmural dispersion of repolarization, which is vulnerable to ventricular fibrillation induction [66–72].

In a recent case report of Brugada syndrome patients who experienced several episodes of ventricular fibrillation and retained implantable cardioverter defibrillators (ICDs), it was found that the ventricular fibrillation episode was completely abolished after being treated with cilostazol at 200 mg/day [12]. However, when cilostazol was discontinued, ventricular fibrillation recurred even when being treated at 100 mg/day. After 200 mg/day of cilostazol was readministered, ventricular fibrillation disappeared. In a 1-year follow-up in three ICD implanted patients, no ventricular fibrillation was observed with cilostazol treatment [12]. This report strongly indicates the need for further investigation regarding the beneficial effects of cilostazol in preventing ventricular tachyarrhythmia. Supporting this notion is a recent report by Kanlop *et al.* [73] who demonstrated in swine that intravenous administration of cilostazol (6 mg/kg) could increase the ventricular fibrillation threshold, as well as improve defibrillation efficacy.

Several mechanisms have been proposed to explain the effects of cilostazol in preventing ventricular fibrillation in those cases with Brugada syndrome. Cilostazol has the ability to accelerate heart rate, which can suppress the transient outward K^+ channel (I_{to}) [12]. If I_{to} is suppressed, other channels that always open following the I_{to} will be decelerated. This effect can prolong the action potential duration. Also, cilostazol can increase intracellular cAMP, which can lead to an increase in intracellular Ca^{2+} . This effect could preserve the dome of an action potential and normalizes the ST segment, thus preventing arrhythmia [12].

The sympathovagal imbalance during a nocturnal period indicated by an increased vagal tone and a decreased sympathetic tone has been proposed as a major factor in aggravating arrhythmia in Brugada patients [74,75]. HRV in Brugada patients demonstrated a nocturnal standard deviation of averaged $N-N$ intervals (SDANN), an ultra-low-frequency (ULF) and a low-frequency component were lowered, whereas a high-frequency component was increased in patients who showed deleterious symptoms [74,75]. The results also demonstrated the association between a ST segment elevation following ventricular fibrillation events and an increased vagal activity [74,75]. It is possible that the inhibition of adenosine uptake by cilostazol could prevent vagal hyperactivity in Brugada syndrome. Furthermore, the PDE3 inhibitory effects of cilostazol could improve the sympathetic tone through the adenylate cyclase pathway by increasing cAMP levels. These dual mechanisms could, therefore, maintain a sympathovagal balance in Brugada syndrome. However, the hypothesis that cilostazol can improve HRV in Brugada patients needs to be validated in future studies.

In contrast to the report by Tsuchiya [12], Abud *et al.* [76] have demonstrated that cilostazol failed to prevent ventricular fibrillation in Brugada patients. As the ventricular tachyarrhythmia in Brugada syndrome could originate in the ventricle or in the atrium, as atrial fibrillation precipitating ventricular fibrillation [77], it is possible that the different genotypes of Brugada syndrome, as presenting with or without atrial fibrillation [76,78], might affect the result of cilostazol treatment in different ways. Therefore, it may be concluded that cilostazol could be useful for treating ventricular fibrillation in Brugada syndrome if the arrhythmia originates in the ventricle. However, in atrial fibrillation-precipitated ventricular fibrillation cases, cilostazol may not prevent the occurrence of ventricular fibrillation [76].

Conclusion

Cilostazol has been shown to affect cardiac electrophysiological properties by increasing inward Ca^{2+} current and decreasing outward potassium current (I_{to}), thus, minimizing the ventricular arrhythmia induction. Cilostazol increases intracellular cAMP in myocardial cells to a lesser degree, compared with other PDE3 inhibitors, and inhibits adenosine uptake in myocardial cells. It has also been shown to be beneficial in bradyarrhythmias. Furthermore, despite its positive chronotropic effect, recent reports have indicated that it did not increase major cardiovascular events when used in patients with coronary artery or peripheral artery diseases. All of these effects of cilostazol could be beneficial in arrhythmia prevention, even in heart failure. Future investigations on the effects of cilostazol in the heart failure model are needed to warrant its possible clinical benefits.

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PPAR γ activator, rosiglitazone: Is it beneficial or harmful to the cardiovascular system?

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against arteriosclerosis by normalizing the metabolic disorders and reducing chronic inflammation of the vascular system. Despite these benefits, inconsistent findings have been reported, and growing evidence has demonstrated adverse effects of rosiglitazone on the cardiovascular system, including increased risk of acute myocardial infarction, heart failure and chronic heart failure. As a result, rosiglitazone has been recently withdrawn from EU countries. Nevertheless, the effect of rosiglitazone on ischemic heart disease has not yet been firmly established. Future prospective clinical trials designed for the specific purpose of establishing the cardiovascular benefit or risk of rosiglitazone would be the best way to resolve the uncertainties regarding the safety of rosiglitazone in patients with heart disease.

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Key words: Rosiglitazone; Ischemic reperfusion injury; Heart disease; Type II diabetic; Thiazolidinediones

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Abstract

Rosiglitazone is a synthetic agonist of peroxisome proliferator-activated receptor γ which is used to improve insulin resistance in patients with type II diabetes. Rosiglitazone exerts its glucose-lowering effects by improving insulin sensitivity. Data from various studies in the past decade suggest that the therapeutic effects of rosiglitazone reach far beyond its use as an insulin sensitizer since it also has other benefits on the cardiovascular system such as improvement of contractile dysfunction, inhibition of the inflammatory response by reducing neutrophil and macrophage accumulation, and the protection of myocardial injury during ischemic/reperfusion in different animal models. Previous clinical studies in type II diabetes patients demonstrated that rosiglitazone played an important role in protecting

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INTRODUCTION

Type II diabetes mellitus (T2DM) is a disease whose incidence is dramatically increasing and requires continuing medical management in many countries^[1,2]. T2DM is characterized by insulin resistance and impaired glucose tolerance^[3]. The development of T2DM involves 3 metabolic defects that include insulin resistance, alterations in hepatic glucose production and β -cell deficiency^[4]. The

earliest defect seen in the development of T2DM is insulin resistance^[4]. In this state, the β -cells produce large amounts of insulin reaching a supraphysiologic level as a compensatory response to peripheral tissue insulin resistance. If insulin resistance is left untreated, the β -cells begin to fail to produce insulin, resulting in a state of relative insulin deficiency^[4]. T2DM is known to develop after this phase. T2DM patients have a 2- to 4-fold increased risk of developing coronary artery disease, unstable angina and myocardial infarction (MI)^[5,6]. Furthermore, T2DM patients have been shown to have a worse prognosis than non-diabetic patients after a cardiovascular event^[7,8].

Thiazolidinediones (TZDs) are an oral medication developed to reduce insulin resistance in T2DM patients and have been used since 1997^[9]. TZDs exert their properties by stimulating a nuclear hormone receptor, the peroxisome proliferator-activated receptor γ (PPAR γ)^[10]. Troglitazone was the first drug developed but was withdrawn from the market due to liver toxicity^[9]. Currently, pioglitazone and rosiglitazone are the only compounds that are available for clinical use^[9]. However, the effects of rosiglitazone have been controversial regarding cardiovascular effects in both animal and clinical studies^[11-59]. On the positive side, rosiglitazone has been shown to exert its potent insulin sensitization by improving insulin resistance in T2DM^[10,60]. Various studies demonstrated that the therapeutic effects of rosiglitazone could reach far beyond its action as an insulin sensitizer because it has other therapeutic effects on many organs especially in the cardiovascular system in both animal models and humans^[11-59]. Nevertheless, in the past decade growing evidence from both basic and clinical studies indicates that rosiglitazone could be harmful to the heart^[11-25]. Because of its serious undesired effects, rosiglitazone has been recently withdrawn from the EU market^[61], and is under close monitoring by the US Food and Drug Administration^[62,63].

In this review, we aim to summarize and discuss the overall benefits as well as the adverse effects of rosiglitazone on the heart from both pre-clinical and clinical reports. Understanding the inconsistent findings as well as the limitations found in each study using rosiglitazone should allow investigators to carefully design future studies that hopefully can clarify previous inconsistent findings, and to indicate whether rosiglitazone should be used in patients.

BENEFICIAL EFFECTS OF ROSIGLITAZONE ON THE CARDIOVASCULAR SYSTEM

Previous studies reported that rosiglitazone had beneficial effects on the cardiovascular system in *in vitro*, *in vivo* and clinical studies. These beneficial effects of rosiglitazone are summarized in Table 1. In rat models of ischemia/reperfusion (I/R) injury, pretreatment with rosiglitazone

reduced the infarct size and improved ischemia/reperfusion-induced myocardial contractile dysfunction^[26,46,48]. Rosiglitazone treatment also improved left ventricular (LV) systolic pressure and positive and negative maximal values of the first derivative of LV pressure (dP/dt) during I/R injury^[32,46]. In addition, in both obese and normal rats rosiglitazone could decrease systolic blood pressure, improve contractile function and normalize the insulin level^[31,44,45]. These findings suggested that rosiglitazone could prevent the development of hypertension associated with insulin resistance. This notion was supported by the finding that rosiglitazone treatment could enhance nitric oxide (NO)-mediated arteriolar dilation^[28]. Furthermore the accumulation of neutrophils and macrophages and expression of monocyte chemoattractant factor (MCP)-1 in the ischemic heart was diminished by rosiglitazone^[46]. Likewise, rosiglitazone treatment in diabetic rat and mouse models reduced the blood levels of glucose, triglycerides, and free fatty acids, and enhanced cardiac glucose oxidation in the ischemic myocardium^[26,50]. Rosiglitazone treatment also reduced myocardial apoptosis and infarction size post I/R injury by restoring the balance between the pro-apoptotic and anti-apoptotic mitogen-activated protein kinase (MAPK) signaling pathway, increasing phosphatidylinositol-3-kinase-Akt phosphorylation, and inhibiting p42/44 MAPK^[26,35,38,41,59].

In *in vitro* studies, incubation of a rat cardiomyoblast cell line with rosiglitazone demonstrated cardioprotective effects against oxidative stress, and the antioxidant enzyme heme oxygenase 1 was upregulated in these cells after rosiglitazone treatment^[40]. Furthermore, rosiglitazone could inhibit cardiac fibroblast proliferation, increase connective tissue growth factor expression and decrease NO production induced by advanced glycation endproducts in cultured neonatal rat cardiac fibroblasts^[37]. In addition, rosiglitazone could prevent cardiac hypertrophy by inhibiting angiotensin II^[27,32,54].

Many clinical studies reported that the beneficial effects of rosiglitazone on the cardiovascular system were similar to those from animal studies. Rosiglitazone therapy has been shown to reduce cardiovascular complications associated with T2DM^[47,49,58]. Data from preliminary studies in patients who underwent coronary angioplasty and stent implantation demonstrated that rosiglitazone treatment for 6 mo led to a lower occurrence of restenosis and a lower degree of stenosis of the luminal diameter after angioplasty^[64]. Furthermore, a study in patients with T2DM demonstrated that treatment with 4 mg/d of rosiglitazone for 12 wk decreased not only insulin resistance but also pulse wave velocity, which is a direct parameter of arterial stiffness in patients with diabetes and coronary arteries disease (CAD)^[58]. Rosiglitazone has been shown to reduce plasma levels of C-reactive protein^[5,51,56,58], matrix metalloproteinase-9 and MCP-1^[58] in T2DM patients, suggesting that rosiglitazone plays an important role in protecting against arteriosclerosis by normalizing metabolic disorders and reducing chronic inflammation of the vascular system. Rosiglitazone treat-

Table 1 Reports of the beneficial effects of rosiglitazone on the cardiovascular system in pre-clinical and clinical studies

Models	Dose of rosiglitazone	Major findings	Interpretation	Ref.
Cultured neonatal rat cardiomyocytes	5 µmol/L; pretreated for 30 min before stimulation with Ang II (1 µmol/L) for 48 h	Inhibited Ang II-induced upregulation of skeletal α -actin and ANP genes, and prevent an increase in cell surface area	Rosiglitazone involved in the inhibition of cardiac hypertrophy	[27]
Isolated and cultured neonatal rat ventricular myocytes	1, 5, 10 µmol/L; pretreated for 48 h	Accelerated Ca^{2+} transient decay rates Increased SERCA2 mRNA levels Upregulation of IL-6 secretion Enhanced TNF- α - and lipopolysaccharide-induced NF- κ B-dependent transcription	Cardioprotective effects of rosiglitazone may be mediated <i>via</i> NF- κ B	[43]
Isolated and cultured adult rat ventricular myocytes	10^{-8} - 10^{-5} mol; pretreated for 24 h	Did not increase protein synthesis Did not attenuate hypertrophic response to noradrenaline, phorbol-12-myristate13-acetate and endothelin-1	Rosiglitazone did not directly induce cardiomyocyte hypertrophy in cardiomyocytes	[30]
Cultured neonatal rat ventricular myocytes	10 µmol/L; pretreated for 24 h	Inhibited the endothelin-1-induced increase in protein synthesis, surface area, calcineurin enzymatic activity, and protein expression Inhibited the nuclear translocation of NFATc4 Enhanced the association between PPAR γ and calcineurin/nuclear factor of activated T-cells	Rosiglitazone inhibited endothelin-1-induced cardiac hypertrophy <i>via</i> calcineurin/nuclear factor of activated T-cells pathway	[29]
Cultured rat cardiomyoblast cell line H9c2(2-1)	100 µmol/L; pretreated for 24 h	Increased expression of heme oxygenase 1 Increased cell viability under oxidative stress induced by H $_2$ O $_2$	Rosiglitazone had cardioprotective effects against oxidative stress	[40]
Cultured neonatal rat cardiac fibroblasts	0.1, 1, 10 µmol/L; pretreated for 48 h	Inhibited cardiac fibroblast proliferation Increased connective tissue growth factor expression Decreased nitric oxide production induced by advanced glycation endproducts	Rosiglitazone could prevent myocardial fibrosis	[37]
Cultured neonatal rat ventricular myocytes	1 µmol/L; pretreated for 30 min prior to H $_2$ O $_2$ treatment	Decrease cell apoptosis Increase Bcl-2 protein content	Rosiglitazone protected cells from oxidative stress through upregulating Bcl-2 expression	[42]
Cultured neonatal rat cardiac myocytes	0.1, 1, 3, 10, 30 µmol/L; pretreated for 30 min before hypoxia	Decreased cytoplasmic accumulation of histone-associated DNA fragments Increased reoxygenation-induced rephosphorylation of Akt Did not alter phosphorylation of the MAP kinases ERK1/2 and c-Jun N-terminal kinase	Rosiglitazone protected cardiac myocytes against I/R injury by facilitating Akt rephosphorylation	[35]
Fatty Zucker rats	7-7.5 µmol/L per kilogram po; 9-12 wk	Decreased systolic blood pressure Decreased fasting hyperinsulinemia Improved mesenteric arteries contraction and relaxation	Rosiglitazone prevented the development of HT and endothelial dysfunction associated with insulin resistance	[45]
Rats with I/R injury	3 mg/kg per day po; pretreated for 7 d; 1 and 3 mg/kg iv given during I/R	Improved left ventricular systolic pressure, dP/dt_{max} and dP/dt_{min} Reduced neutrophils and macrophages accumulation Reduced the infarct size Downregulation of CD11b/CD18 Upregulation of L-selectin on neutrophils and monocytes	Rosiglitazone decreased infarct size and improved contractile dysfunction during I/R possibly <i>via</i> inhibition of the inflammatory response	[46]
Fatty Zucker rats with I/R injury (<i>Ex vivo</i> model)	3 mg/kg po; 7 or 14 d prior to isolated perfuse heart study	Normalized the insulin resistance Restored GLUT4 protein levels Improved contractile function Prevented greater loss of ATP	Rosiglitazone protected obese rat heart from I/R injury	[44]
I/R injury in isolated perfused normal and STZ-induced diabetic rat hearts (<i>Ex vivo</i> model)	1 µmol/L given prior to ischemia; 10 µmol/kg per day po after STZ injection for 4 wk	Inhibited activating protein-1 DNA-binding activity Inhibited of Jun NH $_2$ -terminal kinase phosphorylation Reduced lactate levels and lactate dehydrogenase activity	Rosiglitazone attenuated postischemic myocardial injury in isolated rat heart	[34]
Sprague-Dawley rats	5 mg/kg per day po; 7 d	Reduced systolic blood pressure reduced vascular DNA synthesis, expression of cyclin D1 and cdk4, AT $_1$ receptors, vascular cell adhesion molecule-1, and platelet and endothelial cell adhesion molecule, and NF- κ B activity	Rosiglitazone prevented the development of hypertension and endothelial dysfunction	[31]
T2DM mice	3 mg/kg per day po; 7 d	Did not affect serum glucose and insulin Increased serum 8-isoprostane and dihydroethyline-detectable superoxide production Enhanced catalase and reduced NAD(P)H oxidase activity Did not affect SOD activity	Rosiglitazone enhanced nitric oxide mediation of coronary arteriolar dilations <i>via</i> attenuating oxidative stress in T2DM mice	[28]

Hypercholesterolemic New Zealand rabbits with I/R injury	3 mg/kg per day po; 5 wk prior to I/R	Attenuated postischemic myocardial nitrative stress Restored a beneficial balance between pro- and anti-apoptotic MAPK signaling Reduced postischemic myocardial apoptosis Improved cardiac functional recovery	Rosiglitazone attenuated arteriosclerosis and prevented I/R-induced myocardial apoptosis	[38]
Zucker diabetic fatty rats with I/R injury	3 mg/kg per day po; 8 d prior to I/R	Reduced blood glucose, triglycerides, and free fatty acids levels Enhanced cardiac glucose oxidation Increased Akt phosphorylation (Akt-pS473) and its downstream targets (glycogen synthase kinase-3 β and FKHR-pS256) (forkhead transcription factor) Reduced apoptotic cardiomyocytes and myocardial infarct size	Rosiglitazone protected heart against I/R injury	[26]
Sprague-Dawley rats with I/R injury	3 mg/kg per day po; 7 d prior to I/R	Reduced infarct size Decreased myocardial expression of AT ₁ receptors Increased AT ₂ mRNA and protein expression Inhibited p42/44 MAPK Did not alter Akt1 expression	Rosiglitazone attenuated myocardial I/R injury possibly <i>via</i> increase expression of AT ₂ and inhibition of p42/44 MAPK	[41]
Sprague-Dawley rats with I/R injury	3 mg/kg per day po for 8 wk prior to I/R	Improved left ventricular dP/dt _{max} and dP/dt _{min} Inhibited myocardial angiotensin II and aldosterone No significant effects on myocardial AT ₁ and AT ₂ mRNA	Rosiglitazone had a beneficial effect on post-infarct ventricular remodeling, but had a neutral effect on mortality	[32]
WT and eNOS knockout mice with I/R injury	3 mg/kg ip; pretreated for 45 min prior to I/R	WT mice: increased the recovery of left ventricular function and coronary flow following ischemia eNOS knockout mice: suppressed the recovery of myocardial function following ischemia	Rosiglitazone protected the heart against I/R injury <i>via</i> nitric oxide by phosphorylation of eNOS	[48]
Isolated perfused hearts from T2DM mice	23 mg/kg per day po; pretreated for 5 wk	Normalized plasma glucose and lipid concentrations Restored rates of cardiac glucose and fatty acid oxidation Improved cardiac efficiency due to decrease in unloaded myocardial oxygen consumption Improved functional recovery after low-flow ischemia	Rosiglitazone improved cardiac efficiency and ventricular function	[50]
WT and APN knockdown/knockout mice with myocardial infarction	20 mg/kg per day po; pretreated 72 h prior to MI and continuously treated until 7 and 14 d	Improved the postischemic survival rate of WT mice at 14 d of treatment Increased adipocyte APN expression Elevated plasma APN levels Reduced infarct size Decreased apoptosis and oxidative stress Improved cardiac function	APN was crucial for cardioprotective effects of rosiglitazone in myocardial infarction	[57]
Hypercholesterolemic rats	4 mg/kg per day po; pretreated for 5 mo	Reduced Ang II level Upregulated AT ₂ Improved lipid metabolism	Rosiglitazone protected the heart against cardiac hypertrophy <i>via</i> improved lipid profile, reduced Ang-II and increase AT ₂ expression	[54]
Mice with I/R injury	3 mg/kg per day po; pretreated for 14 d prior to I/R	Reduced ratio of infarct size to ischemic area (area at risk) Reduced the occurrence ventricular fibrillation Attenuated cardiac apoptosis Increased levels of p-Akt and p-GSK-3 α	Cardioprotective effects of rosiglitazone against I/R injury were mediated <i>via</i> a PI3K/ Akt/GSK-3 α -dependent pathway	[59]
T2DM patients (n = 21)	4 mg/d; 6 mo	Weight loss (first 12 wk) Decreased waist circumference Decreased systolic and diastolic blood pressure Reduced HbA1c	Rosiglitazone amplified some of the positive benefits of lifestyle intervention	[55]
Randomized, double-blind, placebo-controlled study in T2DM (n = 357)	4 or 8 mg/d; 26 wk	Reduced C-reactive protein, matrix metalloproteinase-9 and white blood cell levels Did not alter interleukin-6 level	Rosiglitazone had beneficial effects on overall cardiovascular risk	[49]
Randomized, double-blind in CAD patients without diabetes (n = 40, control = 44)	4 mg/d for 8 wk followed by 8 mg/d for 4 wk	Reduced E-selectin Reduced von Willebrand Reduced C-reactive protein & fibrinogen Reduced homeostasis model of insulin resistance index Elevation of LDL and triglyceride level	Rosiglitazone reduces markers of endothelial cell activation and levels of acute-phase reactants in CAD patients without DM	[56]
Randomized, double-blind, placebo-controlled study in T2DM with CAD patients (n = 54)	4-8 mg/d; 16 wk	Improved glycemic control and whole-body insulin sensitivity Increased myocardial glucose uptake in both ischemic and non-ischemic regions	Rosiglitazone facilitated myocardial glucose storage and utilization in T2DM with CAD patients	[36]
Randomized controlled trial in patients with impaired glucose tolerance (n = 2365, control = 2634)	8 mg/d; 3 yr	Facilitated normoglycemic Did not alter cardiovascular event	Rosiglitazone reduced incidence of T2DM and increased normoglycemia	[47]

Randomized, double-blind, placebo-controlled trial in patients with T2DM (<i>n</i> = 70, control = 16)	8 mg/d; 6 mo	Decreased plasma glucose and HbA1c with a trend to decrease HOMA index Decreased C-peptide and fasting insulin Reduced C-reactive protein Improved endothelium-dependent dilation	Rosiglitazone improved endothelial function and C-reactive protein in patients with T2DM ^[51]
Randomized, controlled trial in patient with T2DM with CAD (Rosiglitazone; <i>n</i> = 25)	4 mg/d; 12 wk	Decreased insulin resistance Decreased pulse wave velocity Reduced plasma levels of C-reactive protein and monocyte chemoattractant protein 1	Rosiglitazone prevented arteriosclerosis by normalizing metabolic disorders and reducing chronic inflammation of the vascular system ^[58]
Prospective and cross-sectional study in T2DM (Rosiglitazone; <i>n</i> = 22, metformin/rosiglitazone; <i>n</i> = 100)	Treated with rosiglitazone 6 mo	Decreased endotoxin Increased adiponectin levels	Lower endotoxin and higher adiponectin in the groups treated with rosiglitazone may be responsible for the improved insulin sensitivity ^[39]
Comprehensive meta-analysis of randomized clinical trials (<i>n</i> = 42922, control = 45483)	Results of 164 trials with duration > 4 wk	The OR for all-cause and cardiovascular mortality with rosiglitazone was 0.93 and 0.94, respectively The OR for nonfatal MI and heart failure with rosiglitazone was 1.14 (0.9-1.45) and 1.69 (1.21-2.36), respectively The risk of heart failure was higher when rosiglitazone was administered as add-on therapy to insulin	Rosiglitazone did not increase risk of MI or cardiovascular mortality ^[52]

Ang: Angiotensin; ANP: Atrial natriuretic peptide; OR: Odds ratio; T2DM: Type 2 diabetes mellitus; Hb: Hemoglobin; HOMA: Homeostatic Model of Insulin Resistance; CAD: Coronary artery disease; WT: Wild-type; APN: Adiponectin; eNOS: Endothelial nitric oxide synthase; GSK: Glycogen synthase kinase; LDL: Low density lipoprotein; AT: Angiotensin receptor type; SOD: Superoxide dismutase; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol-3-kinase; STZ: Streptozotocin; I/R: Ischemia/reperfusion; TNF: Tumor necrosis factor; IL: Interleukin; NF: Nuclear factor; SERCA: sarcoendoplasmic reticulum calcium ATPase; HT: Hypertension; NFAT: nuclear factor of activated T cells; ERK: Extracellular signal-regulated kinase; PPAR: Peroxisome proliferator-activated receptor; MI: Myocardial infarction.

ment in patients with T2DM with/without CAD has also been shown to improve myocardial glucose uptake and utilization^[36,47]. Rosiglitazone decreased both systolic and diastolic pressure^[53,55], suggesting that this drug could improve systolic and diastolic function. All of these findings indicate that in addition to improving insulin resistance in T2DM patients, rosiglitazone also has the beneficial effects on overall cardiovascular risk.

ADVERSE EFFECTS OF ROSIGLITAZONE ON THE CARDIOVASCULAR SYSTEM

Despite these previously mentioned beneficial effects of rosiglitazone on the cardiovascular system, growing evidence indicates other adverse cardiovascular outcomes. The effect of rosiglitazone in increasing mortality in post-MI rats was first reported by Lygate *et al.*^[21] in 2003. Later, more studies, including clinical trials, demonstrated undesirable effects of rosiglitazone on the cardiovascular system. These findings suggested that rosiglitazone treatment may be harmful and should be used with caution in cardiovascular patients. A summary of reports on the adverse effects of rosiglitazone in various models as well as clinical studies are shown in Table 2.

Rosiglitazone treatment has been shown to induce apoptotic cell death in cultured vascular smooth muscle cell by increasing caspase 3 activity and the cytoplasmic histone-associated DNA fragmentation *via* the proapoptotic extracellular signal-regulated kinase 1/2-independent pathway^[17]. Likewise, in an *in vivo* I/R injury model, it has been demonstrated that rosiglitazone therapy for 8 wk in non-diabetic rats with MI did not reduce either LV in-

fart size or LV hypertrophy, and increased mortality rate after I/R injury^[21]. These findings suggested that rosiglitazone did not have cardioprotective effects in myocardial I/R injury. Furthermore, rosiglitazone treatment has been shown to increase cardiac phosphorylation of the p38MAPK signaling pathway^[15], suggesting that rosiglitazone could facilitate cardiomyocyte apoptosis. In addition, rosiglitazone has been shown to be associated with an increased incidence of cardiac hypertrophy due to the increased expression of atrial natriuretic peptide, B-type natriuretic peptide, collagen I and III and fibronectin^[16], leading to cardiac hypertrophy. The deterioration in cardiac function was also found in mice and rats when treated with rosiglitazone^[12,24].

In a large animal model, which is more similar to a human, a recent study in swine has demonstrated that intravenous administration of rosiglitazone at clinically relevant doses attenuated epicardial monophasic action potential shortening during ischemia, possibly *via* blockade of cardiac ATP-sensitive potassium channels, and increased the propensity for ventricular fibrillation^[20].

Growing evidence from recent clinical trials suggest that rosiglitazone could have serious harmful effects on the cardiovascular system^[11,13,14,18,19,22,23,65]. The meta-analysis by Nissen *et al.*^[11] was the first report raising concerns about the cardiovascular safety profile of rosiglitazone. In a meta-analysis, Nissen *et al.*^[11] demonstrated that T2DM patients who received rosiglitazone treatment had a significantly increased risk of MI, heart failure and cardiovascular mortality. Although the method and statistical analysis used in this study have been criticized^[14,52,66], the subsequent meta-analyses showed similar concerns

Table 2 Reports of the adverse effects of rosiglitazone on the cardiovascular system in pre-clinical and clinical studies

Model	Dose of rosiglitazone	Major findings	Interpretation	Ref.
Isolated and cultured vascular smooth muscle cells	1-10 μ mol/L; incubated for 24 h	Induced cell death in a concentration-dependent manner Increased caspase 3 activity and the cytoplasmic histone-associated DNA fragmentation PD98059 (MAPKK inhibitor) did not abolish rosiglitazone induced ERK1/2 activation (proapoptotic effects)	Rosiglitazone induced apoptotic cell death through an ERK1/2-independent pathway	[17]
Rats with I/R injury	3 mg/kg per day po; pretreated for 14 d prior to I/R	Did not reduce left ventricular infarct size or hypertrophy Increased mortality rate Improved ejection fraction and prevented an increase left ventricular end diastolic pressure	Rosiglitazone did not prevent left ventricular remodeling, but was associated with increased mortality after myocardial infarction	[21]
Swine with I/R injury	3 mg/kg per day po; pretreated for 8 d prior to I/R	Increased expression of PPAR γ Had no effect on myocardial contractile function Did not alter substrate uptake and proinflammatory cytokines expression	Rosiglitazone had no cardioprotective effects in a swine model of myocardial I/R injury	[25]
PPAR γ -knockout (CM-PGKO) mouse	10 mg/kg per day po; 4 wk	Increased phosphorylation of p38 mitogen-activated protein kinase Induced phosphorylation of extracellular signal-related kinase 1/2 Did not affect phosphorylation of c-Jun N-terminal kinases Induced cardiac hypertrophy	Rosiglitazone caused cardiac hypertrophy at least partially independent of PPAR γ in cardiomyocytes	[15]
Wild type and PPAR γ overexpression mice	10 mg/kg per day po; 15 d	Increased lipid accumulation Increased size of the heart Decreased fractional shortening Increased CD36 expression	Rosiglitazone and PPAR γ overexpression could be harmful to cardiac function	[24]
Swine with I/R injury	0.1, 1.0 10 mg/kg iv; pretreated for 60 min	Attenuated MAP shortening during ischemia by blocking cardiac KATP channels Increased propensity for ventricular fibrillation during myocardial ischemia	Rosiglitazone promoted onset of ventricular fibrillation during cardiac ischemia	[20]
Sprague-Dawley rats	15 mg/kg per day po; 21 d	Induced eccentric heart hypertrophy associated with increased expression of ANP, BNP, collagen I and III and fibronectin Reduced heart rate and increased stroke volume Increased heart glycogen content, myofibrillar protein content and turnover Reduced glycogen phosphorylase expression and activity	Rosiglitazone induced cardiac hypertrophy <i>via</i> the mTOR pathway	[16]
Meta-analysis in T2DM (<i>n</i> = 15565, control = 12282)	Received rosiglitazone more than 24 wk	Increased the risk of myocardial infarction Increased cardiovascular death incidence	Rosiglitazone increased in the risk of myocardial infarction and borderline increased in risk of cardiovascular death	[11]
RECORD study (<i>n</i> = 4447)	Received rosiglitazone with mean follow-up time of 3.75 yr	Increased the risk of heart failure	Rosiglitazone increased risk of heart failure, but did not increase the risk of cardiovascular death or all cause mortality	[18]
RECORD study (<i>n</i> = 4447)	Received rosiglitazone with mean follow-up time of 5.5 yr	Increased the risk of heart failure	Rosiglitazone increased risk of heart failure Suggestion of contraindication for rosiglitazone to be used in patients developing symptomatic heart failure	[65]
Case-control analysis of a retrospective cohort study (<i>n</i> = 159026)	Treated with TZDs at least 1 yr	Increased risk of heart failure Increased mortality Increased risk of acute myocardial infarction	Rosiglitazone was associated with risk of heart failure, acute myocardial infarction, and mortality	[19]
Retrospective, double-blind, randomized clinical studies with rosiglitazone (<i>n</i> = 14237)	Received rosiglitazone 24-52 wk	Increased heart failure incidence Increased events of myocardial ischemia	Rosiglitazone increased the risk of heart failure and myocardial infarction	[13]
A meta-analysis of randomized controlled trials (<i>n</i> = 6421, control = 7870)	Received rosiglitazone at least 12 mo	Increased risk of myocardial infarction and heart failure No increased risk of cardiovascular mortality	Rosiglitazone increased risk of myocardial infarction and heart failure, without increased risk of cardiovascular mortality	[23]

I/R: Ischemia/reperfusion; PPAR: Peroxisome proliferator-activated receptor; T2DM: Type 2 diabetes mellitus; RECORD: Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes; ANP: Atrial natriuretic peptide; BNP: B-type natriuretic peptide; ERK: Extracellular signal-regulated kinase; TZD: Thiazolidinedione; mTOR: Mammalian Target of Rapamycin pathway

regarding MI and heart failure, but not cardiovascular mortality^[23,52,65].

Lipscombe *et al.*^[19] also demonstrated that rosiglitazone therapy in patients with T2DM was associated with a significantly increased risk of congestive heart failure, acute MI, and death. Similarly, results from a meta-analysis demonstrated that rosiglitazone treatment for at least 12 mo was associated with a significantly increased risk of MI and heart failure^[23]. A retrospective analysis also suggested that rosiglitazone may increase the risk of heart failure^[13]. These data from the clinical trials and meta-analysis in recent years strongly indicated that rosiglitazone could have adverse effects on the cardiovascular outcome due to increased risk of MI and heart failure, resulting in increased mortality in patients treated over a long period with rosiglitazone^[14,18,22,23]. A meta-analysis demonstrated that patients treated with both rosiglitazone and pioglitazone had a 1.7-fold increase in risk of congestive heart failure with a slightly greater increase in risk with rosiglitazone than with pioglitazone (1.3-fold)^[67].

The association between TZDs and heart failure is well recognized as a class effect. An increased plasma volume rather than direct effects on cardiac function is thought to be the mechanism responsible for heart failure^[12]. Fluid retention is mediated through increased sodium reabsorption of the renal PPAR γ -dependent pathway in the collecting tubules^[68].

Unlike the mechanism responsible for heart failure, the mechanism of increased MI risk of rosiglitazone is still controversial. An unfavorable effect of the lipid profile has been proposed, in which rosiglitazone increases low density lipoprotein cholesterol to a greater extent than pioglitazone, and decreases the triglyceride level to a smaller extent than pioglitazone^[69].

DISCREPANCY IN FINDINGS FROM ROSIGLITAZONE USE

As summarized in Table 1 for the beneficial effects and Table 2 for the adverse effects of rosiglitazone, these controversial reports are still debated. Although each side for and against the use of rosiglitazone has its own supporting documentation, the growing number of reports of serious adverse cardiovascular effects cannot be taken lightly. It is possible that the controversy on the cardiovascular effects of rosiglitazone could be due to differences in species which could have different drug metabolism, different experimental models, different drug administration methods as well as different time intervals of drug treatment which relates to the effects of the drug. The differences in patients' clinical characteristics may also contribute to the differences in outcomes, in which older patients with preexisting cardiovascular disease are more likely to have serious cardiovascular events.

Regardless of this controversy, since evidence from clinical reports indicated potential cardiovascular risks of rosiglitazone, the European Medicines Agency suggested that the anti-diabetes drug rosiglitazone (Avandia[®])

should be suspended from the EU market due to its excessive cardiovascular risk^[61,70]. As a result, rosiglitazone has been withdrawn from the EU market^[61]. However, rosiglitazone is still available in the US but remains under close monitoring from the US Food and Drug Administration^[61-63,71].

CONCLUSION

Rosiglitazone is a potent agent in the treatment of hyperglycemia in patients with T2DM because it is an insulin sensitizer and improves glucose uptake. Despite previous reports on its beneficial effects, growing evidence indicates that rosiglitazone increases cardiovascular risks in patients taking this drug. Although this drug has been withdrawn from the EU market, it is still can be used elsewhere. It is important that future large clinical trials should be done to evaluate the definitive cardiovascular outcome of the drug and the interplay between rosiglitazone and other available anti-hyperglycemic agents. In addition, large meta-analyses are also essential and must be carefully interpreted in order to elucidate the effects of rosiglitazone on cardiovascular risks and outcomes.

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Calcium channels and iron uptake into the heart

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(TTCC) have been shown to play an important role in the diseased heart. Although TTCC and iron uptake in cardiomyocytes has not been investigated greatly, a recent finding indicated that TTCC could be an important portal in thalassemic hearts. In this review, comprehensive findings collected from previous studies as well as a discussion of the controversy regarding iron uptake mechanisms into cardiomyocytes *via* calcium channels are presented with the hope that understanding the cellular iron uptake mechanism in cardiomyocytes will lead to improved treatment and prevention strategies, particularly in iron-overloaded patients.

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Key words: Cardiomyocytes; L-type calcium channel; T-type calcium channels; Iron overload; Thalassemia

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Abstract

Iron overload can lead to iron deposits in many tissues, particularly in the heart. It has also been shown to be associated with elevated oxidative stress in tissues. Elevated cardiac iron deposits can lead to iron overload cardiomyopathy, a condition which provokes mortality due to heart failure in iron-overloaded patients. Currently, the mechanism of iron uptake into cardiomyocytes is still not clearly understood. Growing evidence suggests L-type Ca^{2+} channels (LTCCs) as a possible pathway for ferrous iron (Fe^{2+}) uptake into cardiomyocytes under iron overload conditions. Nevertheless, controversy still exists since some findings on pharmacological interventions and those using different cell types do not support LTCC's role as a portal for iron uptake in cardiac cells. Recently, T-type Ca^{2+} channels

INTRODUCTION

Iron (Fe) is an essential element for all living organisms and plays a central role in many Fe-containing proteins such as in iron storage proteins (ferritin and hemosiderin), energy metabolism (cytochromes, mitochondrial aconitase and Fe-S proteins of the electron transport chain), cellular respiration (hemoglobin and myoglobin), and DNA synthesis (ribonucleotide reductase)^[1-3]. However, under iron overload conditions the regulatory mechanism which keeps the balance between iron uptake

and iron excretion could be disrupted, causing an elevation of non-transferrin bound iron (NTBI) in the plasma of iron-overloaded patients^[4,5]. NTBI is toxic and participates in the production of harmful hydroxyl radicals, which could cause severe cellular damage and organ dysfunction^[1,2,6,7].

An excess of plasma iron can lead to iron accumulation in many organs including the heart^[5]. Excessive iron accumulation in the heart can cause cardiac cellular damage known as iron-overload cardiomyopathy. This cardiac complication causes 71% of all deaths in thalassemia major patients^[8]. Although iron chelation therapy is widely used for treating iron overload patients, iron overload cardiomyopathy is still the most common cause of mortality in these patients^[9,10]. Even though the fundamental mechanisms for excessive iron uptake in the heart have been investigated for decades, the precise mechanism underlying cardiomyocyte dysfunction induced by iron overload is not clearly understood. Although several NTBI transporters have been proposed and are responsible for cellular iron uptake, recent evidence suggests that calcium channels may play an important role as a portal for cardiac iron uptake^[11].

In this review, the role of L-type Ca^{2+} channels (LTCC) as well as T-type Ca^{2+} channels (TTCC) as iron transporters into the heart are presented. The consistent findings as well as discrepancies of results among various studies on iron uptake into cardiomyocytes *via* these calcium channels under various conditions are comprehensively reviewed and discussed.

LTCCS AS A PORTAL FOR IRON UPTAKE INTO CARDIOMYOCYTES

The L-type Ca^{2+} channel is a voltage-gated ion channel that plays a central role in cardiac and smooth muscle contraction^[12]. LTCCs are heterotetrameric polypeptide complexes that are composed of $\alpha 1$, $\alpha 2/\delta$, β , and, in some tissues, γ subunits^[12]. The Ca^{2+} channel $\alpha 1$ subunit (170-240 ku) is organized into four homologous motifs (I-IV), with six transmembrane segments (S1-S6)^[12]. Recently, 10 $\alpha 1$ subunit genes have been identified including Cav1.1 ($\alpha 1\text{S}$), 1.2 ($\alpha 1\text{C}$), 1.3 ($\alpha 1\text{D}$), 1.4 ($\alpha 1\text{F}$), Cav2.1 ($\alpha 1\text{A}$), 2.2 ($\alpha 1\text{B}$), 2.3 ($\alpha 1\text{E}$), Cav3.1 ($\alpha 1\text{G}$), 3.2 ($\alpha 1\text{H}$), and 3.3 ($\alpha 1\text{I}$). For LTCCs, these can be divided into 4 classes: Cav1.1 ($\alpha 1\text{S}$), 1.2 ($\alpha 1\text{C}$), 1.3 ($\alpha 1\text{D}$), and 1.4 ($\alpha 1\text{F}$). In cardiac muscles, only the $\alpha 1\text{C}$ (dihydropyridine-sensitive) subunit is expressed in high levels and is also called a high-voltage-activated channel^[12]. LTCCs can be found in the heart and are primarily used for Ca^{2+} transport as well as playing an important role in the electrical activity of the heart. However, previous studies have shown that LTCCs can also transport other divalent cations including Fe^{2+} ^[13-15].

Several findings have been shown to support the role of LTCC in myocardial iron transport^[11,15]. A study in an iron loaded perfused rat heart showed that iron uptake was increased by the LTCC agonist, Bay K 8644 and iron

uptake was inhibited by the LTCC blocker, nifedipine^[15]. Oudit *et al*^[16] demonstrated that treatments with LTCC blockers such as amlodipine and verapamil could lead to the inhibition of LTCC current in cardiomyocytes, reduced myocardial iron accumulation, decreased oxidative stress and improved survival in iron-loaded mice. In addition, iron overloaded transgenic mice with cardiac-specific overexpression of LTCC were shown to have increased myocardial iron accumulation and oxidative stress, resulting in impaired cardiac function in comparison with control mice^[16]. Furthermore, since the LTCC does not contain iron responsive elements (IREs) in the LTCC mRNA, it is not regulated by cellular iron levels under an iron overload condition. As a result, L-type Ca^{2+} currents were not decreased in iron overload conditions^[16], confirming that the expression of LTCC was not regulated by the IRE. Furthermore, it has been shown in iron overloaded rats that the LTCC blocker diazepam could reduce mortality from iron overload without inhibition of iron absorption or urinary iron excretion^[17].

In addition to the heart, a previous study also demonstrated that LTCC blockers verapamil and amlodipine did not decrease iron accumulation in the liver of mice with iron overload, and hypothesized that this was due to the fact that hepatocytes express minimal levels of LTCC^[16]. However, a recent study by Ludwiczek and colleagues demonstrated that the LTCC blocker nifedipine could reduce iron accumulation in the liver of wild-type mice, but had no effect in divalent metal transporter 1 (DMT1) deficient mice, suggesting that this effect of nifedipine-mediated modulation of iron transport is *via* DMT1^[18]. Nevertheless, these findings suggest that nifedipine could possibly be beneficial in iron overload cardiomyopathy.

DISCREPANCIES IN FINDINGS ON IRON UPTAKE INTO CARDIOMYOCYTES *VIA* LTCC

It is important to realize that not all reports regarding the mechanisms of iron uptake *via* LTCC are consistent. Despite strong evidence supporting the role of LTCC as a route for NTBI transport in the heart, Parkes and colleagues demonstrated otherwise^[19]. In cultured rat neonatal myocytes, they demonstrated that LTCC blockers (nifedipine, verapamil, and diltiazem) did not alter iron uptake in these cells^[19]. Our recent findings also demonstrated that the LTCC blocker verapamil could not prevent iron uptake into cultured adult mouse cardiomyocytes^[20].

Several reasons to explain these inconsistent results may be drawn from previous reports. Most studies that support the role of LTCC for iron uptake in cardiomyocytes used freshly prepared cardiomyocytes taken from isolated perfused hearts^[15] or *in vivo*^[16]. However, a report that failed to show the role of LTCC in iron uptake into cardiomyocytes used cultured cardiomyocytes^[19,20].

In cultured cardiomyocytes, it is possible that LTCC

may not fully develop compared with isolated cardiomyocytes obtained from the heart. If fully developed, it is also possible that the LTCC in cultured cardiomyocytes may not function properly. Furthermore, the ages of cultured cardiomyocytes could have played a role in this discrepancy.

In the light of these inconsistent findings, it is possible that cardiomyocytes obtained from different methods may have different cellular characteristics and properties. All of these proposed hypotheses have not been tested and will need to be further investigated to elucidate the definite mechanism of iron uptake into the heart and resolve these existing discrepancies.

TTCC AS A PORTAL FOR IRON UPTAKE INTO CARDIOMYOCYTES

TTCC have three isoforms: Cav3.1 ($\alpha 1G$), 3.2 ($\alpha 1H$), and 3.3 ($\alpha 1I$) that are localized to the brain, kidney, and heart and are also called low-voltage-activated channels^[21]. It has been shown that only Cav3.1 and Cav3.2 are expressed in the heart^[21]. TTCCs have been reported to be functionally expressed only in embryonic hearts and disappear in adults^[22]. TTCC can be found abundantly only in sinoatrial pacemaker cells and Purkinje fibers of many species in adult hearts and are important for the maintenance of pacemaker activity^[21,23]. However, TTCC currents and expression have been demonstrated to reappear and play an important pathological role in diseased hearts with conditions such as ventricular hypertrophy^[21,24,25] and post-myocardial infarction^[26]. The increased TTCC expression has been shown to contribute to the progression of heart failure^[21].

Growing evidence indicates that TTCC blockers could be beneficial in diseased hearts. Recently, Horiba and colleagues demonstrated that the blockade of Ca^{2+} entry into cardiomyocytes *via* TTCC using the TTCC blocker efonidipine could block signal transduction involved in cardiac hypertrophy^[27]. In addition, a study in a mouse model of dilated cardiomyopathy has shown that a TTCC blocker could restore the resting membrane potential, and reduce the number of premature ventricular contractions and ventricular tachycardia, thus reducing the incidence of sudden death in these mice^[28]. These findings suggest that TTCC blockade may be potentially useful for the prevention of sudden death in patients with heart failure^[28]. It is known that iron overload conditions can lead to increased iron uptake into cardiomyocytes, resulting in cardiac hypertrophy and failure^[29-32]. However, it is not known if TTCC blockers could be cardioprotective in this type of cardiomyopathy.

Recently, our study using cultured cardiomyocytes taken from the heart of thalassemic mice demonstrated that intracellular iron accumulation in cultured ventricular myocytes of thalassemic mice was significantly higher than in wild type (WT) cells^[20]. These findings suggest that thalassemic cardiomyocytes could have pathways which can greatly uptake iron into the cells more than that in

WT cells. In addition, under an iron overloaded condition, our results demonstrated that the TTCC blocker, efonidipine, could prevent iron uptake into cultured thalassemic cardiomyocytes^[20]. Although efonidipine is not a selective TTCC blocker and could also block LTCC, its efficacy in blocking TTCC is greater than that of LTCC^[21]. In that study, since verapamil could not prevent iron uptake when efonidipine could, these findings suggested that TTCC could play a significant role in iron uptake into cardiomyocytes in this thalassemic cardiomyocyte model^[20]. Moreover, our microarray data demonstrated that the TTCC genes were up-regulated in thalassemic hearts, which is well correlated with the iron uptake results, suggesting that TTCCs could play an important role in iron uptake in thalassemic hearts, and that their re-expression could be due to the pathological state of a thalassemic heart itself or from the iron-overloaded condition, or both.

Since iron overload patients can develop cardiomyopathy and heart failure^[33-35], it is important that the association between iron overload, TTCC expression/function and cardiac complications be determined. Future studies in both basic and clinical research are needed to warrant the clinical usefulness of TTCC blockers in the prevention and treatment of iron overload cardiomyopathy particularly in thalassemia patients.

CONCLUSION

Iron overload is a serious and fatal complication in many diseases including iron-overload cardiomyopathy in thalassemia patients. Although pathways for cellular iron uptake have been investigated for many decades, its mechanism is still not clearly understood. In the past few years, findings regarding new possible pathways for cellular iron uptake have been suspected, including LTCC and TTCC. However, their definite roles as iron transporters in cardiomyocytes are still debated. Understanding the mechanism by which iron enters cardiac cells is very important, since it will provide us with the knowledge to be used in developing better treatment and prevention strategies in iron overloaded patients.

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ORIGINAL ARTICLE

T-type calcium channel as a portal of iron uptake into cardiomyocytes of beta-thalassemic mice

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Abstract

Objectives: Iron-overload condition can be found in β -thalassemic patients with regular blood transfusion, leading to iron deposition in various organs including the heart. Elevated cardiac iron causes iron-overload cardiomyopathy, a condition that provokes mortality because of heart failure in patients with thalassemia. Previous studies demonstrated that myocardial iron uptake may occur via L-type calcium channels (LTCCs). However, direct evidence regarding the claimed pathway in thalassemic cardiomyocytes has never been investigated. **Methods:** Hearts from genetic-altered β -thalassemic mice and adult wild-type mice were used for cultured ventricular cardiomyocytes. Blockers for LTCC, T-type calcium channel (TTCC), transferrin receptor1 (TfR1), and divalent metal transporter1 (DMT1) were used, and quantification of cellular iron uptake under various iron loading conditions was performed by Calcein-AM fluorescence assay. Microarray analysis was performed to investigate gene expressions in the hearts of these mice. **Results:** This study demonstrated that iron uptake under iron-overload conditions in the cultured ventricular myocytes of thalassemic mice was greater than that of wild-type cells ($P < 0.01$). TTCC blocker, efonidipine, and an iron chelator, deferoxamine, could prevent iron uptake into cultured cardiomyocytes, whereas blockers of TfR1, DMT1, and LTCC could not. Microarray analysis from thalassemic hearts demonstrated highly up-regulated genes of TTCC, zinc transporter, and transferrin receptor2. **Conclusions:** Our findings indicated that iron uptake mechanisms in cultured thalassemic cardiomyocytes are mainly mediated by TTCC, suggesting that TTCC is the important pathway for iron uptake in this cultured thalassemic cardiomyocyte model.

Key words cardiomyocytes; T-type calcium channel; L-type calcium channel; iron overload; thalassemia

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Iron overload conditions can be found in patients with thalassemia who receive regular blood transfusion (1, 2). Under physiologic condition, plasma transferrin binds to plasma iron, resulting in iron uptake into cells via endocytosis of transferrin–transferrin receptor pathway, thus reducing plasma free-iron level (3). Under iron excess condition, non-transferrin-bound iron (NTBI) can be found in plasma and can lead to iron accumulation in various organs (4–6). In the heart, iron deposit can lead

to iron overload cardiomyopathy, a condition that is mainly responsible for cardiac dysfunction and mortality in patients with thalassemia (5–7). Although pathways of iron entry into the heart have been investigated for decades, the precise mechanisms by which NTBI gets into cardiomyocytes under iron overload conditions are still not clearly understood.

Several cellular iron transporters such as divalent metal transporter-1 (DMT1) and transferrin receptor

(TfR1) have been shown to be portals for iron transport into cells such as enterocytes and hepatocytes (3). However, in the heart, it has been shown that under iron-loading conditions, TfR1 or DMT1 mRNA and protein expressions were suppressed (8–11), suggesting that they may not be the major portals for iron entry in cardiomyocytes under iron-overload conditions. However, its role in thalassemic cardiomyocytes is not known.

In the past decades, L-type calcium channels (LTCC) has been proposed as a possible portal for iron uptake in cardiomyocytes (12, 13). The role of LTCC in mediating iron uptake into cardiomyocytes has been demonstrated using LTCC agonist to increase iron uptake in isolated perfused rat heart (12) and LTCC blockers to reduce intracellular myocardial iron accumulation in an *in vivo* model (13). Despite this evidence regarding the role of LTCC as an iron portal, inconsistent findings exist that failed to demonstrate LTCC as a portal for iron uptake into cardiomyocytes (14). Furthermore, NTBI transportation via LTCC into thalassemic cardiomyocytes has never been investigated.

In the heart, T-type Ca^{2+} channels (TTCC) have been reported to exist and contribute to cardiac electrical activity during early embryonic state (15). However, under physiologic condition, they normally disappear shortly after birth and cannot be found in the cardiomyocytes (16). However, TTCC current has been shown to reappear in ventricular myocytes under some pathological processes such as ventricular hypertrophy (17) and postmyocardial infarction (18). However, the re-expression of TTCC in thalassemic hearts as well as the role of TTCC as a portal for iron uptake in ventricular thalassemic cardiomyocytes has never been investigated.

It is not known whether iron uptake mechanism in thalassemic cardiomyocytes would be similar or different than that in normal cardiomyocytes. Therefore, in this study, we sought to determine the iron uptake mechanisms using cultured thalassemic cardiomyocytes prepared from the hearts of adult β -thalassemic mice. We investigated the effects of LTCC and TTCC blockers, TfR1 blocker, DMT1 blocker, and iron chelator on iron uptake into thalassemic cardiomyocytes. Iron uptake was determined under various iron-loaded concentrations. In addition, microarray analysis on thalassemic hearts was performed to identify the up-regulation and down-regulation of genes that regulate iron transport and cardiac ion channels, compared to the wild-type hearts.

Materials and methods

Animal models

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Fac-

ulty of Medicine, Chiang Mai University (No. 44/2552) in compliance with NIH guidelines. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Three types of adult C57/BL6 mice (9–12 months old): wild type ($\mu\beta^{+/+}$, WT, $n = 42$), heterozygous β^{KO} type ($\mu\beta^{\text{th-3/+}}$, HT, $n = 43$), and double heterozygous β^{KO} with HbE ($\mu\beta^{\text{E/+}}$, $\mu\beta^{\text{th-3/+}}$, DH, $n = 38$), were used in this study (19, 20). All animals were housed in temperature- and humidity-controlled rooms with 12-h light/dark cycles. We have shown previously that HT mice had reduced hemoglobin level, increased serum iron (SI), increased reactive oxygen species (ROS) production, and depressed heart rate variability (HRV) (21), the clinical pictures that are similar to thalassemia intermedia in patients with thalassemia. Furthermore, our previous study demonstrated that the DH mice with depressed HRV had normal hemoglobin level, SI, and ROS production, indicating the altered cardiac autonomic control (21). Each mouse was genetically characterized prior to its entry into the study (19, 20).

Cultured cardiomyocytes

Primary cultured cardiomyocytes were obtained from HT, DH, and WT adult C57/BL6 mice at 9–12 months of age. Each mouse was anesthetized with isoflurane (Abbott Laboratories, Chicago, IL, USA) and the heart was removed. The ventricles were cut and incubated in Ca^{2+} - Mg^{2+} free Hank's balanced salt solution containing 2 mg/ml collagenase type 2 (PAA Laboratories, New Bedford, MA, USA) for 15 min. Cardiomyocytes were isolated by incubation with 0.25% trypsin–EDTA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. The cell suspension was centrifuged at 521 g for 5 min. After the centrifugation, the cell pellets were mixed and resuspended in completed M199 medium containing 20% fetal bovine serum with 1% penicillin–streptomycin (22). The cell suspension was plated on culture flask and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 10 d. Although these 10-d-old cardiomyocytes have been shown to undergo a morphological transformation and increase cell size, their cardiac phenotype is well preserved (23–25).

Immunocytochemistry methods

To identify the cardiomyocytes in cultured cells, the immunocytochemistry methods were used (26). All genotypes of cultured cardiomyocytes (WT, HT, and DH) were trypsinized, washed, and centrifuged at 3000 g for 5 min at 25°C. Then, the cells were plated on coverslip overnight, before they were fixed with 4% paraformaldehyde for 30 min and kept frozen until they were used for

immunocytochemistry. The cells were washed three times with Tris-buffered saline (TBS) and incubated for 1 h with blocking solution (BS) containing 1.5% normal goat serum in TBS. To identify the cardiomyocytes, those fixed cells were subject to immunostaining with primary antibodies directed against myosin light chain-2 (MLC-2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GATA-binding protein-4 (GATA-4) (Santa Cruz Biotechnology). Both MLC-2 and GATA-4 have been shown to be the markers for cardiomyocytes (27).

Iron treatment of the cardiomyocytes

Ferric ammonium citrate (FAC) at various concentrations ranging from 0 to 2284.8 μM (0–160 $\mu\text{g/ml}$) co-administered with 1 mM ascorbic acid (AA), which represents ascorbate-reduced form of iron (Fe^{2+}), was added to the culture medium of all types of cardiomyocytes for 48 h. The amount of iron uptake into cultured cells was detected using Calcein-AM fluorescence assay (28, 29).

Pharmacological interventions of iron uptake into cardiomyocytes

To determine the effect of iron chelator desferoxamine (DFO: Novartis Ltd., East Hanover, CA, USA) on iron-loaded cardiomyocytes, the cells were loaded with a combination of 285.6 μM FAC + 1 mM AA (or 20 $\mu\text{g/ml}$ Fe^{2+}), i.e. a concentration similar to the plasma iron level in patients with thalassemia (8), and DFO at concentrations of 142.8 and 285.6 μM for 48 h (11, 14).

To determine the roles of LTCC, TTCC, TfR1, and DMT1 on iron uptake, the cells were exposed to (i) various concentrations of LTCC blocker (verapamil) (Sigma-Aldrich) ranging from 0 to 80 μM and (ii) various concentrations of TTCC blocker (efonidipine) (Sigma-Aldrich) ranging from 0 to 20 μM . Although efonidipine can block both LTCC and TTCC, it has been shown that efonidipine could block TTCC more effectively than LTCC (30). Comparisons between the effects of verapamil and efonidipine on iron uptake would allow us to differentiate the effects of these two calcium channel blockers, (iii) anti-TfR1 antibody (Santa Cruz Biotechnology) at 5 and 10 $\mu\text{g/ml}$ and (iv) ebselen [DMT1 blocker (31)] at 12.5 and 25 μM , for 60 min, before loaded with FAC + 1 mM AA, which represents Fe^{2+} , at 285.6 μM for 48 h (14). Then, the cells were analyzed for the iron uptake into cardiomyocytes by using Calcein-AM fluorescence assay (28, 29).

Cell viability determination with methyl thiazol tetrazolium (MTT) assay

In the present study, MTT assay was used to determine the viability of cells and the toxicity of all substances

used in cultured cardiomyocytes (32). After the cardiomyocytes had been plated in a multiwell microtiter plate at 5000 cells/well and incubated at 37°C for 24 h, they were treated with FAC + 1 mM AA solutions at various concentrations of 0–2284.8 μM (Fe^{2+} , 0–160 $\mu\text{g/ml}$), or iron chelator solutions (DFO) at concentrations of 0–285.6 μM , or LTCC blocker solutions (verapamil) at concentrations of 0–80 μM , or TTCC blocker solutions (efonidipine) at concentrations of 0–20 μM , or anti-TfR1 antibody at 5–10 $\mu\text{g/ml}$, or ebselen at 12.5–25 μM , and plated at 37°C for 48 h. After each treatment, cultured cardiomyocytes were treated with MTT reagents to investigate the cell viability. The MTT reagent at 5 mg/ml (15 μL) was added to the treated cells and further incubated for 4 h. The reaction product, blue-colored formazan, was extracted with dimethyl sulfoxide (DMSO) (200 μL), and the absorbance was read at 540/630 nm using a microtiter plate reader (Synergy HT BIO-TEK, Winooski, VT, USA). Viable cells reduced MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] from a yellow water-soluble dye to an insoluble formazan product of dark blue color (32). The number of viable cells was normalized and compared with untreated cells, and the data were expressed as percentage of cell viability (32). All samples were assayed in triplicate, and the mean for each experiment was determined.

Calcein-AM fluorescence assay

To determine the amount of iron uptake into cardiomyocytes, the Calcein-AM fluorescence assay was used (28, 29). Calcein-AM could easily diffuse into the cell through the cell membrane of viable cells owing to its enhanced hydrophobicity compared to Calcein. After Calcein-AM permeated into the cytoplasm, it was hydrolyzed by esterases to become Calcein, which remained inside the cell. Fluorescence intensity of Calcein was quenched by labile iron inside the cell (28, 29). If the labile iron level inside the cells was high, the fluorescence intensity would be low and *vice versa*. After the iron-loaded treatments, cells were washed with phosphate-buffered saline (PBS) and incubated with the fluorescent metal sensor, calcein-AM (5 μM) (Sigma-Aldrich), at 37°C for 30 min. Cells were washed with PBS and were measured for the fluorescence intensity with fluorescence plate reader at 485-nm excitation and 530-nm emission filters (28, 29). Calcein-loaded cells that were shown in the form of fluorescence intensity (FI) were inversely correlated with intracellular iron. During the assay, cell viability was always greater than 95% and was unchanged throughout the process.

RNA isolation for microarray analysis

The heart samples of HT ($n = 5$) and WT ($n = 4$) mice were removed. The RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA) was used to obtain total heart RNA, and the RNase-free DNase (Qiagen) was used to remove the residual DNA from the sample. The optical density (OD) measurements at 260 and 280 nm were used to determine RNA concentration and purity (33). All samples had an OD260/OD280 ratio of 1.80 or higher.

Microarray studies were performed by Codelink™ Expression Bioarray System (Amersham Biosciences, Buckinghamshire, UK). Heart specimens derived from HT and WT C57BL/6 mice were subject to total RNA extraction. In each sample, 100 ng of total RNA was amplified using the Codelink Expression Assay Reagent kit (Amersham Biosciences), following the manufacturer's instructions. The *in vitro* transcription reaction was conducted with labeling of the cRNA by biotinylation. The labeled and amplified cRNA was hybridized to Codelink Uniset Mouse 20K I Bioarray slide (20 000 genes, Amersham Biosciences) at 37°C for 18 h, according to Codelink hybridization protocol. The arrays were washed and then stained with 2 µg/ml cyanine5-streptavidin (Amersham Biosciences). The GenePix Array Scanner (Molecular Devices, Sunnyvale, CA, USA) was used to scan the arrays. Samples were analyzed using Wayne State University Intelligent Biosystems Software (Department of Computer Science, Wayne State University, MI, USA).

Statistical analysis

All iron uptake experiments were performed in triplicate. Data were reported as the mean $\pm$ standard error of mean (SEM) and were processed using SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) release 13.0 for Windows. One-way ANOVA analyses and Student's *t*-test were performed for group comparisons. *P*-value < 0.05 was considered statistical significance.

Results

Identification of cardiomyocytes and effects of iron overload in cardiomyocytes

Positive staining of both MLC-2 and GATA-4 was found in all types (WT, HT, and DH) of cultured cells, indicating that these cells were cardiomyocytes (Fig. 1). Negative control without primary antibody incubation was also performed and demonstrated that no positive staining was observed. At the baseline level, intracellular iron accumulation in the cultured ventricular myocytes of thalassemic (both HT and DH) mice was

significantly higher than those of WT cells (Fig. 2). Under iron-overload condition, iron uptake into all types of cardiomyocytes was in a dose-dependent manner (Fig. 2).

Effects of iron chelator, deferoxamine (DFO), on iron uptake in cardiomyocytes

When DFO was added, it could chelate ascorbate-reduced form of iron from culture medium and led to an increase in Calcein fluorescence intensity, indicating that DFO treatment could decrease iron entry into cultured cardiomyocytes (Fig. 3). This effect of DFO was also in a dose-dependent manner. This finding suggested that DFO could inhibit ascorbate-reduced form of iron uptake into all types of cultured cardiomyocytes and confirmed that the Calcein-AM method used in this study is an appropriate method for measuring intracellular labile iron in thalassemic cardiomyocytes.

Effect of LTCC blocker, verapamil, on iron uptake in cardiomyocytes

To investigate whether LTCC was a possible portal for iron uptake into cardiomyocytes in this cultured cardiomyocyte model, verapamil, the LTCC blocker, co-administered with FAC + AA was added to all types of cultured cardiomyocytes. Our results demonstrated that verapamil, at various concentrations, could not prevent ascorbate-reduced form of iron uptake into any types of cardiomyocytes (Fig. 4). These findings suggested that iron uptake mechanisms in cultured cardiomyocytes were not mainly mediated by LTCC.

Effects of TfR1 and DMT1 on iron uptake into cardiomyocytes

Although previous studies demonstrated that TfR1 and DMT1 expressions in the heart were limited under iron-overload condition (8–11), those studies were carried in normal cardiomyocytes. Their roles in thalassemic cardiomyocytes have never been tested. To test whether TfR1 and DMT1 are involved in iron uptake into thalassemic cardiomyocytes, anti-TfR1 antibody and ebselen (DMT1 blocker) (34) were used to determine the roles of TfR1 and DMT1 for iron uptake into iron-loaded cardiomyocytes. Our results demonstrated that anti-TfR1 antibody at concentrations of 5 and 10 µg/ml and ebselen at concentration of 12.5 and 25 µM could not prevent iron uptake into any types of cardiomyocytes (Fig. 5). These findings indicate that TfR1 and DMT1 were not the common pathways for iron uptake into cultured cardiomyocytes under iron-overload condition.

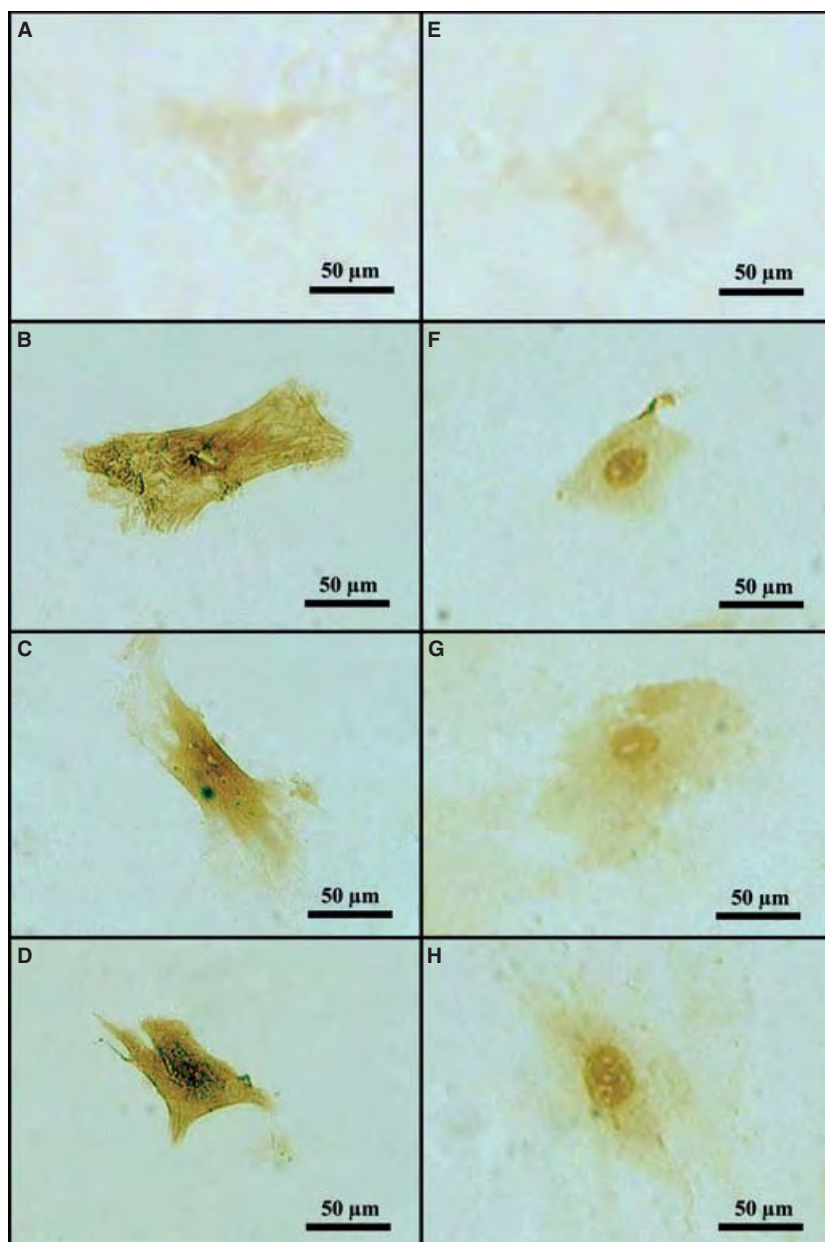


Figure 1 Immunoperoxidase staining of cultured cardiomyocytes. Negative control without primary antibody for myosin light chain 2 (MLC-2) is shown in (A). Positive MLC-2 staining can be seen in the cytoplasm of cardiomyocytes taken from (B) wild type (WT), (C) double heterozygous β^{KO} with HbE (DH), and (D) heterozygous β^{KO} type (HT). Negative control without primary antibody for GATA-binding protein 4 (GATA-4) is shown in (E). Positive GATA-4 staining can be seen in the nucleus of cardiomyocytes taken from WT (F), DH (G), and HT (H).

Effects of TTCC blocker, efonidipine, on iron uptake in cardiomyocytes

The role of TTCC on ascorbate-reduced form of iron transportation in ventricular thalassemic cardiomyocytes has never been investigated. Therefore, we further investigated whether TTCC was a possible portal for iron uptake in this cultured cardiomyocyte model. To test this hypothesis, efonidipine, the TTCC blocker, co-administered with FAC + AA was added to all types of cultured cardiomyocytes. Our results demonstrated that efonidipine, at all tested concentrations, could prevent ascorbate-reduced form of iron uptake into all types of cardiomyocytes ($P < 0.05$) (Fig. 6). Moreover, efonidi-

pine at 20 μM could prevent iron uptake into HT cardiomyocytes greater than that in DH and WT cells ($P < 0.05$). Furthermore, efonidipine at 10 μM could prevent iron uptake into HT cells greater than that in WT cells ($P < 0.05$). These findings suggested that iron uptake mechanisms in cultured thalassemic cardiomyocytes were mainly mediated by TTCC.

Cytotoxic effects of treated reagents in cardiomyocytes

The cytotoxic effect of all reagents used in this study (FAC + AA, DFO, verapamil, efonidipine, anti-TfR1

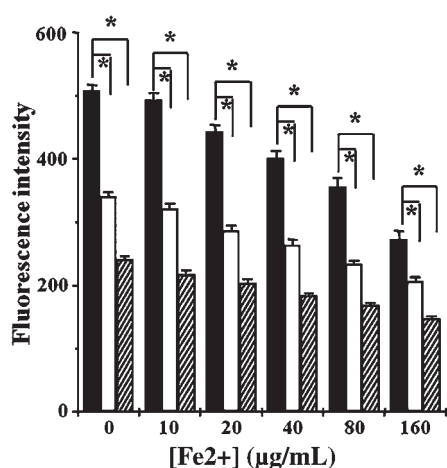


Figure 2 Iron uptake into cardiomyocytes ($n = 9/\text{group}$). Cardiomyocytes from WT (black bar), DH (white bar), and HT (dashed bar) were plated and loaded with various concentrations of ferric ammonium citrate with 1 mM ascorbic acid (FAC $\pm$ AA) for 48 h. Iron uptake into cardiomyocytes was determined using Calcein-AM fluorescence assay. Data were presented as mean $\pm$ SEM. * $P < 0.01$ vs. WT cells.

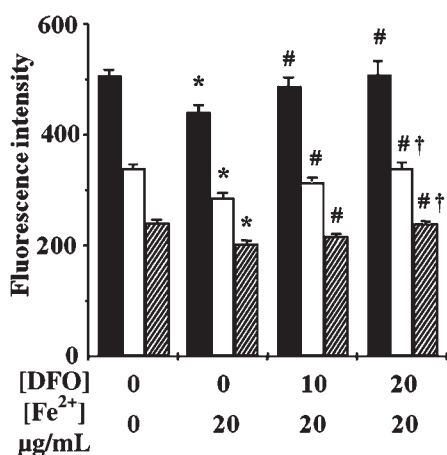


Figure 3 Effects of an iron chelator, desferoxamine (DFO), on iron uptake into cardiomyocytes ($n = 9/\text{group}$). Cardiomyocytes from WT (black bar), DH (white bar), and HT (dashed bar) were plated and loaded with the combination of 285.6 μM (20 $\mu\text{g}/\text{ml}$) ferric ammonium citrate with 1 mM ascorbic acid (FAC $\pm$ AA) and various concentrations of DFO (0–285.6 μM) for 48 h. Cellular iron uptake into cardiomyocytes was determined using Calcein-AM fluorescence assay. Data were presented as mean $\pm$ SEM. * $P < 0.05$ vs. control (non-iron-treated group) of the same cell type, # $P < 0.05$ vs. iron-treated group of the same cell type without DFO, † $P < 0.01$ vs. DFO 10- $\mu\text{g}/\text{ml}$ group of the same cell type.

antibody, and ebselen) on cardiomyocytes was tested by the MTT assay. We found that cell viability of cardiomyocytes was greater than 95% in all treatments, indi-

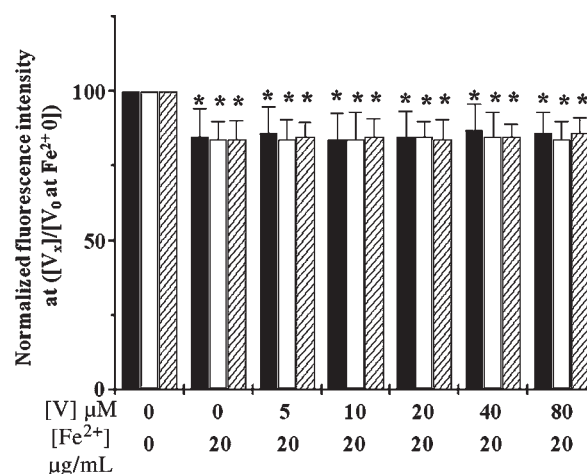


Figure 4 Effects of LTCC blocker, verapamil (V), on iron uptake into cardiomyocytes ($n = 9/\text{group}$). Cardiomyocytes from WT (black bar), DH (white bar), and HT (dashed bar) were plated and exposed to various concentrations of verapamil (0–80 μM) for 60 min before loaded with 285.6 μM (20 $\mu\text{g}/\text{ml}$) ferric ammonium citrate with 1 mM ascorbic acid (FAC $\pm$ AA) for 48 h. Cellular iron uptake was determined using Calcein-AM fluorescence assay. Data were presented as mean $\pm$ SEM. * $P < 0.05$ vs. control (non-iron-treated group) of the same cell type.

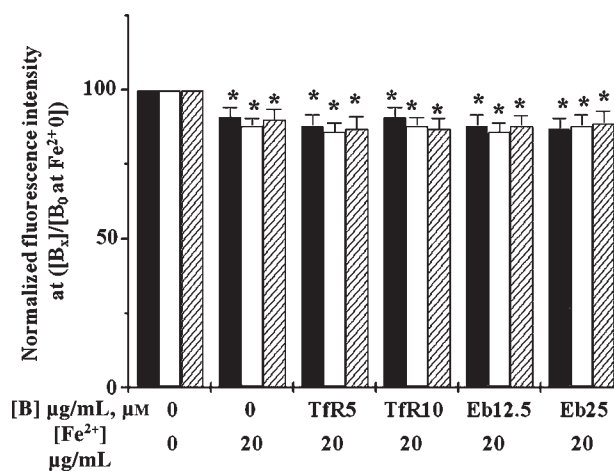


Figure 5 Effects of anti-Tfr1 antibody and divalent metal transporter1 (DMT1) blocker on iron uptake into cardiomyocytes ($n = 6/\text{group}$). Cardiomyocytes from WT (black bar), DH (white bar), and HT (dashed bar) were plated and the cells were exposed to various concentrations of anti-Tfr1 antibody (aTfr, 5 and 10 $\mu\text{g}/\text{ml}$) or Ebselen (DMT1 blocker, 12.5 and 25 μM) for 60 min, before loaded with 285.6 μM (20 $\mu\text{g}/\text{ml}$) ferric ammonium citrate with 1 mM ascorbic acid (FAC $\pm$ AA) for 48 h. Cellular iron uptake was determined using Calcein-AM fluorescence assay. Data were presented as mean $\pm$ SEM. * $P < 0.05$ vs. control (non-iron-treated group) of the same cell type.

cating that there was no cytotoxic effect on cultured cardiomyocytes treated with various concentrations of reagents used in this study.

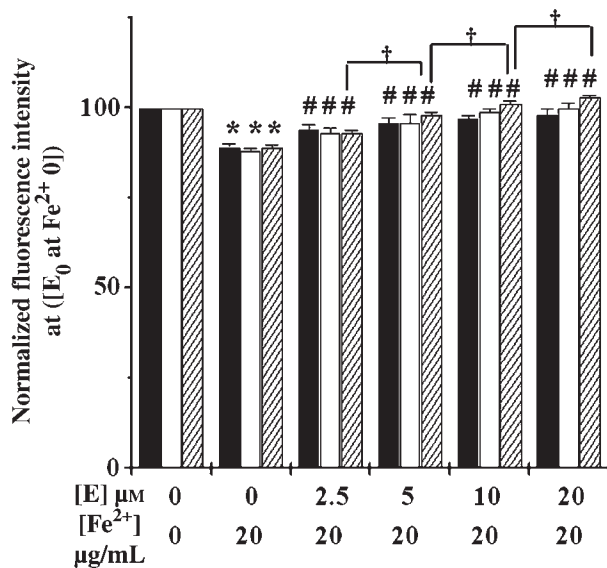


Figure 6 Effects of T-type calcium channel blocker, efonidipine (E), on iron uptake into cardiomyocytes ($n = 5/\text{group}$). Cardiomyocytes from WT (black bar), DH (white bar), and HT (dashed bar) were plated and exposed to various concentrations of efonidipine (0–20 μM) for 60 min, before loaded with 285.6 μM (20 $\mu\text{g/mL}$) ferric ammonium citrate with 1 mM ascorbic acid (FAC $\pm$ AA) for 48 h. Cellular iron uptake was determined using Calcein-AM fluorescence assay. Data were presented as mean $\pm$ SEM. * $P < 0.05$ vs. control (non-iron-treated group) of the same cell type. # $P < 0.05$ vs. iron-treated group of the same cell type without efonidipine. † $P < 0.01$ vs. the other efonidipine concentration of the same cell-type group.

Identification and validation of changes in gene expressions in thalassemic hearts

Microarray study was performed to investigate gene expressions in the heart tissues of the HT thalassemic mice, compared to those in WT mice. In the HT hearts, a number of transporters and ion channels were up-regulated (Table 1). Up-regulations of different subunits of calcium channel were demonstrated in the HT hearts. These include up-regulated TTCC genes such as voltage-dependent T-type alpha 1G subunit (Cacna1g) and voltage-dependent T-type alpha 1H subunit (Cacna1h) calcium channels, which were increased in the HT hearts, compared to those in WT. However, LTCC genes in HT hearts were not different from those in WT hearts. In addition, the up-regulations of zinc transporter such as solute carrier family 39 (zinc transporter) member 5 (Slc39a5), solute carrier family 39 (zinc transporter) member 3 (Slc39a3), solute carrier family 30 (zinc transporter) member 6 (Slc30a6), and solute carrier family 39 (zinc transporter) member 8 (Slc39a8) were found. Transferrin receptor2 (TfR2) and musculus mRNA for transferrin receptor were also up-regulated in HT hearts, compared to those in the WT hearts.

Table 1 Genes with up-regulated expression in HT heart tissues, compared to WT

Gene name	Accession	Fold change
Calcium channel mRNA	AF217002.1	1.519295
Calcium channel, voltage-dependent, T-type, alpha 1G subunit (Cacna1g)	NM_009783.1	1.463021
Calcium channel, voltage-dependent, T-type, alpha 1H subunit (Cacna1h)	NM_021415.2	1.281072
Calcium channel, voltage-dependent, alpha 2/delta subunit 2 (Cacna2d2)	NM_020263.2	2.391459
Solute carrier family 30 (zinc transporter), member 6 (Slc30a6)	NM_144798.2	1.675099
Solute carrier family 39 (zinc transporter), member 5 (Slc39a5)	NM_028051.1	2.159819
Solute carrier family 39 (zinc transporter), member 8 (Slc39a8)	NM_026228.2	1.753658
Solute carrier family 39 (zinc transporter), member 3 (Slc39a3)	NM_134135.1	1.787308
Musculus mRNA for transferrin receptor	X57349.1	1.557613
Transferrin receptor 2 (TfR2)	NM_015799.2	1.602854

Table 2 Genes with down-regulated expression in HT heart tissues, compared to WT

Gene name	Accession	Fold change
Calcium channel, voltage-dependent, gamma subunit 3 (Cacng3)	NM_019430.1	-6.718658
Calcium channel, voltage-dependent, gamma subunit 7 (Cacng7)	NM_133189.1	-5.312569
Solute carrier family 11 [proton-coupled divalent metal ion transporters (DMT)], member 1 (Slc11a1)	NM_013612.1	-1.491265
Solute carrier family 11 [proton-coupled divalent metal ion transporters (DMT)], member 2 (Slc11a2)	NM_008732.1	-1.693117
Solute carrier family 30 (zinc transporter), member 7 (Slc30a7)	NM_023214.3	-2.242076
Solute carrier family 40 [iron-regulated transporter (ferroportin)], member 1 (Slc40a1)	NM_016917.1	-1.421172

DMT, divalent metal transporter.

Down-regulations of DMT family were observed in the HT hearts (Table 2), such as solute carrier family 11 (proton-coupled DMT), member 1 (Slc11a1), and solute carrier family 11 (proton-coupled DMT), member 2 (Slc11a2). These down-regulations of DMT genes agreed well with our data, showing that DMT1 blocker could not prevent iron uptake into thalassemic cardiomyocytes. Furthermore, we also found the down-regulation of iron exporter ferroportin or solute carrier family 40 [iron-regulated transporter (ferroportin)], member 1 (Slc40a1) in the HT hearts.

Discussion

Iron-overload cardiomyopathy has been responsible for cardiovascular morbidity and mortality in patients with thalassemia for many decades (1, 2). Currently, the definite mechanism of iron entry into cardiomyocytes is not clearly understood. The precise mechanism of iron uptake into the heart is crucial as it could provide new approaches for the treatment of iron-overloaded patients. Although a number of studies have been carried out to investigate the iron uptake mechanism in the heart, they were performed in normal hearts. It is not known whether iron entry into cardiac cells is similar or different in thalassemic cardiomyocytes. Our study is the first study that a mouse model of β -thalassemia, in which the HT mice showed a pathophysiology resembling that of human patients with β -thalassemia intermedia (19, 20, 35), was used to investigate the possible pathways of iron uptake into thalassemic cardiomyocytes.

In the present study, the major findings are that (i) iron accumulation at the baseline in the cultured ventricular cardiomyocytes of thalassemic mice was significantly higher than that of WT cells; (ii) DFO could prevent iron uptake into the cells; (iii) the iron uptake into cardiomyocytes could not be blocked by LTCC blocker (verapamil), anti-TfR1 antibody nor DMT1 blocker; (iv) the iron uptake into cardiomyocytes could be prevented by TTCC blocker, efonidipine; and (v) up-regulated genes of TTCC, zinc transporter, and TfR2 were found in thalassemic cardiomyocytes.

We previously demonstrated that HT mice had a significant increase in serum iron, compared to the DH and WT mice (21). In the present study, the baseline of iron accumulation in HT cardiomyocytes which was greater than that in WT could be directly attributed to the high level of serum iron and high iron accumulation in heart tissue (36). Under the normal condition, iron uptake (iron accumulation) into the cultured ventricular cardiomyocytes of both types of thalassemic mice was significantly greater than that of WT cells. These findings suggest that thalassemic cardiomyocytes could have pathways that can greatly uptake iron into the cells more than that in the WT cells. To confirm that iron is uptaken into all types of cultured cardiomyocytes, iron chelator DFO was used and the results showed that DFO could block iron uptake into all types of cultured cardiomyocytes.

Verapamil did not block iron uptake in cultured thalassemic cardiomyocytes

Calcium channel blockers have been widely used to treat cardiovascular diseases for many decades (37, 38). Recent evidence demonstrated that, under the conditions of iron overload, LTCC may be a critical transporter of

iron into cardiomyocytes (12, 13). In the present study, by using the LTCC blocker, our results demonstrated that verapamil could not block iron uptake into any types of cultured cardiomyocytes. These findings suggested that an iron uptake mechanism in cultured cardiomyocytes is not mainly via LTCC and indicated that it may be mediated through other pathways rather than LTCC. Our findings were consistent with a previous study in providing evidence for ascorbate-reduced form of iron transport in the heart (14). Parkes *et al.* (14) demonstrated that the applications of LTCC blockers (nifedipine, verapamil, diltiazem) failed to alter the iron uptake rates in cultured rat neonatal myocytes. However, several studies using isolated perfused hearts (12) or *in vivo* heart models (13) demonstrated that LTCC is a possible pathway for iron uptake in the heart. The discrepancy in findings regarding the effects of LTCC blocker on iron uptake between ours and others could be attributed to the different experimental models used in those studies. It is also possible that the function or activity in transporting iron of LTCC in our cultured cardiomyocytes could be different than that in the *in vivo* or isolated cardiomyocyte models reported in the previous studies (12, 13).

TfR1 and DMT1 were not the main portals for iron uptake in cultured thalassemic cardiomyocytes

Because LTCC blocker could not prevent iron uptake into cardiomyocytes, free excess iron should enter the cells via other pathways. The two most common and possible pathways are via DMT1 and TfR1. In the hearts, previous studies demonstrated the evidence that both DMT1 and TfR1 were not the portals for iron uptake in the heart under iron-overload condition (8, 9). However, their role as iron transporters in thalassemic cardiomyocytes has never been investigated. In the present study, anti-TfR1 antibody and ebselen, i.e. DMT1 blocker, were used to prevent iron uptake via TfR1 and DMT1. The results showed that neither anti-TfR1 antibody nor ebselen could block iron uptake into any types of cultured cardiomyocytes. These findings suggested that TfR1 and DMT1 did not play a major role in iron uptake into cultured cardiomyocytes under iron-overload conditions. These findings were consistent with a previous study which demonstrated that TfR1 and/or DMT1 mRNA as well as their protein expressions were suppressed in cultured neonatal rat cardiomyocytes (8) and in normal adult rat hearts (9) under iron-loaded conditions. Supporting these findings is also from our microarray data which demonstrated that DMT1 and 2 mRNA expressions in the HT heart were down-regulated, compared to that in the WT hearts.

Efonidipine could prevent iron uptake in cultured thalassemic cardiomyocytes

TTCCs are abundantly expressed in the embryonic cardiomyocytes, but their expression is suppressed in the adult cells (16). However, TTCC has been shown to re-express in pathologic hearts such as hypertrophied and failing ventricles, and the resultant T-type Ca^{2+} currents ($I_{\text{Ca,T}}$) are thought to be involved in the pathological process (17, 18). In our cultured cardiomyocyte model, the results showed that TTCC blocker, efonidipine, could prevent iron uptake into cultured cardiomyocytes, with most effectiveness in HT cardiomyocytes. Although efonidipine is not a selective TTCC blocker and could also block LTCC, its efficacy in blocking TTCC is greater than that of LTCC (30). Because verapamil could not prevent iron uptake when efonidipine could, these findings suggested that TTCC could play a significant role in iron uptake into cardiomyocytes in this model. Moreover, our microarray data demonstrated that the TTCC genes were up-regulated in the HT hearts, which is well correlated with iron uptake result, suggesting that TTCCs play an important role in iron uptake in thalassemic hearts, and that their re-expression could be attributed to the pathological state of thalassemic hearts itself or from the iron-overload condition, or both.

Microarray findings indicate other possible iron uptake pathways

As our study demonstrated that TTCC was the main portal for iron uptake in cultured thalassemic cardiomyocytes, we used the microarray assay to investigate the changes of genes of all possible iron transporters in the hearts of thalassemic mice, especially the TTCC genes. Despite enormous amount of information obtained from the microarray assay, we found that several iron transporters in the hearts, including TTCC, TfR2, and zinc transporter, were markedly up-regulated in the HT hearts, whereas ferroportin was down-regulated in the HT hearts. The findings that TTCC genes were upregulated in the thalassemic hearts correlated well with the pharmacological study in the present study that TTCC blocker could prevent iron uptake into the hearts and may well explain the greater amount of iron uptake found in cultured thalassemic cardiomyocytes, compared to the wild-type cells. In addition to TTCC up-regulated genes, microarray data also provided other possible pathways for iron uptake into these cultured thalassemic cardiomyocytes. These include the up-regulated zinc transporter and TfR2 genes. With these valuable data from microarray analysis, future studies are required to investigate the roles of zinc transporter and TfR2 in cellular iron uptake in both normal and thalassemic hearts.

Study limitation

Our finding that TTCC was a major portal for iron entry in the cultured ventricular myocytes of thalassemic mice could be a disease- and species-specific finding and that it could be different in a different study model. Furthermore, because our findings were based on the *in vitro* study, it is possible that similar or different results can be observed in an *in vivo* model. Future *in vivo* studies are needed to warrant the findings obtained from our *in vitro* model.

Conclusion

This study demonstrated that iron uptake under iron-overload condition in the cultured ventricular myocytes of thalassemic mice was greater than that of wild-type cells. TTCC could be one of the main portals for iron uptake into cultured thalassemic cardiomyocytes, as demonstrated by the prevention of iron uptake by TTCC blocker and the up-regulated genes of TTCC from thalassemic mouse hearts. However, microarray data also provide other possible pathways such as zinc transporter and TfR2, as indicated by an up-regulation of these genes in thalassemic hearts. Future studies are required to investigate the possible roles of these transporters in iron uptake mechanisms in thalassemic hearts.

Conflicts of interest statement

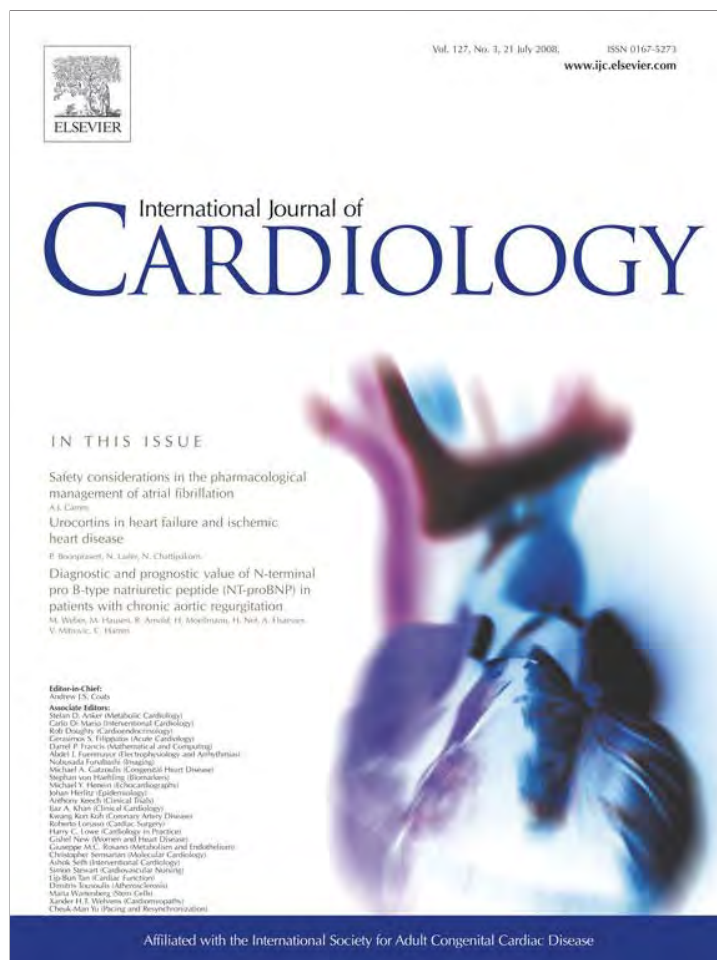
The authors declare that there are no conflicts of interest.

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Review

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Abstract

Urocortins, a novel member of the corticotrophin-releasing factor (CRF) family, have been shown in animal and human studies to possess several beneficial effects in stress, cardiovascular and renal function, and inflammatory responses via CRF receptors. In the heart, urocortins have been demonstrated to produce cardioprotective effects during ischemia and reperfusion injury. Urocortins have also exerted effects on hemodynamic, endocrine and renal parameters in experimental animal heart failure models. In humans, plasma urocortin levels have been shown to significantly increase in systolic heart failure patients. This growing evidence suggests that urocortins may have a prognostic value as well as being a potential therapeutic treatment for heart failure and myocardial infarction patients. Currently, only a few clinical studies on urocortins are available. In this review article, the role of urocortins in the heart has been summarized. Their possible beneficial roles in heart failure and myocardial infarction have been discussed, based on relevant published articles from both basic and clinical studies available to date.

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Keywords: Urocortins; Corticotropin-releasing factor; CRF receptor; Heart failure; Myocardial infarction; Ischemia–reperfusion injury

1. Introduction

Heart failure and ischemic heart disease are common cardiac syndromes, in which an increased incidence has been shown in most nations around the world. The progress of these clinical syndromes can lead to left ventricular dysfunction; including systolic and diastolic dysfunction, and sudden cardiac death. To prevent the progression of the diseases, their identification at an early stage is crucial, since late stage heart failure has been shown to have poor prognosis with high mortality [1]. In past decades, several cardiac

markers were shown to have a predictive value regarding the progression and prognosis in myocardial infarction and heart failure patients. These markers included NT-proBNP [2], cardiac troponin [3], hs-CRP [4], and creatine kinase [5]. Recently, the novel cardioprotective peptides, “urocortins”, have been demonstrated to possess potential value as an early diagnostic marker in heart failure [6]. In this review article, the roles of urocortins as an early cardiac marker as well as their effects in heart failure and ischemic heart disease from animal and clinical studies are presented. In addition, the roles of urocortins, which point to a future clinical research and therapeutic application, are also discussed.

2. Urocortins

Urocortins, 40 amino acid peptides, are a member of the hypothalamic corticotropin-releasing factor (CRF) family. The CRF family consists of CRF, fish urotensin I, frog sauvagine, urocortin I, urocortin II (stresscopin-related peptide, SRP), and

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urocortin III (stresscopin, SCP) [7–10]. The actions of the CRF family peptides are mediated via CRF receptors that are derived from two distinct genes; CRF type1 receptor and CRF type2 receptor [11,12]. Rat urocortin I was first discovered in 1995 from a discrete midbrain region [9]. Its structure is related to urotensin (65% sequence identified) and corticotropin-releasing factor (CRF) (45% sequence identified) [9]. Human urocortin I was identified later by the molecular cloning technique [10]. Regarding its sequence, it was 63% identical to fish urotensin I and 43% identical to CRF at the amino acid level. In rat, the rank orders of mRNA expression of CRF and urocortin I as well as their receptors in each of the tissue types have been shown as follows: the majority of CRF mRNA was found in the brain, whereas the majority of urocortin I mRNA was found in both the heart and brain [13]. Recently, new members of this family, urocortin II and urocortin III, have been identified from human libraries and the genome database [23]. Urocortin II and urocortin III have approximately 20–40% homology with CRF and urocortin I. The homology between urocortin II and urocortin III is approximately 40%. In 2001, human versions of these novel urocortins were also isolated, including a stresscopin-related peptide, which is equivalent to urocortin II, and a stresscopin that is equivalent to urocortin III [7,8,14].

Although urocortins share moderate sequence identity with one another and with CRF, each urocortin has a unique anatomical distribution under the control of different genes. In humans, urocortin I is detected in the brain, placenta, gastrointestinal tract, synovial tissue, lymphocytes, adipose tissue, endothelial cells, immunological tissues, and heart [15–21]. In the brain, urocortin I was detectable at the Edinger–Wesphal nucleus, the lateral superior olive, the supraoptic nucleus (SON), the lateral hypothalamic area, and most caudal part of several brainstem and spinal cord motoneuron nuclei [12,22]. In the nervous system, urocortin I has been shown to enhance anxiety and suppress appetite via the CRF type1 receptor [23]. The finding of urocortin mRNA expression in synovium tissues in rheumatoid arthritis and osteoarthritis patients suggests that urocortin may also act as an immune-inflammatory mediator [19]. Urocortin I activates the pituitary–adrenal axis inducing the release of adrenocorticotrophic hormone (ACTH), and increasing plasma cortisol, and atrial natriuretic peptide in animal models [24–27]. In humans, the biological effects of urocortin I infusion in healthy males include increased plasma level of ACTH and cortisol. These effects of urocortin I are mediated via the CRF type1 receptor [28]. Similar to urocortin I, urocortin II is expressed in the heart and discrete areas of the central nervous system of rats, including stress-related cell groups in the hypothalamus, brainstem and spinal cord [8,29]. Urocortin III is also expressed in the brain and peripheral tissues such as gastrointestinal tract, pancreas, and skin. However, the expression of urocortin III is considerably lower than that of urocortin II [7,30–32]. In humans, urocortin III was present in both the ventricles and atria [33]. However, the highest levels were

found in all regions of the pituitary and hypothalamus [32]. Urocortin III was also distributed in other tissues, such as the lung, pancreas, liver, spleen and skeletal muscle, and present in human plasma and urine [33].

3. CRF receptors for urocortins

CRF receptors consist of two types of seven-transmembrane-spanning G-protein receptors, which are coupled to adenylyl cyclase: CRF type1 and CRF type2 receptors. The CRF type1 receptor is found predominantly in the pituitary and brain regions, whereas the CRF type2 receptor is highly expressed in peripheral tissues including the heart [8,13,29,34]. The CRF type1 receptor has a single functional form: CRF type1 (alpha) receptor, whereas the CRF type2 receptor has three isoforms: CRF type2 (alpha) receptor, CRF type2 (beta) receptor and CRF type2 (gamma) receptor [11,35,36]. The CRF type1 receptor implicates in adrenocorticotrophic hormone (ACTH) release and anxiety-like effect, while the CRF type2 receptor contributes to anxiolysis, appetite-inhibition, vasodilatation, positive inotropic action on the myocardium, and dearousal effects [12]. CRF and urocortin I can bind to both CRF type1 and type2 receptors [9]. Urocortin II and urocortin III bind to only the CRF type2 receptor [7,8]. The CRF type2 receptor binds to urocortin I, urocortin II and urocortin III, with approximately 40-fold greater affinity than CRF [9].

CRF type2 receptors have different tissue distributions. In rats, the CRF type2 (alpha) receptor is highly expressed in the brain, while the CRF type2 (beta) receptor is expressed in the brain, heart, lung, gastrointestinal tract, and skeletal muscle [37]. The CRF type2 (gamma) receptor has only been detected in the brain [38]. The cDNA sequence in the protein-coding region of rat tissue is 94% identical to the CRF type2 (beta) receptor [39,40]. The CRF type2 (beta) receptor has 10-fold higher affinity to urocortin than CRF in rat models [9], whereas the CRF type1 receptor shows little ligand selectivity for urocortin [10]. In the rat's heart, only the CRF type2 (beta) receptor is found. The high level of urocortin mRNA detected in the heart was related to the high expression of CRF type2 (beta) receptor coupling in the heart tissue of rats [13]. In the human heart, however, only the alpha type of CRF type2 receptor is found [41,42].

4. Urocortins and the heart

Kimura et al. reported that urocortins and the CRF type2 (alpha) receptor were detected in all chambers of four human hearts, and detected with highest intensity in the left ventricle, whereas CRF was not detected in cardiac myocytes [17]. In their study, detection of the CRF type1 receptor was weak in the left atrium, left ventricle and right ventricle [17]. Since the CRF type2 (alpha) receptor was detected predominantly in the left ventricle, it has been suggested that urocortins, not CRF, may have an important role on cardiac function [17]. By using radioligand binding techniques, Wiley and Davenport

demonstrated that urocortins have a high affinity to the CRF type2 (alpha) receptor in human left ventricular myocardium, intramyocardial blood vessels and within the medial layer of internal mammary arteries [43].

Currently, urocortins are overwhelmingly shown to affect cardiovascular physiology in normal animals, and pacing-induced heart failure in animal models and clinical studies [26,27,44,45]. The cardiovascular effects of urocortins include a dose-dependent increase in heart rate, cardiac output, and coronary blood flow [26]. Interestingly, urocortins have been shown to possess an anti-apoptotic effect in myocardium that has undergone ischemia–reperfusion injury [26,44,45]. Urocortin and CRF also exerted coronary vasodilatation and positive inotropic effect. These physiological effects of urocortin have been shown to last longer than the action of CRF [46].

The high dose administration of urocortin I in rats has been reported to decrease arterial pressure via vasodilatation, as revealed by a decrease in total peripheral resistance and slightly increased cardiac output [47]. The dilator action of urocortin I is mostly manifested in the heart and stomach [48]. In mice, urocortin I increases cardiac contractility, heart rate, and vasodilatation [46]. However, these effects were not found in CRF type2 receptor-deficient mice [49,50]. These data indicate that actions of urocortin I in peripheral tissues are expressed via the CRF type2 receptor. Urocortin I has also been shown to enhance cardiac output, protect myocytes from ischemia–reperfusion injury, stimulate cardiac natriuretic peptide secretion, induce vasodilatation, especially in the coronary arteries of normal and heart failure models [26,27,44–46,51–54], and decrease left atrial pressure and peripheral vascular resistance [27,27,47].

Urocortin II exerts positive inotropic effect via CRF type2 receptor-mediated stimulation and enhances contractility of rabbit ventricular myocytes [55]. Urocortin III causes an increase in cardiac output and a decrease in peripheral resistance and left atrial pressure in heart failure sheep [56]. Since these cardiovascular effects are beneficial in reducing preload and afterload to the heart, researchers are currently investigating the roles of urocortins in heart failure using animal models as well as heart failure patients [6,27,47,56,57].

5. Urocortins and heart failure

Rademaker et al. investigated the effects of urocortin administration in both normal and pacing-induced heart failure sheep [27,47,56,57]. Urocortin I, urocortin II and urocortin III induced an immediate increase in cardiac output and a decrease in peripheral resistance and left atrial pressure. In normal sheep, however, only slight changes in peripheral resistance and atrial pressure were found. Interestingly, urocortin I, urocortin II and urocortin III caused a dose-dependent decrease in plasma vasopressin, endothelin-1, renin, aldosterone, epinephrine, plasma atrial and brain natriuretic peptide levels in heart failure sheep, whereas adrenocorticotrophic hormone and cortisol levels were increased. These urocortins also increased urine output,

sodium excretion, and creatinine clearance [47,56,57]. Urocortin infusion in normal sheep resulted in a similar rise in cortisol and vasopressin, a decrease in aldosterone, and no significant effects on plasma renin activity and natriuretic peptides. Nevertheless, long-term peripheral and central administration of urocortin caused an increase in arterial pressure and heart rate in 15 normal ewes [58]. Furthermore, Mackay et al. also demonstrated that urocortin II caused a reduction in arterial pressure and increased heart rate in fifty adult heart failure rats [59]. These reports, using animal models, support the role of urocortins in arterial pressure and intravascular volume homeostasis in heart failure.

Currently, there are only a few clinical studies of urocortin available. In a cross-sectional study by Ng et al., investigation was carried out on plasma urocortin in 119 patients with systolic heart failure and 212 healthy individuals (LVEF > 50%) [6]. They reported that urocortin was elevated in systolic heart failure (LVEF ≤ 45%), especially in NYHA functional classes I–II [6]. The relative increase in plasma urocortin level was greater in males than in females, but the level decreased with increasing age, especially in heart failure groups. The decreased urocortin levels, with increasing NYHA class, were reinforced by a significant correlation between urocortin and ejection fraction in heart failure patients. From these findings, it was suggested that the urocortin level complemented NT-proBNP in the early diagnosis of mild clinical heart failure [6]. An increased plasma urocortin level in mild stage systolic heart failure, and the reduced level when heart failure progresses, could have potential clinical benefits if warranted, since these findings may be used for the diagnosis of early heart failure and as a prognostic indicator for heart failure progression. Recently, Davis et al. investigated the effects of urocortin II infusion in 8 healthy unmedicated men [60]. They found that urocortin II induced an increase in cardiac output, heart rate, and left ventricular ejection fraction, while decreasing systemic vascular resistance. These effects also increased plasma renin activity, angiotensin II and norepinephrine [60]. Although the clinical benefits of urocortins are still unclear, due to the small number of clinical studies, the growing body of evidence indicates the need to warrant their clinical significance by performing more clinical studies in the future, including those that investigate the plasma profile of urocortin in heart failure patients with long-term follow up.

6. Urocortins and myocardial ischemia

Apoptosis is known to implicate the pathogenesis of cardiovascular diseases such as myocardial infarction [61]. It causes the loss of cardiac myocytes and worsens the cardiac function. While hypoxia induces both necrotic and apoptotic forms of cell death in cardiac myocytes, the degree of cardiac dysfunction after ischemia–reperfusion injury reflects the level of myocardial injury and cell death [62].

In experimental studies, the increased urocortin level had been expressed when cardiac cells were exposed to thermal shock and ischemia [63,64]. Brar et al. studied urocortin

against the damaging effects of ischemia–reperfusion injury in rat hearts [44]. They demonstrated that exogenous urocortin caused increased cardiac contractility and vasodilatation, especially in coronary arteries, and protected the heart during ischemia by reducing the number of cell deaths induced by hypoxia [44]. Urocortin prevented neonatal rat myocytes from cell death when it was administered prior to stimulated hypoxia/ischemia, and at the point of reoxygenation after stimulated hypoxia/ischemia [44]. It acted via a p42/p44 MAPK-dependent signaling pathway in *in vitro* and *in vivo* adult rat hearts with ischemia–reperfusion injury [54]. In cardiac myocytes of rats, Okosi et al. demonstrated that the urocortin mRNA level was increased by thermal shock and that exogenous urocortin protected cardiac myocytes from cell death induced by hypoxia [64]. Exogenous urocortin also protected cardiac myocytes against necrotic cell death from a reduction in LDH release [64]. Other protective roles of urocortin in ischemia–reperfusion injury include inhibiting free radical activities, preventing mitochondrial damage, and activating the phosphatidylinositol 3-OH kinase and PKC-epsilon in mice [65–69]. The infarction size and mean arterial pressure were significantly decreased in rat hearts that received urocortin [54,69].

Urocortin increased interleukin-6 levels from peripheral blood mononuclear cells [19]. In mice, it reduced lipopolysaccharide-induced serum TNF-alpha and interleukin-1 beta levels [19,70]. Honjo et al. demonstrated that urocortin mRNA expression is also upregulated by TNF-alpha and IFN-gamma [18], suggesting that urocortin could have an anti-oxidative effect against oxidative stress in inflammatory lesions [18]. Urocortin has also been shown to reduce necrotic and apoptotic cell death in isolated rat hearts exposed to ischemia–reperfusion injury, and partially prevent the depletion of cellular energy stores, with enhancement of ventricular function [62].

In murine hearts, Brar et al. demonstrated that urocortin II and urocortin III protected murine cardiomyocytes from ischemia–reperfusion injury and reduced the percentage of infarct size [29]. These effects have been demonstrated to act via the ERK1/2-p42 signaling pathway and CRF type2 receptor mediated in the heart [29].

7. Future role and clinical application of urocortins

Previous studies have demonstrated the beneficial effects of urocortin, including coronary vasodilatation, increased cardiac contractility, coronary blood flow and conductance, cardiac output, and heart rate as well as protection against ischemia–reperfusion injury [26,27,29,44,45,47,56,57,63,64]. Theoretically, these effects are useful in the treatment of cardiovascular disorders, particularly heart failure and conditions associated with ischemia–reperfusion such as myocardial infarction. Practically, the role of urocortins in heart failure and ischemic heart disease is still unclear. Therefore, caution should be taken despite growing evidence of the beneficial effects of urocortins. For example, exogenous urocortin I could cause

undesired effects when treating ischemia–reperfusion injury, since it could increase the plasma ACTH level via CRF type1 receptor activation [28]. Since most reports were performed in experimental animal models, with only a few clinical studies, large prospective clinical studies with long-term follow up are needed to warrant the clinical significance of urocortins.

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Cilostazol attenuates ventricular arrhythmia induction and improves defibrillation efficacy in swine

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Abstract: Previous reports demonstrated that cilostazol, a phosphodiesterase 3 inhibitor, affected cellular electrophysiology and reduced episodes of ventricular fibrillation (VF) in patients with Brugada syndrome. However, its effects on VF induction and defibrillation efficacy have never been investigated. We tested the hypothesis that cilostazol increases the VF threshold (VFT) and decreases the upper limit of vulnerability (ULV) and the defibrillation threshold (DFT). A total of 48 pigs were randomly assigned to defibrillation and VF induction studies. The diastolic pacing threshold (DPT), VFT, ULV, DFT, and effective refractory period were determined before and after the infusion of cilostazol at 6 mg/kg, 3 mg/kg, or vehicle. The DPT was significantly increased after administration of 3 and 6 mg/kg cilostazol. The ULV and DFT were significantly decreased after administration of 6 mg/kg cilostazol only. The ULV in the 6 mg/kg group had 12% lower peak voltage and 25% lower total energy, and the DFT had 13% lower peak voltage and 25% lower total energy. The VFT was not altered in any experimental group. This study shows that cilostazol administration significantly increased the DPT, which was associated with significantly reduced DFT and ULV.

Key words: cilostazol, phosphodiesterase 3, ventricular fibrillation threshold, defibrillation threshold, upper limit of vulnerability, arrhythmia inducibility.

Résumé : Des rapports antérieurs ont démontré que le cilostazol, un inhibiteur de la phosphodiestérase III, influe sur l'électrophysiologie cellulaire et réduit les épisodes de fibrillation ventriculaire (FV) dans le syndrome de Brugada. Toutefois ses effets sur l'induction de la FV et l'efficacité de la défibrillation n'ont jamais été examinés. Nous avons vérifié l'hypothèse que le cilostazol augmente le seuil de FV (SFV), et diminue la limite maximale de vulnérabilité (LMV) et le seuil de défibrillation (SDF). Nous avons réparti 48 porcs au hasard dans des groupes d'induction de fibrillation ventriculaire et de défibrillation. Nous avons déterminé le seuil de stimulation diastolique (SSD), la période réfractaire efficace, le SVF, la LMV et le SDF, avant et après la perfusion 6 mg/kg de cilostazol, de 3 mg/kg, ou d'un véhicule. L'administration de cilostazol a augmenté le SDF de manière significative. La perfusion de 6 mg/kg a augmenté significativement la LMV et le SDF. La LSV du groupe 6 mg/kg a eu une tension de crête de 12 % inférieure et une énergie totale de 25 % inférieure. Le SDF de ce groupe a eu une tension de crête de 13 % inférieure et une énergie totale de 25 % inférieure. Le SFV est demeuré stable chez tous les groupes expérimentaux. Ainsi, l'administration de cilostazol a augmenté significativement le SSD, ce qui a été associé à une réduction significative du SDF et de la LMV.

Mots-clés : cilostazol, phosphodiestérase 3, seuil de fibrillation ventriculaire, défibrillation, limite maximale de vulnérabilité, inductibilité d'arythmies.

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Introduction

Cilostazol is a selective phosphodiesterase 3 (PDE3) inhibitor (Cone et al. 1999). Because of its potent effect on vasodilatation and antiplatelet aggregation, cilostazol was approved by the US Food and Drug Administration for claudication treatment (Dawson et al. 1998). The antiarrhythmic effects of cilostazol were first presented in atrial bradyarrhythmia (Atarashi et al. 1998). Previous studies demonstrated that cilostazol affected the automaticity of the heart by increasing heart rates in patients and improving symptoms in bradycardic atrial fibrillation, sick sinus syndrome, and Wenckebach type atrioventricular (AV) block (Madias 2003). In patients with third-degree AV block, cilostazol increased heart rates and increased the ventricular escape rate without the abolishment of AV block or a change in the fre-

quency of premature ventricular beats (Kodama-Takahashi et al. 2003). Also, cilostazol has been shown to decrease atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) concentrations (Madias 2003), hormones presenting in AV asynchrony and heart failure (Mair et al. 2001).

In a recent case report on Brugada syndrome patients, it was shown that ventricular fibrillation (VF) episodes were completely abolished after oral administration of cilostazol at 200 mg/day (Tsuchiya et al. 2002). However, VF recurred when cilostazol was discontinued or reduced to 100 mg/day. In a 1-year follow-up of 3 patients with implanted cardioverter defibrillators, no VF occurred during cilostazol treatment (Tsuchiya et al. 2002). Recently, cilostazol has been shown to directly activate mitochondrial Ca^{2+} -activated K^{+} channels, thus protecting the myocardium from ischemic and (or) reperfusion injury (Fukasawa et al. 2008).

Despite this possible beneficial effect, direct investigation has never been tested regarding the effects of cilostazol on VF induction and defibrillation. In the present study, we tested the hypotheses that (i) cilostazol decreases VF inducibility by increasing VF threshold (VFT) and decreasing the upper limit of vulnerability (ULV), and that (ii) cilostazol improves defibrillation efficacy by decreasing the defibrillation threshold (DFT).

Materials and methods

Animal preparation and electrode placement

Forty-eight male and female Landrace pigs (25–30 kg) were used in this study. The animals were cared for in accordance with the *Guide to the Care and Use of Experimental Animals* (Olfert et al. 1993) and with the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University. In each pig, anesthesia was induced with a combination of atropine (0.04 mg/kg), telazol (4.4 mg/kg), and xylazine (2.2 mg/kg) and maintained with 1.5%–3.0% isoflurane delivered in 100% oxygen (Shinlapawittayatorn et al. 2006). The surface electrocardiogram (lead II), arterial oxygen tension (PaO_2), end-tidal CO_2 , femoral arterial blood pressure (BP), core temperature, respiratory rate, blood gases, and electrolytes were monitored throughout the study. Under artificial respiration, a catheter with a 34 mm platinum-coated titanium coil electrode (Guidant) was inserted into the right ventricular (RV) apex. A 68 mm electrode catheter was placed at the junction between the right atrium and the superior vena cava (Chattipakorn et al. 2000a). These 2 electrodes were used for shock delivery. The acute experiments were divided into 2 series: a VF induction study (series 1, $n = 24$) and a defibrillation study (series 2, $n = 24$). Pigs were randomly designated to 1 of these 2 series. In each series, the pigs were divided into 3 groups ($n = 8$ each). All parameters in the VF induction and defibrillation studies were determined before and after cilostazol or vehicle administration. In these cases, both BP and heart rate (HR) were determined from the data recorded before VFT, ULV, and DFT determination before drug or saline administration. The drug was infused for 50 min. At the end of the infusion, we waited for another 20 min before the determination of electrophysiological parameters (i.e., BP and HR) began.

Diastolic pacing threshold and effective refractory period measurement

A train of 10 S1 stimuli was delivered through a pacing electrode with a stimulation pulse of 5 ms at the tip of an RV catheter, beginning at 0.1 mA, with 500 ms intervals. The pacing current was increased in 0.1 mA increments until all S1 stimuli elicited a ventricular response. This current was defined as the diastolic pacing threshold (DPT). The effective refractory period (ERP) was determined by delivering an S2 stimulus after the last S1 stimulus. The S1–S2 interval was initially set at 350 ms and was reduced in 10 ms steps until an S2 stimulus was unable to elicit a ventricular response. This S1–S2 interval was defined as an ERP (Kanlop et al. 2008).

VF induction and ULV determination protocol

The interval between the last S1 and the peak T wave was determined 3 times from the lead II electrocardiogram. The average of these intervals was used as a coupling interval to deliver an S2 stimulus (S1–S2 coupling interval) (Chattipakorn et al. 2000b, 2000c, 2006; Rodríguez and Trayanova 2003; Swerdlow et al. 2003). The S2 shock was biphasic and was delivered by shocking electrodes with a Ventak external cardioverter-defibrillator (ECD) (Guidant). The initial shock strength was set at 100 V. If the shock induced VF, the shock strength was decreased in 10 V steps until VF was not induced (Compos et al. 1997; Malkin and Hoffmeister 2000). If the shock did not induce VF, the strength was increased in 10 V steps until VF was induced (Compos et al. 1997; Malkin and Hoffmeister 2000). The lowest shock strength required to induce VF is defined as the VFT (Lakireddy et al. 2006).

For ULV determination, the coupling interval between the last S1 and the peak T wave was determined again. An S2 stimulus was then delivered at an initial shock strength of 400 V. The shock strength was increased or decreased after a 3-reversal up/down protocol (Chattipakorn et al. 2000b). The lowest shock strength above the VFT that was unable to induce VF was defined as the ULV. There was a minimum interval of 4 min between VF episodes to allow the heart to return to physiological conditions (Sungnoon et al. 2008).

Defibrillation protocol

VF was induced by delivering a 60 Hz AC current through the tip of the RV electrode. Defibrillation shocks were delivered with defibrillation electrodes after VF was induced for 10 s. For episodes in which defibrillation was unsuccessful, a rescue shock was delivered to restore sinus rhythm. A minimum interval of 4 min was allowed between VF episodes. The defibrillation shock strength was initially set at 400 V and was increased or decreased after the 3-reversal up/down protocol (Chattipakorn et al. 2000a). The lowest shock strength required for successful defibrillation after the third reversal was defined as the DFT.

Cilostazol preparation

Cilostazol solution was prepared as an aqueous stock solution (Hakaim et al. 1999; Saitoh et al. 1993). A 50 mg cilostazol (Pletal) tablet was dissolved in normal saline just before the experiment to form a concentration

Table 1. Electrophysiological and hemodynamic parameters measured before and after drug or saline administration in Landrace pigs.

	Group I, cilostazol 6 mg/kg		Group II, cilostazol 3 mg/kg		Group III, control	
	Before	After	Before	After	Before	After
Diastolic pacing threshold, mA	0.40±0.06	1.40±0.20**	0.40±0.07	0.90±0.08**	0.40±0.07	0.6±0.07
Effective refractory period, ms	273±9	293±9	233±8	253±12	234±8	246±6
S1–S2 coupling interval, ms	308±11	324±14	262±8	265±15	258±10	266±11
Systolic blood pressure, mm Hg	121±6	113±5	122±7	120±5	120±5	123±9
Diastolic blood pressure, mm Hg	78±6	79±6	82±6	78±3	74±5	77±8
Heart rate, beats/min	86±3	86±5	99±5	106±4	99±5	103±3

Note: The control group received 100 mL saline (vehicle). Values are means of 8 animals per group. **, Significant at $p = 0.01$ vs. before drug administration (within group).

of 0.5 mg/mL. The solution was then filtered using Whatman No. 1 filter paper until a clear solution was obtained.

A previous study reported that the pharmacological effects were demonstrated with an infusion of cilostazol 3 mg/kg body weight in the *in vivo* model (Hakaim et al. 1999). Therefore, in the present study, we started by intravenously administering cilostazol at a dose of 6 mg/kg, 3 mg/kg, or vehicle (100 mL saline) in groups I, II, and III, respectively, over 50 min, to investigate their effects on fibrillation induction and defibrillation efficacy.

Statistical analysis

Values are expressed as means ± SE in both VF induction and defibrillation studies. Analysis of covariance (ANCOVA) was used to assess whether the inequality of the baseline values affected the results after treatment. Comparisons of variables before and after drug or saline administration (within-group comparison) were performed individually in each group by using the paired 2-tailed Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

The basic cardiac electrophysiological and hemodynamic parameters (DPT, ERP, S1–S2 coupling interval, HR, systolic BP (SBP), and diastolic BP (DBP)) in all experimental groups are shown in Table 1. After the drug administration in groups I (cilostazol 6 mg/kg) and II (cilostazol 3 mg/kg), the DPT (tested DPT) was significantly higher ($p = 0.01$ in both groups) than the DPT before drug administration (control DPT). In group III, vehicle (saline) did not change the DPT compared with baseline. The ERPs were not different in any of the experimental groups. The S1–S2 coupling interval before and after drug administration was not different in any of the groups. Both concentrations of cilostazol and vehicle administration did not change systolic BP, diastolic BP, or HR.

In the VF induction study, the number of VF episodes requiring defibrillation shocks before drug administration was 7.4 ± 0.5 , 6.8 ± 0.4 , and 7.0 ± 0.3 episodes in groups I, II, and III, respectively, and after drug administration was 6.3 ± 0.3 , 7.0 ± 0.6 , and 7.1 ± 0.3 episodes, respectively. It has been shown that the VF episodes were not significantly different in any of the groups. The peak voltage, total energy, impedance, and pulse width of VFT before and after drug or vehicle administration were not changed in any of the experimental groups (Fig. 1). From the analysis of covariance, the tested ULV was found to be affected by the base-

line ULV. Despite this effect, the tested ULV was still significantly ($p < 0.05$) decreased after 6 mg/kg cilostazol administration (Figs. 2A, 2B). In the 6 mg/kg group, peak voltage was reduced by 12% and total energy was reduced by 25%. The impedance and pulse width were not significantly changed in any of the experimental groups (Figs. 2C, 2D). Furthermore, in group I, the ULV–VFT window width, calculated by subtracting the VFT from the ULV, was shown to significantly decrease compared with the ULV–VFT window width before drug administration. This effect was not shown in groups II or III. (Table 2)

In the defibrillation study, before drug administration, VF was induced 7.1 ± 0.6 , 7.5 ± 1.0 , and 9.1 ± 0.5 episodes in groups I, II, and III, respectively; after drug administration, VF was induced 6.9 ± 1.0 , 6.9 ± 0.6 , and 9.0 ± 0.5 episodes, respectively. It has been shown that the VF episodes were not significantly different in any of the groups. The tested DFT was affected by the baseline DFT. However, 6 mg/kg cilostazol significantly decreased the DFT from the baseline (Figs. 3A, 3B). In the 6 mg/kg group, peak voltage was reduced by approximately 13% and total energy was reduced by approximately 25%. The peak voltage and total energy for the DFT before and after drug or saline administration were not different in groups II or III. The impedance and pulse width were not significantly changed in any of the groups (Figs. 3C, 3D).

Discussion

The major findings of this study are as follows: (i) administration of both 3 and 6 mg/kg cilostazol significantly increased the DPT; and (ii) a 6 mg/kg cilostazol infusion significantly decreased the ULV and the DFT.

Effects of cilostazol on myocardial stabilization

In the past, there was disagreement over the use of PDE3 inhibitors in patients with cardiac disease, since the inhibitors facilitate the incidence of arrhythmia in this group of patients (Feldman et al. 1993; Packer et al. 1991). Previous studies investigated the effects of PDE3 inhibitors, such as amrinone, milrinone, and enoximone, on cardiac electrophysiological parameters by measuring HR, QT_c (corrected QT interval), and atrial or ventricular ERP (Goldstein et al. 1986; Miles et al. 1989; Naccarelli et al. 1984). However, the effects of cilostazol on the DPT and ERP have never been investigated.

It is known that PDE3 has many isoforms, and that PDE3 inhibitory drugs demonstrate different effects depending on

Fig. 1. Effects of cilostazol on electrophysiological parameters for the ventricular fibrillation threshold before and after drug administration ($n = 8$ pigs per group).

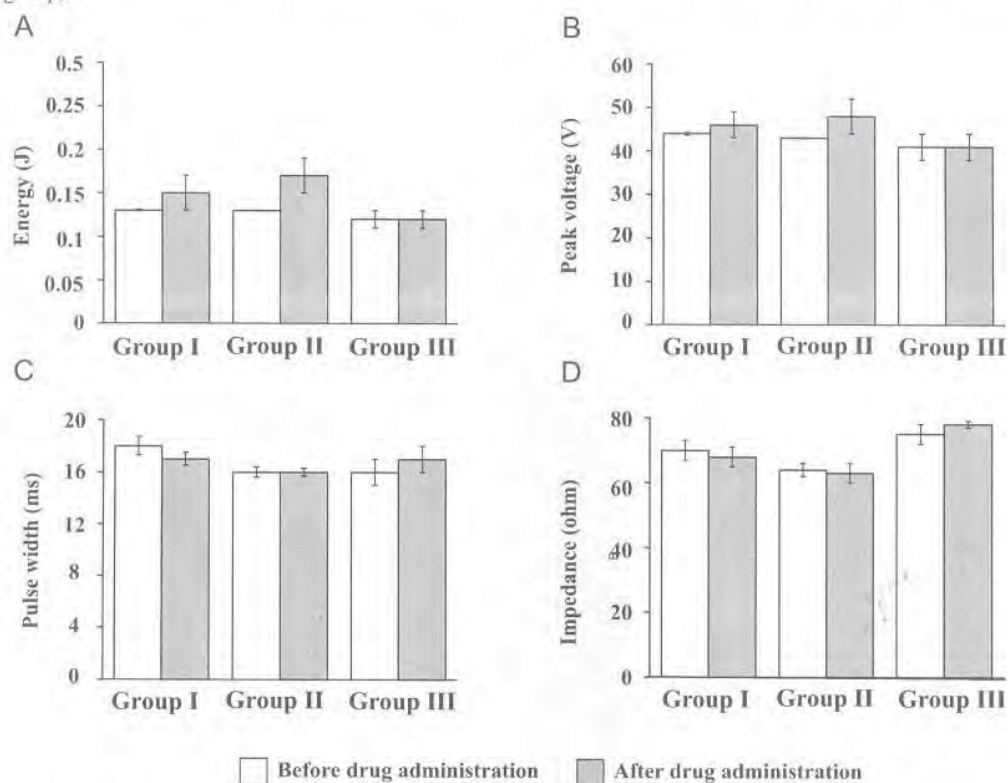


Fig. 2. Effects of cilostazol on electrophysiological parameters for the upper limit of vulnerability before and after drug administration. *, Significant at $p < 0.05$ vs. before drug administration (within group, $n = 8$).

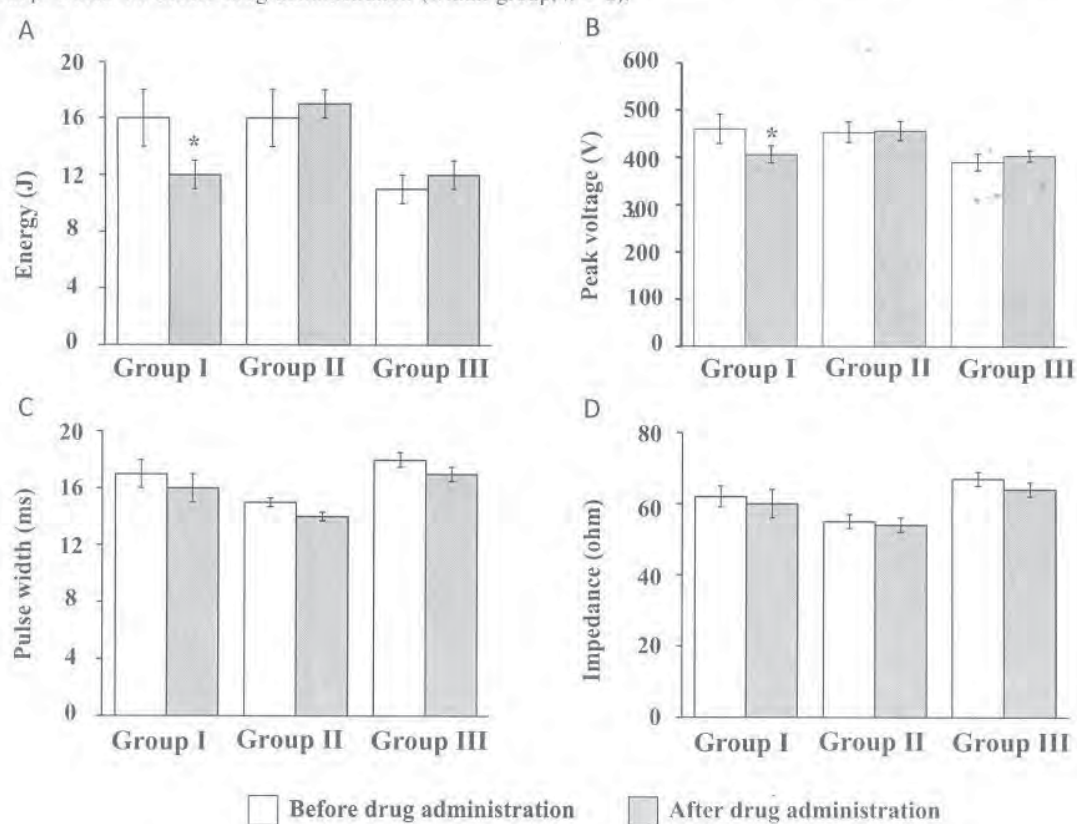
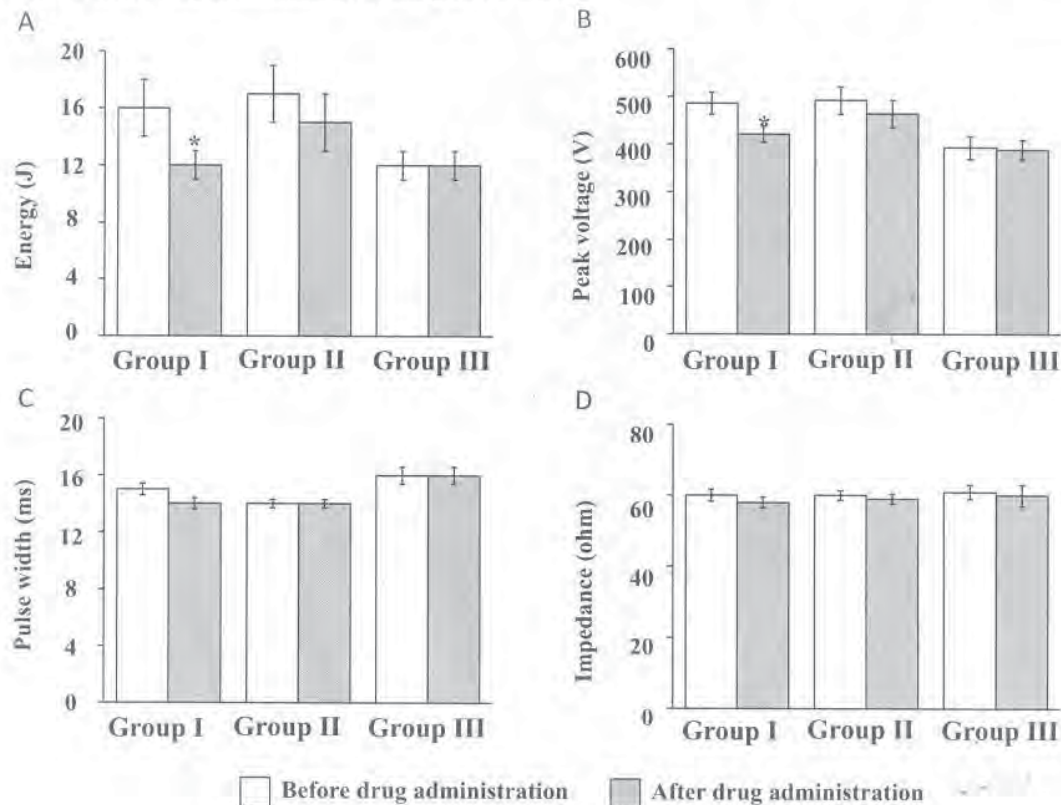


Table 2. The mean ventricular fibrillation induction window width (ULV–VFT) in the 3 treatment groups.

	Group I, cilostazol 6 mg/kg		Group II, cilostazol 3 mg/kg		Group III, control	
	Before	After	Before	After	Before	After
Voltage, V	416±31	360±16*	410±22	407±20	349±19	362±13
Energy, J	16±2	12±1*	17±1	17±1	11±3	12±2

Note: ULV, upper limit of vulnerability; VFT, ventricular fibrillation threshold. Values are means of 8 animals per group.
 *, Significant at $p < 0.05$ vs. before drug administration (within group).

Fig. 3. Effects of cilostazol on electrophysiologic parameters for the defibrillation threshold before and after drug administration. *, Significant at $p < 0.05$ vs. before drug administration (within group, $n = 8$).

the PDE3 isoform they inhibit (Atarashi et al. 1998; Cone et al. 1999; Hambleton et al. 2005). In this study, ERP was not changed from baseline after cilostazol administration. This finding is similar to that reported previously using other PDE3 inhibitors (Goldstein et al. 1986; Miles et al. 1989; Naccarelli et al. 1984). However, the effect of a PDE3 inhibitor on the DPT has never been investigated. In our study, cilostazol increased the DPT. It is possible that the effect of cilostazol on the DPT is similar to or different from previously tested PDE3 inhibitors, because of the different isoforms that each drug inhibits. This hypothesis remains to be tested.

Recently, Fukasawa and colleagues (2008) demonstrated that cilostazol promotes the electrical stability of myocardium in ischemic and (or) reperfusion injury in isolated hearts by directly activating mitochondrial Ca^{2+} -activated K^+ (mitoK_{Ca}) channels, leading to an attenuation of Ca^{2+} overload in mitochondria and the stabilization of Ca^{2+} homeostasis. Furthermore, in normal hearts, cilostazol is known to

activate L-type Ca^{2+} channels using a cAMP-activated protein kinase A pathway (Verde et al. 1999).

Effects of cilostazol on defibrillation efficacy

In this study, we demonstrated that cilostazol 6 mg/kg increased defibrillation efficacy by significantly decreasing the DFT. The DFT was not significantly changed after the infusion of cilostazol 3 mg/kg. Since uniform dispersion of refractoriness is an important determinant of successful defibrillation, one possible explanation for this observation is that cilostazol decreases the degree of dispersion of refractoriness in a dose-dependent manner. Future studies are needed to test this hypothesis.

Effects of cilostazol on VF induction

In the present study, the administration of cilostazol 6 mg/kg significantly decreased the ULV, and the proportion of ULV reduction was similar to the proportion of DFT reduction. Previous studies have demonstrated a close correlation between

the ULV and the DFT (Green et al. 2003; Rodríguez and Trayanova 2003). Our study has shown that both dosages of cilostazol increased the DPT. It is possible that the DPT increased by cilostazol decreases the sensitivity of the myocardium to any premature stimulus. According to the ULV hypothesis for defibrillation, shocks weaker than the ULV fail to defibrillate because they fall into the vulnerable period, thus reinitiating VF (Chen et al. 1986). Our results are consistent with such reports. Regarding the reentrant mechanism, the important factors for VF initiation and sustained VF are the initiation of new wave fronts, an increased dispersion of refractoriness that leads to unidirectional block, and a decreased conduction velocity (Rodríguez and Trayanova 2003).

Study limitations

This study was performed in normal pig hearts. The effects of cilostazol on VF induction or defibrillation may be different in diseased hearts, such as those in patients with Brugada syndrome or myocardial infarction. Also, it is possible that cilostazol 6 mg/kg increases the VFT, but that the VFT protocol used in our study was not sensitive enough (10 V steps) to detect it. This may explain why VFT did not significantly change after cilostazol administration. Since the plasma level of cilostazol was not measured, the relationship between plasma concentration and the observed effects remains to be investigated. The definite mechanism responsible for this dose-dependent effect of cilostazol on the ULV and the DFT was not elucidated in this study because of technical limitations. Our DPT and ERP results do not predict total electrical stability, since they were determined at only a single site. Although it is possible that DPT increases with time because of electrode contact changes or tissue damage, it is likely that the increase in DPT by cilostazol in our study was due to the effect of the drug itself because saline did not change the DPT. Furthermore, the vascular effect of cilostazol was not observed in this study. It is possible that these effects were masked by the effects of the deep general anesthesia with fluid maintenance that was used to keep the animal under physiological conditions throughout the study.

Conclusion

Cilostazol alters cardiac electrophysiology by increasing the DPT and decreasing the ULV and DFT. These findings indicate that cilostazol can decrease VF inducibility by reducing the ULV and can improve defibrillation efficacy by reducing the shock energy required to successfully defibrillate the heart.

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Reversible acetylcholinesterase inhibitory effect of *Tabernaemontana divaricata* extract on synaptic transmission in rat CA1 hippocampus

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Background & objectives: Acetylcholinesterase inhibitors (AChE-Is) are used for the treatment of Alzheimer's disease (AD). These drugs including galanthamine have been shown to modulate synaptic activity in hippocampus and improve memory processes. Although *Tabernaemontana divaricata* extract (TDE) has been used as traditional medicine for various pharmacological effects, its effect in enhancing cholinergic activity provides additional benefit to its known effects. We investigated whether TDE can modulate the synaptic function in hippocampus and compared its effects to those of galanthamine.

Methods: Hippocampal slices were prepared from male Wistar rats, functional effects of TDE were characterized by using pharmacological tools and extracellular recordings of field excitatory postsynaptic potentials (fEPSPs).

Results: TDE significantly reduced fEPSPs. The fEPSPs reduction was prevented by atropine, but not pancuronium. These TDE effects were similar to those of galanthamine.

Interpretation & conclusions: Our findings indicate that TDE can effectively modulate synaptic responses in the hippocampus similar to galanthamine, suggesting that this traditional medicine could be beneficial in ageing with ACh deprivation in the brain.

Key words Acetylcholinesterase inhibitor - field excitatory postsynaptic potentials- hippocampus - *Tabernaemontana divaricata*

Acetylcholine (ACh), acting through neuronal muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs), is an important modulator of electrical activity in the brain. It is involved in numerous cellular processes underlying cognition including development, synaptic transmission, neuronal excitability and systems-level rhythmicity¹⁻³. Stimulation of cholinergic inputs to the hippocampus demonstrates a decrease in the size of synaptic responses in glutamatergic projections

in different regions of the hippocampus^{1,4,8}. An acetylcholinesterase inhibitor (AChE-I), physostigmine, has been shown to enhance cholinergic transmission and depress glutamate release in hippocampal pathways⁹. Modulation of the synaptic strength of excitatory glutamate synapses in the hippocampus is believed to be involved in memory processing¹⁰.

The loss of cholinergic function, particularly in the hippocampus, has been implicated in Alzheimer's

disease (AD)¹¹. Current treatment is mainly based on the use of AChE-Is. Although some beneficial effects of these drugs on cognition have been reported, these are costly and have undesirable side effects¹². Several drugs commonly used today for AD treatment were developed from local and traditional medicine, such as galanthamine, an AChE-I which was developed from alkaloids in 'Snowdrop'¹³.

Tabernaemontana divaricata (L.) R. Br. Ex Roem. & Schult (*T. divaricata*), a garden plant in tropical countries, is a rich source of alkaloids with various pharmacological properties¹⁴. *T. divaricata* has been used in the folk medicine for anti-infection, anti-inflammation, analgesic effect, anti-tumour effect, antioxidative effect and the effect in neuronal activity¹⁵. Ingkaninan *et al*¹⁶ have shown *in vitro* that ethanol extracts from *T. divaricata* root (TDE) at a concentration of 0.1 mg/ml inhibit >90 per cent of AChE activity. Recently, we have demonstrated that TDE acts as a reversible neuronal AChE-I in rats¹⁷. These findings suggest that this traditional medicine could possibly help to improve memory, particularly in ageing with ACh deprivation in the brain. Despite this advancement in our understanding of the effects of TDE, its functional effect in the hippocampus, a brain region critical for learning and memory, has never been investigated. Therefore, in the present study we tested whether TDE can modulate dendritic field excitatory postsynaptic potentials (fEPSPs), which indicate the neuronal synaptic function, in *Cornu Ammonis* 1 (CA1) hippocampal slices of normal rats as found in current AChE-I drugs used for AD therapy. We also compared the effects of TDE with galanthamine, a well-known AChE-I drug used to treat AD patients.

Material & Methods

Plant materials and *T. divaricata* extract: *T. divaricata* (collection no. Changwijiit 0020 at the PBM herbarium, Fac. Pharmaceutical Sciences, Mahidol University, Thailand) was collected from Phitsanulok, Thailand. Roots were extracted as described previously¹⁷. To validate the quality of TDE in each experiment, each lot was analyzed for inhibitory effects of AChE activity *in vitro* and *in vivo* before being used.

Hippocampal slice preparation: The study protocol was approved from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee. Hippocampal slices (400 μ m) were prepared from 4-5 wk old male Wistar rats obtained from the National Animal Center, Salaya Campus, Mahidol

University, Thailand (n=23) using standard methods. Rats were anaesthetized with halothane, decapitated, and the brain removed and placed in ice-cold "high sucrose" artificial CSF (aCSF) containing (mM): NaCl 85; KCl 2.5; MgSO₄ 4; CaCl₂ 0.5; NaH₂PO₄ 1.25; NaHCO₃ 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95 per cent O₂/5 per cent CO₂ (pH 7.4). This solution enhanced neuronal survival during the slicing procedure. Hippocampal slices were cut using a vibratome (Vibratome Company, St. Louis, MO, USA). Following a 30-min post-slice incubation in high sucrose aCSF, slices were transferred to a standard aCSF solution containing (mM): NaCl 119; KCl 2.5; CaCl₂ 2.5; MgSO₄ 1.3; NaH₂PO₄ 1; NaHCO₃ 26; and glucose 10, saturated with 95 per cent O₂/5 per cent CO₂ (pH 7.4) for an additional 30 min. For recordings, the slices were transferred to a submersion recording chamber and continuously perfused at 3-4 ml/min with standard aCSF warmed to 25-28°C.

Stimulation and recording: CA1 fEPSPs were recorded (Axopatch 200B, Axon Instruments, CA, USA) using standard methods described previously¹⁸. A stainless steel bipolar stimulating electrode (FHC, Bowdoinham, ME, USA) was placed in stratum radiatum to stimulate the Schaffer collaterals. A glass microelectrode (Sutter Instrument, Novato, CA, USA) filled with 2M of NaCl (Sigma, St. Louis, MO, USA) was placed in CA1 stratum radiatum to record fEPSPs. The stimulus frequency was 0.1 Hz. The stimulus intensity was adjusted to yield a fEPSP of 0.8-1.0mV in amplitude and produce ~50 per cent of maximal fEPSP responses. The delivery of two stimuli in rapid succession (50 msec interstimulus interval) elicited paired-pulse facilitation (PPF).

Drug application: Substances used in this experiment included TDE (dissolved in ethanol), atropine, ACh and galanthamine (dissolved in ddH₂O). Appropriate concentrations of specific substances in solution were determined experimentally¹⁷. In baseline and wash conditions, hippocampal slices were perfused with standard aCSF. The same amount of ethanol was added to standard aCSF in baseline and wash conditions as was used for dissolving TDE. All chemicals were applied to the slices in a bath chamber via gravity perfusion. All chemicals were obtained from Sigma, St. Louis, MO, USA.

Data analysis: Data were filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using pClamp 9.2 software (Axon Instruments, CA, USA). The initial slope of the fEPSP was measured and plotted vs. time. The initial slope is the first slope of the field

potential detected following stimulus artifact and fiber valley. The initial slope is used for measurement of synaptic strength in the synaptic experiment. Statistical significance between the groups was determined with the Student's *t*-test. Only experiments with less than a 10 per cent change in the original baseline were included in the analysis.

Results

TDE's effect on fEPSPs was recorded in the stratum radiatum of the CA1 hippocampus, in response to stimulation of Schaffer collaterals. Paired-pulse facilitation (PPF), in response to paired stimulation pulses, was used to clarify Schaffer collateral pathways (Fig. 1A). TDE (60 µg/ml) reduced the size of the responses beginning 2–3 min after the start of infusion, with maximum effects appearing over the following 5–7 min. In our set-up, compounds added to the infusion line required approximately 1 min to reach the slices. The fEPSP depression was prominent and did not appear to be accompanied by distortion of waveform (Fig. 1A, inset). The fEPSP responses returned to

the same level as baseline responses after 15 min of washout (Fig. 1A). The mean depression of fEPSPs following the application of 60 µg/ml of TDE was 47 ± 4 per cent ($n=7$, Fig. 1B). We used 60 µg/ml of TDE in this study since this concentration demonstrated maximum effect on the depression of fEPSPs in this study. TDE with concentrations higher than 10 µg/ml transiently reduced synaptic responses. The range of fEPSP depression in TDE concentrations of 10–100 µg/ml was 46–75 per cent ($n=5$ per dose, the dose response curve of TDE was not shown).

The effects of 60 µg/ml of TDE were completely blocked by 10 µM atropine, an mAChR antagonist (Fig. 2A and 2B), suggesting that the TDE effect was mediated by mAChRs. We used a high concentration of atropine (10 µM) since it can completely block the TDE effect on reducing synaptic response in all hippocampal slices. To investigate whether the nAChRs were involved in the synaptic modulation of TDE, pancuronium

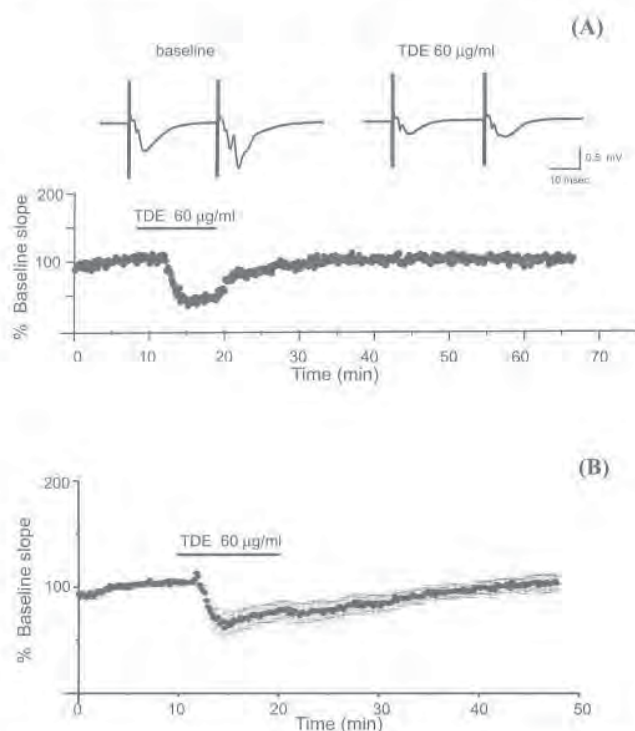


Fig. 1. TDE effects on CA1 hippocampal responses. (A) TDE-induced depression of synaptic responses. Traces were obtained from the experiment shown in 1A (inset). Each trace shows the average of 20 consecutive sweeps, recorded 2 min before (baseline) and during TDE application (TDE 60 µg/ml). (B) Average of 7 TDE applications shown as mean $\pm$ SEM.

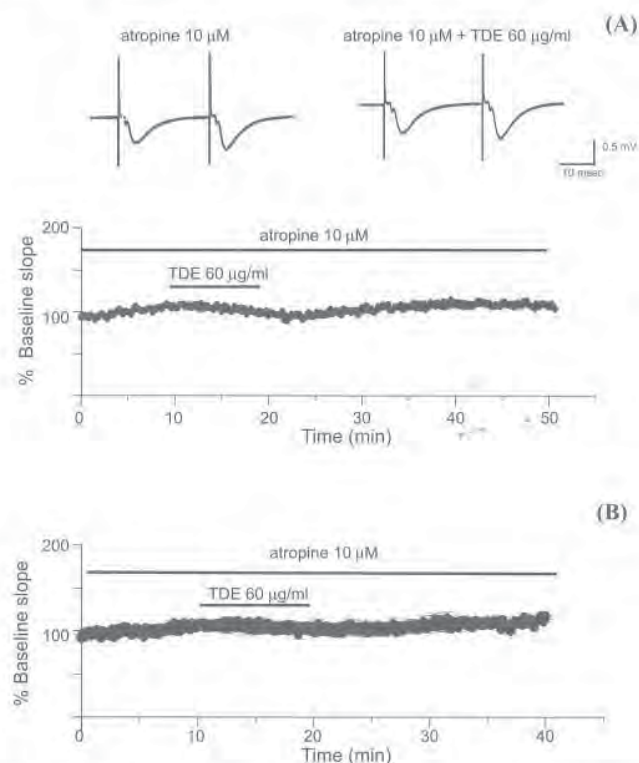


Fig. 2. The non selective muscarinic antagonist, atropine (10 µM), completely blocked the acute synaptic depression caused by TDE. (A) Atropine blocked the TDE-induced synaptic depression. Traces were obtained from the experiment shown in 2A (inset). Each trace shows the average of 20 consecutive sweeps, recorded 2 min before the application of TDE (atropine 10 µM) and during the application of TDE (atropine 10 µM + TDE 60 µg/ml application). (B) Average of 7 TDE applications shown as mean $\pm$ SEM.

bromide, a non selective nAChR antagonist, was used. In contrast to the effect of atropine, the TDE-induced reduction in Schaffer collateral fEPSPs persisted despite pretreatment of the slices with pancuronium bromide (100 μ M). The mean depression of fEPSPs following the application of 60 μ g/ml of TDE with and without 100 μ M pancuronium was not significantly different ($n=6$). We used a high concentration of pancuronium (100 μ M, the dose response curve of pancuronium with 60 μ g/ml was not shown in this study) to completely block all subtypes of nAChR in hippocampal regions without having any effects on synaptic transmission¹⁹.

The TDE effect of fEPSPs that we observed in CA1 was similar to the effect of galanthamine, a reversible AChE-I, and of ACh. One μ M galanthamine application transiently depressed fEPSPs (Fig. 3). The mean

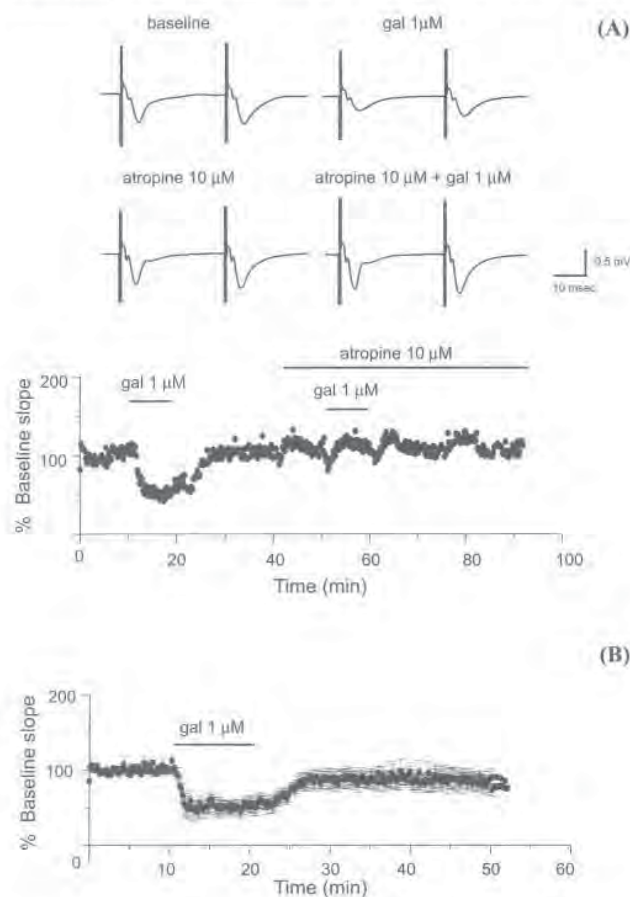


Fig. 3. Effect of 1 μ M galanthamine (gal) on CA1 hippocampal responses. (A) Galanthamine-induced depression of synaptic responses. Depression was blocked by atropine (10 μ M). Traces were obtained from the experiment shown in 3A (inset). Each trace shows the average of 20 consecutive sweeps. (B) Average of 4 galanthamine applications shown as mean $\pm$ SEM.

fEPSP depression following the application of 1 μ M galanthamine was 42 ± 8 per cent (Fig. 3B, $n=4$). We used 1 μ M galanthamine in this study since it has been shown to modulate glutamatergic synaptic transmission²⁰. The effect of galanthamine in suppressing fEPSPs was also completely blocked by 10 μ M atropine (Fig. 3A). Our findings suggest that TDE acts to suppress CA1 synaptic responses as same as galanthamine does.

ACh (1 mM) transiently depressed fEPSPs, similar to that of TDE application (Fig. 4). The ACh effect in suppressing fEPSPs was also completely blocked by 10 μ M atropine (Fig. 4A). The mean fEPSP depression following the application of 1 mM ACh was 77 ± 3 per

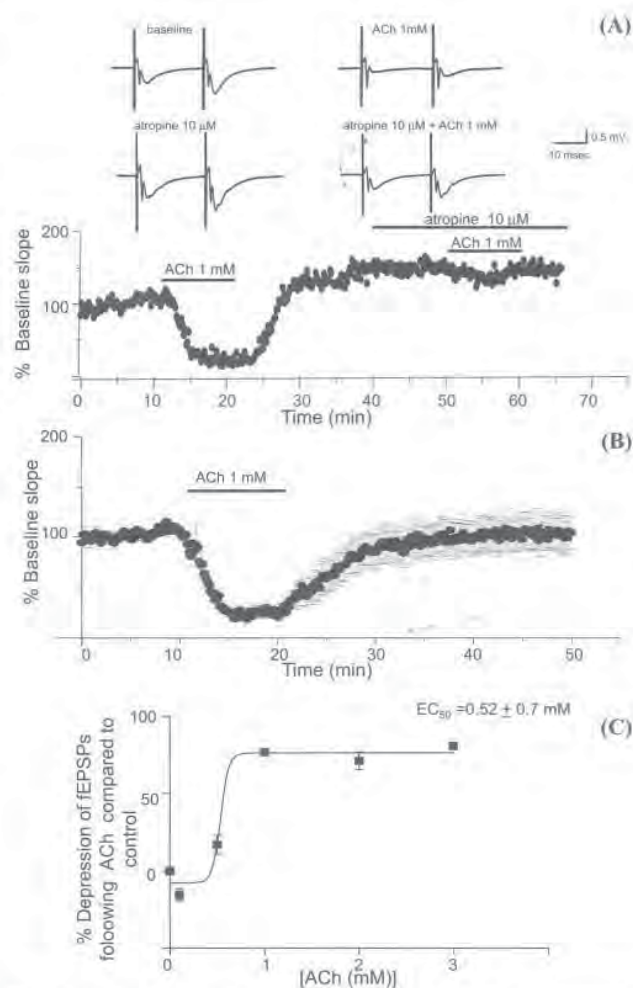


Fig. 4. Acetylcholine (ACh) caused a large depression of hippocampal synaptic response. Its effect was blocked by atropine. (A) ACh-induced depression of synaptic responses. ACh effect was blocked by atropine. Traces were obtained from the experiment shown in 4A (inset). Each trace shows an average of 20 consecutive sweeps. (B) Average of 7 ACh applications shown as mean $\pm$ SEM. (C) The dose response of ACh in depressing synaptic responses ($n=5$).

cent ($n=7$, Fig. 4B). We used 1 mM ACh since it had maximal effect on fEPSPs depression (Fig. 4C). The depression of fEPSPs by ACh was dose-dependent and the EC_{50} in fEPSPs depression occurred at 0.52 ± 0.7 mM of ACh ($n=5$ per dose, Fig. 4C).

The depression of synaptic transmission caused by both TDE and galanthamine application was less and slower than that by ACh (Fig. 5A). This finding suggests that TDE depresses fEPSPs in a similar way to galanthamine and TDE might not directly suppress fEPSPs in the same fashion as ACh application. Fig. 5B demonstrates the paired-pulse facilitation (PPF), a simple and sensitive measure of changes in presynaptic neurotransmitter release probability. We used PPF to test the hypothesis that TDE, galanthamine and ACh reduce synaptic responses by presynaptically depressing glutamatergic release. Mean paired-pulse facilitation of the CA1 hippocampus was increased

during the application of TDE (60 μ g/ml), galanthamine (1 μ M) and ACh (1 mM) (Fig. 5B). Mean facilitation of the response slope with 60 μ g/ml of TDE during infusion was 11 ± 4 per cent greater than that before TDE infusion ($P<0.05$). Mean facilitation of the response slope with 1 μ M galanthamine during infusion was 13 ± 1 per cent greater than before galanthamine infusion ($P<0.05$). The increase of the PPF response slope with 1 mM ACh during infusion was 35 ± 6 per cent greater than that before ACh infusion ($P<0.01$).

Discussion

The major finding of this study was that TDE suppressed synaptic transmission at hippocampal CA1 synapse. We demonstrated that TDE-enhanced cholinergic transmission in hippocampal circuits affected synaptic glutamatergic transmission by depressing neurotransmitter release in a similar manner to galanthamine and ACh. In this study, the TDE effect was blocked by atropine, but not pancuronium, suggesting that TDE may modulate synaptic transmission via the muscarinic cholinergic function.

A massive glutamatergic input from the cortex depends on a collection of afferents releasing neurotransmitters other than glutamate for synchronizing rhythms in the hippocampus. ACh is one of powerful presynaptic modulators at the glutamatergic synapses^{6,21}. The cholinergic innervation in the hippocampus is provided from the medial septal nucleus²². Stimulation of cholinergic innervation in different regions of the hippocampus reduces glutamatergic synaptic transmission^{1,4-8,23}. Much evidence has been provided on cholinergic interaction with glutamatergic transmission^{11,24}. A study showed that coincident glutamatergic and cholinergic inputs transiently depress glutamatergic release at the CA1 synapse³. The interaction between cholinergic and glutamatergic transmission was suggested to be important in memory processing²³.

We have shown that TDE acts as a reversible AChE-I and is capable of increasing neuronal activity in the cerebral cortex, possibly by increasing cholinergic function¹⁷. In this study, we showed that TDE caused a transient depression of synaptic transmission in Schaffer collateral pathways of the CA1 response. The modulation of synaptic response by TDE was similar to the effect of galanthamine and ACh. These findings suggest that TDE possibly elevates the endogenous ACh level at the cholinergic synapses in hippocampal circuits. The synaptic

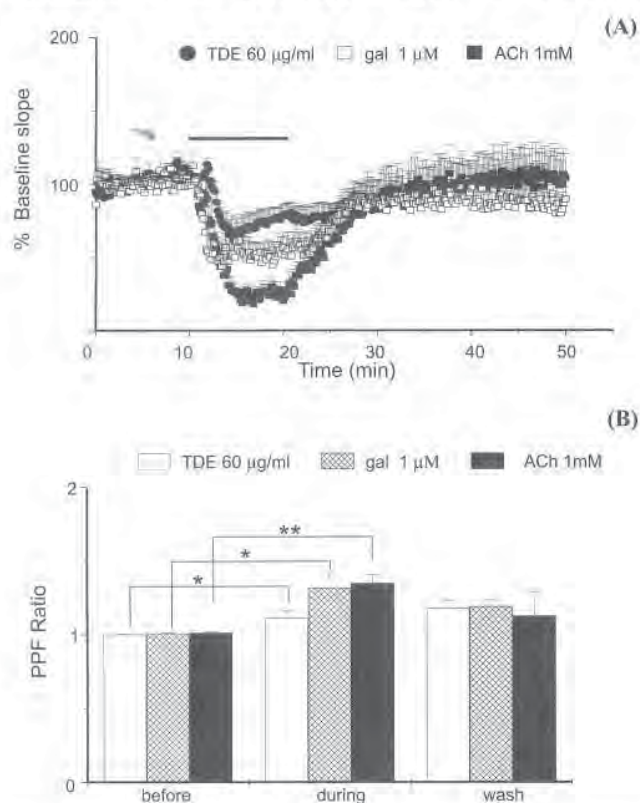


Fig. 5. Comparison of average depression of synaptic responses between 60 μ g/ml of TDE, 1 μ M galanthamine (gal) and 1 mM of ACh. **(A)** TDE- and galanthamine-induced suppression of CA1 hippocampal synaptic responses appeared to be less and slower than ACh-induced suppression. **(B)** TDE, galanthamine and ACh significantly increased paired-pulse facilitation (PPF) (* $P<0.05$, ** $P<0.01$ before comparison during TDE, galanthamine or ACh application).

modulation of TDE may occur via cholinergic regulation of neurotransmission. This possibility is supported by our findings where the transient depression resulting from TDE application was prevented by mAChRs blockade. Atropine alone had no effect on hippocampal synaptic transmission. Also, TDE effects were similar to those of galanthamine, as demonstrated in this study, and those of physostigmine in a previous study⁹.

Changes in a paired-pulse ratio by drug typically indicate a presynaptic mechanism of drug action^{25,26}. TDE acutely reduces the fEPSPs slope and the depression is accompanied by an increase in the paired-pulse ratio, suggesting that TDE transiently depressed synaptic transmission by decreasing neurotransmitter release at the presynaptic site. This effect is similar to those observed in physostigmine application⁹. TDE's effect on the paired-pulse ratio was similar to those of galanthamine and ACh. The paired-pulse results provide insights into the functional changes arising from cholinergic suppression of glutamatergic synapses by TDE. The different fEPSPs depression and PPF effects between ACh and TDE/galanthamine found in this study could be due to the fact that TDE and galanthamine caused an increase in the endogenous ACh level at the synaptic sites. However, the increased amount of ACh in the synaptic regions caused by TDE and galanthamine application was less than the high-concentration exogenous ACh application in our study.

In conclusion, the present findings demonstrated that TDE showed similar effects to those of galanthamine. This important evidence indicates that consumption of this natural product (TDE) could be beneficial in slowing the process of learning and memory loss particularly in old age people or Alzheimer's patients. Our findings also suggest that TDE could possibly be used to develop a new acetylcholinesterase inhibitor for the treatment of Alzheimer's disease. Further, our findings support the beneficial effects of TDE and demonstrate the additional benefits of this traditional medicine on the learning and memory process with future clinical significance.

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ORIGINAL ARTICLE

Heart rate variability in beta-thalassemia patients

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Abstract

Background: Cardiac failure remains the major cause of death in beta-thalassemia major (TM). Reduced heart rate variability (HRV) is associated with a higher risk of arrhythmias after myocardial infarction and heart failure. We evaluated HRV in TM patients and its relationship with hemodynamics and echocardiographic parameters during a 6-month follow-up. **Methods:** Thirty-four TM patients (19 ± 10 yr) and 20 healthy subjects (17 ± 6 yr) were evaluated. Hematologic, biochemical, echocardiographic and HRV parameters were determined at entry and at 6-month follow-up. Time and frequency domain HRV parameters were analyzed from 24-h recorded electrocardiograms. All TM patients received blood transfusion and chelation therapy. **Results:** Both time and frequency domain HRV parameters were markedly reduced in TM patients, compared to the control. The significantly improved HRV was seen in correlation with higher hemoglobin (Hb) level when compared within TM group at different time point. No correlation was seen between HRV and serum ferritin, reactive oxygen species (ROS) and non-transferrin bound iron (NTBI). **Conclusion:** HRV is depressed in TM patients. HRV was significantly correlated with Hb level, suggesting that anemia greatly influences the cardiac autonomic balance.

Key words heart rate variability; thalassemia major; anemia

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Cardiac failure remains the major cause of death in patients with transfusion-dependent β -thalassemia (thalassemia major, TM) (1–3). The major complication of long-term red blood cell transfusion is iron deposition, which in the heart causes a functional impairment (4). This cardiac complication due to iron overload can be prevented by intensive iron chelation (4–6). Currently, many available tests, such as serum ferritin level and endomyocardial biopsy, are not definitely representative (7, 8). Furthermore, both endomyocardial and liver biopsies for iron measurement are invasive. Conventional electrocardiography is not helpful since the majority of patients with asymptomatic cardiomyopathy is virtually absent of any abnormal electrocardiogram (ECG), while echocardiographic finding is thought to be late, already indicating poor prognosis in TM patients (9). Although

T2-star magnetic resonance (MR-T2*) has been demonstrated to be promising in giving an early diagnosis on iron overload cardiomyopathy (10), its use is currently strictly limited by its availability and cost. Due to this limitations, cardiac T2* is currently not considered as a practical approach for general use in evaluating cardiac iron status in thalassemia patients, particularly for those in the second and third-world countries. Furthermore, current cardiac T2* cannot be used to evaluate the cardiac autonomic disturbances occurring in the heart. For biochemical analysis, non-transferrin-bound iron (NTBI) has been shown to be the good theoretical predictor (11, 12). However, it still remains available only in the research lab and the link between NTBI and cardiac iron/dysfunction has not been established clinically (11, 13, 14). Therefore, a reliable non-invasive, low-cost, and

easily access diagnostic test that can be used to detect an early development of cardiac involvement in this group of patients during the early stage of the disease is needed so that intensive chelation can be properly provided.

Recently, heart rate variability (HRV) has been used to determine the cardiac autonomic function in patients with postmyocardial infarction (15) and heart failure (16), and has been demonstrated as a strong risk predictor in those patients. Despite its clinical significance, there are only a few cross-sectional studies investigating HRV in TM patients (17–20). In the present study, HRV was investigated in TM patients, and the relationships between HRV and hematological and biochemical parameters as well as left ventricular functions were studied during a 6-month follow-up period.

Materials and methods

Study protocol

The study protocol was reviewed and approved by the institutional ethic committee of the Faculty of Medicine, Chiang Mai University. All participants gave written informed consent prior to the study entry. Thirty-four TM patients (14 homozygous beta-thalassemia and 20 Hb E/beta-thalassemia) and 20 healthy subjects were prospectively evaluated. All patients were treated with blood transfusion and desferrioxamine for iron chelation. The inclusion criteria for patient entry into the trial were diagnosed TM patients currently treated with regular blood transfusion and age ≥ 10 yr. Exclusion criteria were as follows: patients who were lost to follow up; patients taking any antiarrhythmic drug and/or cardiovascular medication that could affect sympatho-vagal balance in the past 3 months; alanine aminotransferase (ALT) > 300 U/L; serum creatinine > 2 mg/dL; and patients with symptoms and signs of heart disease as evaluated by chest film and ECG. Laboratory profiles, echocardiography as well as 24-h Holter ECG recording for HRV analysis in each patient were collected at the time of entry and at 6-month follow-up. Twenty healthy subjects with matched age (10 males; 17 ± 5 yr) were included as the control group for one-time HRV study. The primary end point of the study was death or ventricular arrhythmias which required intervention during study, and the secondary end point was the ending of a 6-month follow-up.

Heart rate variability measurement

Twenty-four-hour ECG was recorded for HRV determination. The recordings were reviewed and the classification of each beat was manually checked and corrected before the HRV was determined by the analysis

software. HRV parameters were categorized into two categories, time domain and frequency domain.

Time-domain HRV parameters determined in the present study were the standard deviation of all normal sinus R-R intervals in the entire 24-h recording (SDNN), the standard deviation of all average normal sinus R-R intervals for all 5-min segment in the 24-h recordings (SDANN), the average of the standard deviations of all R-R intervals for all 5-min segments in the 24-h recordings (ASDNN), and the root mean square of the mean of the squared differences of two consecutive R-R intervals (rMSSD). Frequency-domain HRV parameters including low-frequency power (LF), high-frequency power (HF), and the LF/HF ratio were also determined.

Laboratory profiles

Hemoglobin (Hb), hematocrit (Hct), serum ferritin, plasma NTBI, and the production of reactive oxygen species (ROS) were determined in each patient at the date of entry and at 6-month follow-up.

Quantification of plasma NTBI

Plasma NTBI was evaluated as previously described by Singh *et al.* (21). At the beginning, a small volume of aluminum chloride solution (a final concentration of $200 \mu\text{M}$) was added to $450\text{-}\mu\text{L}$ plasma and incubated at room temperature for 1 h. After $50 \mu\text{L}$ of 800-mM nitrilotriacetic acid (NTA) (a final concentration of 80 mM) solution pH 7.0 was added, the mixture was incubated for 30 min at room temperature to produce a ferric-nitrilotriacetate complex, $\text{Fe}^{3+}\text{-(NTA)}_2$. Then, the $\text{Fe}^{3+}\text{-(NTA)}_2$ was separated from plasma proteins by spinning plasma mixture through the membrane filter (30-kDa cut-off, polysulfone type, 0.5-mL capacity) at $12\,000\text{ g}$, 15°C for 45 min. Ultrafiltrate was injected into non-metallic $50\text{-}\mu\text{L}$ loop and analyzed with the high performance liquid chromatography (HPLC) technique. HPLC requirements were composed of glass analytical column (ChromSep-ODS1, 100×3.0 mm, $5 \mu\text{m}$) connected with guard column (10×3.0 mm, $5 \mu\text{m}$) and mobile-phase solvent (3 mM CP22 in 19% acetonitrile buffered with 5 mM MOPS pH 7.0). Having been isocratically eluted with the mobile-phase solvent at a flow rate of 1.0 mL/min , NTBI as $\text{Fe}^{3+}\text{-(NTA)}_2$ was fractionated on the column, then immediately on-column derivatized with CP22 to form a $\text{Fe}^{3+}\text{-(CP22)}_3$ complex. Resulting orange-colored $\text{Fe}^{3+}\text{-(CP22)}_3$ product was on-line detected at 450 nm with the SpecMonitor® 2300 flow-cell detector (LDC Milton-Roy Inc., FL, USA). The NTBI peak height was integrated and recorded for further determination of NTBI concentration from the calibration curve. Calibration curve was produced by plotting

obtained peak height values on *y*-axis against iron concentration on *x*-axis. Equation of linear regression line was used to calculate plasma NTBI concentration.

Flow cytometric determination of erythrocyte oxidative stress

The production of ROS was measured using the method previously described (22). Two microliter blood was diluted with 9 mL of Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate buffered saline (D-PBS) to a concentration of 1×10^6 RBCs/mL, and was incubated with 20- μL DCFH-DA (10 mg/mL in methanol) under 5% CO_2 atmosphere at 37°C for 15 min. The solution was centrifuged at 3000 *g* for 15 min at 15°C. RBC pellet was resuspended in 2-mL PBS and centrifuged. Finally, the pellet was resuspended in 1-mL PBS and divided into two cell fractions (500 μL each). The oxidative stress level represented as fluorescence intensity (FI) was determined in the cells with the induction of 3% H_2O_2 (35 μL) by flow cytometry.

Echocardiographic studies

Tissue Doppler and M-mode echocardiography were used to determine cardiac function in all patients. Cardiac chamber was measured by left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD). Cardiac index (CI), left ventricular ejection fraction (LVEF), and fractional shortening (FS) were used to measure the cardiac function.

Statistical analysis

Numerical data are presented as mean $\pm$ standard deviation. Differences between TM patients and the control group were performed using the non-parametric Mann–Whitney *U* test. The Wilcoxon signed rank test was used to test differences between measured parameters at baseline and at follow-up in TM patients. The correlations between the HRV parameters and the hematologic and echocardiographic parameters were determined by Pearson test. A *P* value less than 0.05 was considered statistically significant. Statistical analysis was performed with the use of SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of patients are shown in Table 1. There were no significant differences in age and gender between the TM group and the control group. Twenty-eight patients (82%) underwent splenectomy. In the last

Table 1 Demographic, time-domain and frequency-domain HRV parameters of thalassemia major (TM) patients at entry and the control subjects

Parameters	TM group at entry	Control group	<i>P</i> value
Number of subjects	34	20	–
Age (yr)	19 $\pm$ 10	17 $\pm$ 5	NS
Gender			
Male (%)	17 (50)	10 (50)	NS
Female (%)	17 (50)	10 (50)	NS
Splenectomy (%)	28 (82)	–	–
Number of blood transfusion (last 12 months)	10 $\pm$ 6	–	–
Heart rate (bpm)	94 $\pm$ 11	81 $\pm$ 10	0.000
Hemoglobin (g/dL)	6.7 $\pm$ 1.4	14.7 $\pm$ 1.7	0.000
Hematocrit (%)	21.9 $\pm$ 4.7	43.1 $\pm$ 4.1	0.000
MCV	73.6 $\pm$ 6.2	88.5 $\pm$ 4.5	0.000
BSA-normalized O_2 delivery	457 $\pm$ 123	461 $\pm$ 139	NS
Echocardiographic values			
LVEDD (mm)	49.6 $\pm$ 6.1	50.3 $\pm$ 5.8	NS
LVESD (mm)	31.2 $\pm$ 4.7	30.7 $\pm$ 5.0	NS
Cardiac index	5.2 $\pm$ 1.0	5.4 $\pm$ 1.1	NS
LVEF (%)	63.9 $\pm$ 7.6	65.9 $\pm$ 8.7	NS
FS (%)	35.8 $\pm$ 6.1	36.6 $\pm$ 6.8	NS
Cardiac thoracic ratio (%)	53 $\pm$ 4	52 $\pm$ 4	
Time domain			
SDNN (m s)	92 $\pm$ 25	170 $\pm$ 51	0.000
SDANN (m s)	85 $\pm$ 24	157 $\pm$ 57	0.000
ASDNN (m s)	34 $\pm$ 11	74 $\pm$ 16	0.000
rMSSD (m s)	20 $\pm$ 8	53 $\pm$ 16	0.000
Frequency domain			
LF (m s^2)	12 $\pm$ 4	26 $\pm$ 8	0.000
HF (ms^2)	10 $\pm$ 5	25 $\pm$ 6	0.000
LF/HF ratio	1.3 $\pm$ 0.5	1.0 $\pm$ 0.3	0.031

SDNN, standard deviation of all normal sinus R-R intervals in the entire 24-h recording; SDANN, standard deviation of all averaged normal sinus R-R intervals for all 5-min segment in the 24-h recordings; ASDNN, average of the standard deviations of all R-R intervals for all 5-min segments in the 24-h recordings; rMSSD, root mean square of the mean of the squared differences of two consecutive R-R intervals; LF, low frequency power; HF, high frequency power.

12 months prior to the study entry, all patients received blood transfusion (10 $\pm$ 6 times) and desferrioxamine for iron chelation. Desferrioxamine was planned to be given as per standard protocol. However, due to problems with availability of infusion equipment and patient compliance, only the minority received proper dose. Fourteen of 34 patients received 30–50 mg/kg/d of desferrioxamine regularly (i.e. > 3 times/wk). Mean heart rate of TM patients was significantly higher, whereas Hb and hematocrit (Hct) were significantly lower, compared to the control group. All time-domain HRV parameters (SDNN, SDANN, ASDNN, and rMSSD) were significantly decreased in the TM group, compared to the control group. Frequency-domain HRV parameters, both

LF and HF, of TM group were also significantly decreased while LF/HF ratio was significantly increased compared to the control group. Although 10 TM patients with depressed HRV had asymptomatic mild left ventricular hypertrophy (LVH) diagnosed by echocardiography, there was no difference between HRV of TM patients with and without LVH (not shown).

After 6-month follow-up with the same treatment regimen of blood transfusion (6 ± 2 times/6 months) and iron chelation, none had ventricular arrhythmias or died. Although all HRV parameters at 6-month follow-up were significantly decreased compared to the control group, they were significantly improved compared to the HRV at entry (Table 2). For hematologic parameters, Hb level, serum ferritin, and ROS were significantly higher than the levels at entry. The Hct and NTBI levels were slightly increased but did not reach statistical significance, whereas the heart rate was not altered. All measured echocardiographic parameters (LVEDD, LVESD, CI, LVEF, and FS) were not changed at 6-month follow-up, indicating that cardiac function was not deteriorated during the follow-up period.

Table 2 HRV, hematologic, and echocardiographic parameters of TM patients at entry and at 6-month follow-up

Parameters	TM group at entry	TM group at 6-months	P value
Hematologic values			
Hemoglobin (g/dL)	6.7 ± 1.4	7.2 ± 1.2	0.023
Hematocrit (%)	21.9 ± 4.7	22.6 ± 4.0	NS
MCV	73.6 ± 6.2	74.1 ± 5.6	NS
Serum ferritin (ng/dL)	4492 ± 2710	5296 ± 3867	0.037
NTBI (μm)	5.1 ± 4.2	5.7 ± 3.6	NS
ROS (FI unit)	16 ± 11	34 ± 14	0.000
BSA-normalized O ₂ delivery	457 ± 123	445 ± 92	NS
Echocardiographic values			
LVEDD (mm)	49.6 ± 6.1	49.0 ± 5.8	NS
LVESD (mm)	31.2 ± 4.7	31.7 ± 4.8	NS
Cardiac index	5.2 ± 1.0	5.0 ± 0.9	NS
LVEF (%)	63.9 ± 7.6	62.8 ± 5.7	NS
FS (%)	35.8 ± 6.1	36.1 ± 4.3	NS
Heart rate (bpm)	94 ± 11	95 ± 8	NS
Cardiac thoracic ratio (%)	53 ± 4	53 ± 4	NS
Time domain			
SDNN (m s)	92 ± 25	113 ± 19	0.000
SDANN (m s)	85 ± 24	105 ± 19	0.000
ASDNN (m s)	34 ± 11	43 ± 13	0.003
rMSSD (m s)	20 ± 8	28 ± 15	0.001
Frequency domain			
LF (m s ²)	12 ± 4	14 ± 4	0.023
HF (m s ²)	10 ± 5	14 ± 7	0.002
LF/HF ratio	1.3 ± 0.5	1.2 ± 0.4	0.041

NTBI, non-transferrin-bound iron; ROS, production of reactive oxygen species; LVEF, left ventricular ejection fraction; FS, fractional shortening; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension.

Table 3 The correlations between HRV parameters and Hb and Hct in TM patients

R	Hemoglobin	Hematocrit
Time domain		
SDNN	0.505*	0.554*
SDANN	0.492*	0.493*
ASDNN	0.527*	0.548*
rMSSD	0.309†	0.300†
Frequency domain		
LF	0.478*	0.506*
HF	0.373†	0.285†

* $P < 0.001$; † $P < 0.05$

SDNN, standard deviation of all normal sinus R-R intervals in the entire 24-h recording; SDANN, standard deviation of all averaged normal sinus R-R intervals for all 5-min segment in the 24-h recordings; ASDNN, average of the standard deviations of all R-R intervals for all 5-min segments in the 24-h recordings; rMSSD, root mean square of the mean of the squared differences of two consecutive R-R intervals; LF, low frequency power; HF, high frequency power.

All improved HRV parameters was found to be significantly correlated with Hb and Hct levels (Table 3). However, there were no correlations between HRV parameters and serum ferritin, NTBI, ROS, O₂ delivery as well as the number of blood transfusion prior to the enrollment. None of the echocardiographic parameters was correlated with HRV.

Discussion

In the present study, the major findings include: (1) all time-domain and frequency-domain HRV parameters are significantly depressed in TM patients while LF/HF ratio was significantly increased, compared to the control and (2) within the TM group, improved HRV parameters were correlated with improved Hb and Hct levels.

Sympatho-vagal imbalance can affect cardiac electrophysiology and may lead to increase mortality due to cardiac autonomic dysfunction (23–28). HRV measurement has been used to assess cardiac autonomic dysfunction in various disorders such as myocardial infarction (29–31), heart failure (32–37), cardiac transplantation (38–40), diabetic neuropathy (41), and HIV infection (42). Numerous studies have confirmed that decreased HRV in the time or frequency domain, measured after myocardial infarction and heart failure is associated with increased risk of mortality (23–28). In TM patients, previous cross-sectional studies have shown that both time-domain and frequency-domain HRV parameters were significantly decreased in patients with TM, and that they had no correlation with any hematologic, biochemical or echocardiographic parameters (18, 19). However, no prospective study has been done to investigate HRV and its relationship to the changes of hematologic values during treatment.

In the present study, we determined for the first time a disturbance of HRV indices with a 6-month follow-up period in TM patients. Consistent with previous cross-sectional studies, all time-domain and frequency-domain HRV parameters were depressed in the TM group at the initial study enrollment, compared to the control. These indices indicated that cardiac autonomic functions, both sympathetic and parasympathetic activities, might be impaired in TM patients. Moreover, sympatho-vagal imbalance which is designated by an increased LF/HF ratio is still observed in the TM group. Since heart rate was increased in TM patients, indicating high sympathetic activity and/or low parasympathetic activity, whereas both LF and HF were depressed, this sympatho-vagal imbalance in the TM group could be mainly due to depressed parasympathetic activity. However, it is premature at this time to conclude that HRV is absolutely 'impaired' in this population since it could represent a normal physiologic adaptation to anemia and that the spectrum of abnormalities observed in the TM patients in the present study might be reproduced in the control population when the Hb and Hct are low.

At 6-month follow-up, Hb was significantly improved in these TM patients. As there was no change in the treatment plan, increased Hb and serum ferritin may have been a trial effect when a transfusion plan was strictly followed as patients were closely monitored. All HRV parameters were also significantly improved. In addition, the LF/HF ratio was decreased, indicating an improvement of sympatho-vagal balance. The finding that heart rate was not changed at 6-month follow-up despite increased Hb, LF, HF, and decreased LF/HF ratio further emphasizes the significant contribution of improved cardiac parasympathetic activity in these TM patients.

In the present study, the production of ROS after blood transfusion was significantly increased during the 6-month follow-up period, indicating oxidative damage. Both NTBI, and echocardiographic parameters (LVEDD, LVESV, CI, LVEF, and FS) were not altered at 6-month follow-up, indicating that they may not be sensitive enough to detect early cardiac disturbance in TM patients with preclinical stage of heart disease. Although there were 10 TM patients diagnosed asymptomatic mild LVH with no clinical sign of heart diseases or cardiac dysfunction, HRV parameters of those with and without mild LVH were not significantly different, indicating that asymptomatic mild LVH may be too mild to affect already depressed HRV in TM patients. Despite this finding, the number of asymptomatic mild LVH included in this study was still small and could be premature for this assumption.

Significant correlations between all HRV parameters, and Hb and Hct levels were found in the present study.

However, no correlation between HRV indices and other measured parameters were observed. These findings suggest that blood transfusion therapy not only improved an anemic condition but also mainly increased cardiac parasympathetic activity, represented by an increased HF component. Previous studies in patients with sickle cell anemia and vitamin B-12 deficiency have indicated that chronic anemia may affect the cardiac autonomic dysfunction (43, 44). Similarly, in the present study, improved sympatho-vagal imbalance may be explained by improved anemic condition as seen at 6-month follow-up in TM patients.

It is important to note that the patient population in the present study was significantly undertransfused compared to the European and North American management practices with a mean pre-transfusion Hb level at an average of 2.5 g/dL lower. This disparity could be responsible for the effect of Hb observed in this trial but not others. At the Hb levels observed in the present population, erythropoietic drive was not inhibited and the patients were likely to be hypermetabolic. Furthermore, this degree of anemia could create a chronically elevated cardiac output and possibly responsible for the increased sympatho-vagal tone as well as the LV dilation and LVH observed in some patients in the present study. Also, improved Hb level in this study could be a trial effect, with patients subtly shortening their transfusion intervals (on average) because of closer monitoring. It could also represent improved red cell survival, in which lower levels of circulating free Hb might mediate HRV by affecting NO bioavailability.

Although HRV has been demonstrated in many studies to be a good predictor of mortality, it is possible that the predictive value of depressed HRV can be much different in the TM population than any of the non-anemic disease cohorts where it predicts mortality. Future studies with long-term follow-up are needed to investigate the predictive value of HRV in thalassemia population.

Study limitation

The identification of the amount of iron accumulation in the myocardium was not determined in the present study. Future studies are needed to determine the correlation between HRV parameters and the amount of iron deposit in the heart to warrant its clinical significance. Furthermore, cardiac complication was not observed during a 6-month follow-up period in this study. Therefore, HRV as a predictor of cardiac complication could not be tested. Long-term follow-up is needed to investigate the benefit in risk stratification of HRV. In the present study, many TM patients were under chelated due to the cost and patients' compliances related to chelator administration. Since the use of oral iron chelators was

limited during the study period, all patients in this study received desferrioxamine monotherapy.

Conclusion

In TM patients, HRV was significantly depressed, indicating cardiac autonomic disturbance in these patients. Sympatho-vagal imbalance was markedly improved after Hb level was increased, suggesting that an anemic condition greatly influences cardiac autonomic function in TM patients. Blood transfusion not only maintains the level of Hb, but also reduced HRV disturbance. The improvement of symptho-vagal imbalance, which is designated by decreased LF/HF ratio, could be mainly due to an enhanced cardiac parasympathetic activity.

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Cardiac mortality is associated with low levels of omega-3 and omega-6 fatty acids in the heart of cadavers with a history of coronary heart disease

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Abstract

The benefits of omega-3 (ie, eicosapentaenoic acid and docosahexaenoic acid [DHA]) and omega-6 (ie, linoleic acid and arachidonic acid [AA]) fatty acids on reducing cardiac mortality are still debated. In this study, we tested the hypothesis that high levels of omega-3 and omega-6 fatty acids in heart tissues are associated with low cardiac mortality in Thai cadavers. One hundred fresh cadavers were examined in this study. The cause of death, history of coronary heart disease (CHD), and fish consumption habits were obtained from death certificates, cadaver medical record profiles, and a questionnaire to a person who lived with the subject before death. In each cadaver, biopsies of cardiac tissues were taken from the interventricular septum for measurement of fatty acid. Of the 100 cadavers (average age, 69 ± 13 years), 60 were men. The frequency of fish consumption was directly associated with omega-3 and omega-6 fatty acids in heart tissues ($P < .01$). History of CHD and cause of death (cardiac vs noncardiac) were not significantly associated with levels of omega-3 or omega-6 fatty acids. However, in cadavers with a history of CHD, high levels of omega-3 and omega-6, particularly DHA and AA, were associated with low cardiac mortality ($P < .05$). Fish consumption is associated with levels of omega-3 and omega-6 fatty acids in heart tissues. Although omega-3 and omega-6 fatty acids are not associated with cardiac mortality in the overall studied population, their low levels (especially DHA and AA) in heart tissues are associated with high cardiac mortality in cadavers with a history of CHD.

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Keywords: Cadaver; Omega-3; Omega-6; Fatty acids; Heart; Cardiac mortality

Abbreviations: AA, arachidonic acid; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; LA, linoleic acid; MMC-FA, methyl-7-methoxycoumarin fatty acid; PUFA, polyunsaturated fatty acids; VT, ventricular tachycardia.

1. Introduction

Cardiovascular mortality is the major health concern in most nations around the world. In the past few decades, diet and food supplements have become an important issue in preventive medicine, particularly in cardiovascular diseases

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[1–5]. A growing body of evidence from both basic and clinical studies has demonstrated that omega-3 long-chain polyunsaturated fatty acids (PUFA) prevent cardiac arrhythmia and reduce cardiovascular mortality [1,3,6–16]. These omega-3 fatty acids, including eicosapentaenoic (EPA; C20:5 omega-3) and docosahexaenoic acid (DHA; C22:6 omega-3), are mainly obtained in the diet from the consumption of fatty fish and fish oils. In the heart, both EPA and DHA are incorporated into the cell membrane of cardiomyocytes.

Previous studies have demonstrated that consumption of food supplements containing omega-3 fatty acid significantly increases the amount of EPA and DHA incorporated into the heart tissue [17–19]. Studies using isolated cardiac myocytes demonstrated that during ischemia EPA and DHA are released from myocardial membrane and exert anti-arrhythmic effects by prolonging the refractory periods of cardiac action potentials [20,21]. Clinical studies have also demonstrated an increase of EPA and DHA levels in heart tissues after ingestion of omega-3 supplements and the significance of EPA and DHA in preventing cardiac tachyarrhythmias [17,19].

Unlike omega-3 fatty acids, the cardioprotective roles of omega-6 fatty acids (ie, linoleic acid [LA] and arachidonic acid [AA]) are still debated [22–29]. However, a recent American Heart Association Science Advisory Statement supports the benefits of omega-6 fatty acids in reducing the risk of coronary heart disease (CHD) [30]. Despite these growing bodies of evidence regarding the clinical benefit of omega-3 and omega-6 fatty acids on the heart, information on the significance of the levels of these fatty acids in human heart tissues and their association with mortality is still limited. Furthermore, many studies use food supplements to investigate the role of these long-chained PUFA in reducing cardiovascular mortality and preventing arrhythmia [8,11,16,19,31–33]. The direct association between omega-3 and omega-6 fatty acid levels in human heart tissues and cardiac mortality in the general population without food supplements has never been reported.

Because of a limited source of information regarding the natural levels (ie, without food supplement) of these fatty acids in the heart in the general population and their association with cardiac mortality, it was the intent of this study to directly determine the levels of omega-3 (DHA and EPA) and omega-6 (LA and AA) fatty acids in heart tissues taken from cadavers who died from both cardiac and noncardiac causes. In this study, we tested the hypothesis that the omega-3 and omega-6 fatty acid contents in the heart are associated with fish consumption and that high levels of omega-3 and omega-6 fatty acids in heart tissues are associated with low cardiac mortality. Because it is known that both omega-3 and omega-6 fatty acids are essential in cardiomyocytes and that they exert beneficial effects including arrhythmia prevention and mortality reduction, the direct measurement of omega-3 and omega-6 fatty acids in cardiac tissues would allow us to investigate whether they

would have direct association with cardiac mortality. To reach this goal, heart tissues from cadavers were examined. To seek the association between fish consumption and the levels of omega-3 and omega-6 fatty acids in heart tissues, the history of fish consumption habits in each cadaver prior to death was determined. This study also investigated whether cadavers with high levels of omega-3 and omega-6 fatty acids in heart tissues would be less likely to die from cardiac cause as compared with those with low levels of these fatty acids in heart tissues.

2. Methods and materials

2.1. Cadaver selection

This study was approved by the Ethics Committee at the Faculty of Medicine, Chiang Mai University. Consent was obtained from a spouse or close relative of the deceased. Subjects were cadavers who had died within 24 hours of notification. The bodies were immediately frozen and delivered to the Department of Anatomy, Faculty of Medicine, Chiang Mai University. The cadaver's fish consumption habits were obtained from a spouse/close relative who had lived with the subject for at least 6 months before death. The cause of death and history of heart disease for each cadaver were obtained from the death certificate designated by the attending physician who signed the certificate. If additional information was needed, the attending physician was contacted to confirm the definite cause of death. Cases with undetermined cause of death were excluded from the study group.

Cardiac tissues were collected from 100 cadavers. The tissue (approximately 75 mg) was taken from the interventricular septum of fresh cadaveric hearts [17] at the Department of Anatomy and was immediately frozen at -86°C . The interventricular septum was chosen for a region of biopsy because a previous report demonstrated that the level of omega-3 and omega-6 fatty acids in heart tissues biopsied from this region was correlated with the levels of fatty acids supplement [17].

2.2. Extraction of cardiac lipids

Heart tissues were weighed and lyophilized overnight. After lyophilization, saline solution was used to suspend the tissue samples with 10 to 15 seconds of sonication [17]. Lipid extraction was performed using the technique described previously [34]. In brief, total lipid in the heart tissues was extracted with chloroform/methanol (2:1, vol/vol) solvent in a proportion of 1:5 (wt/vol) in a screw-cap tube overnight. The mixture was centrifuged at 3000 rpm, 4°C for 20 minutes to sediment tissue pellet. Chloroform-extractable lipid (upper layer) was transferred to a new test tube and evaporated to dryness under nitrogen gas stream. Before analysis, residue was reconstituted in methanol, and concentrations of PUFA, cholesterol, and triglyceride were measured as described below.

2.3. Determination of PUFA concentrations

2.3.1. Saponification

Lipids extracted from myocardial tissues were dissolved in methanol and saponified with the 10% potassium hydroxide (KOH) solution in methanol (with the help of phenolphthalein as an indicator) or triethylamine at 80°C for 15 minutes [35]. The solvent was evaporated by a rotary evaporator or by blowing down with nitrogen gas, and the residual fatty acid salt was analyzed using the precolumn derivatization high-performance liquid chromatography (precolumn HPLC) technique.

2.3.2. Precolumn HPLC analysis of omega-3 and omega-6 fatty acids

Isopropanol (AnalaR grade), methanol (AnalaR grade), acetonitrile (HPLC grade), and dichloromethane (AnalaR grade) were purchased from the Merck Chemicals Company (Darmstadt, Germany). Boron trifluoride (14%), butylated hydroxytoluene, methyl acetate, 4-bromomethyl-7-methoxycoumarin, and 18-crown-6 ether were purchased from the Sigma-Aldrich Chemicals Co (St. Louis, Mo). Docosahexaenoic acid, EPA, LA, and AA (Sigma-Aldrich Chemicals Co) were used as reference standards. L- α -phosphatidylcholine diheptadecanoic acid (Sigma-Aldrich Chemicals Co) was used as an internal standard. Butylated hydroxytoluene was added as an antioxidant to all organic solvents at 50 mg/L.

For derivatization, the method was slightly modified from that reported by Wolf and Korf [36,37]. The residual fatty acid salt of tissue extract, internal standard, and dichloromethane were mixed for 15 minutes and evaporated under nitrogen. The derivatizing agent was prepared by mixing 40 μ L of solution A (5 mg of Br-MMC in 5 mL dry acetonitrile), 2 μ L of solution B (13 mg of 18-crown-6 in 5 mL acetonitrile), and 2 mL acetonitrile. The working derivatizing solution (100 μ L) and potassium carbonate (2–4 mg) were then added to the mixture, sonicated rapidly (3 strokes, 30 seconds per stroke), and heated for 15 minutes at 60°C. After cooling, the solution containing methyl-7-methoxycoumarin fatty acid (MMC-FA) derivative was passed through 0.22- μ m nylon-membrane filter and analyzed with the HPLC method.

The HPLC system (Waters Corporation Milford, Mass) with 2 dual-piston high-pressure pumps (Waters 600 Controller with a binary gradient device), Rheodyne 7125 manual injector (20- μ L loop), and fluorescence detector (Waters 474FL Detector) were used in this study. After 20 μ L of sample was injected, the MMC-FA derivatives were adsorbed on the column (NovaPak C18, 7.3% carbon load, 3.9 $\times$ 150 mm, 4 μ m; Part number WAT036975, Waters Corporation) housed in an oven cabinet (Waters Column Oven) at 30°C. The continuous gradient elution (70%–100% acetonitrile) was performed at a flow rate of 1 mL/min from 0 to 30 minutes. Eluates were monitored online with a fluorescence detector ($\lambda_{\text{excitation}}$ 362 nm, $\lambda_{\text{emission}}$ 415 nm). Peak area of the eluted fatty acids was recorded and

integrated with the Waters Millenium 32 HPLC Software. Persisting fatty acids were identified by comparison with known standard fatty acids and quantified from the curves constructed from different concentrations of EPA, DHA, AA, and LA.

High-performance liquid chromatography was used for fatty acid determination in this study for several reasons. Although gas chromatography/mass spectrometry and gas chromatography/flame ionization detection are commonly used for quantitation of fatty acids [38–40], however, the gas chromatograph was not available. In comparison to a gas chromatograph, HPLC with UV or fluorometric detection is easy to operate and can be used as an alternative way to measure very small amounts of fatty acids. Because fatty acids are nonchromophoric and nonfluorophoric, they were labeled with a fluorogenic 4-bromomethyl-7-methoxycoumarin to produce esters of MMC-FA, which are strongly fluorescent ($\lambda_{\text{excitation}}$ 362 nm, $\lambda_{\text{emission}}$ 415 nm). The HPLC analysis established by Wolf and Korf [36] can be adapted to very low levels (nanogram range) of fatty acids. In the past decade, simple and highly sensitive HPLC analysis with fluorescent detection has been used for the analysis of fatty acids in many laboratories [41–43], and the results obtained by the HPLC technique have been shown to be similar to those obtained by gas chromatography analysis [44].

In this study, PUFA from the extracts were identified by comparison with known standard fatty acids, and their concentrations were determined from standard curves. The PUFA composition was reported as weight percent of total PUFA, and the levels of omega-3 and omega-6 fatty acid components as well as the cholesterol and triglyceride levels in each specimen were obtained using this method.

2.4. Determination of triglyceride and cholesterol contents in heart tissues

2.4.1. Analysis of triglyceride concentration

Triglycerides assay was based on the enzymatic determination of glycerol using the enzyme glycerol phosphate oxidase after hydrolysis by lipoprotein lipase [45,46]. In principle, triglyceride was hydrolyzed by lipase enzyme to release glycerol and fatty acids. The glycerol was catalyzed by glycerol kinase and then glycerol phosphate oxidase to produce hydrogen peroxide. The hydrogen peroxide was oxidized by peroxidase and gave electron to 4-aminoantipyrin in the presence of phenol to form a red-colored quinoneimine product. In assay procedure, specimen (10 μ L) was added to triglyceride enzyme reagent (BioTechnical Co, Bangkok, Thailand) containing lipase, glycerol kinase, glycerol phosphate oxidase/horse-radish peroxidase/*p*-aminoantipyrine (1.0 mL) and incubated at 37°C for 10 minutes. Optical density of the product was measured at 505 nm against reagent blank using a double-beam scanning UV-VIS spectrophotometer (PharmaSpec Model UV-1700, Shimadzu Corporation, Kyoto, Japan). Concentrations of the triglyceride were determined from a calibration curve

constructed from different triglyceride concentrations. The amounts of cardiac triglyceride were calculated and expressed as milligram per gram tissue dry weight.

2.4.2. Analysis of total cholesterol concentration

Estimation of total cholesterol concentrations is based on the method established by Richmond [47]. Cholesterol ester was first hydrolyzed by cholesterol esterase to release unesterified cholesterol and fatty acid. The unesterified cholesterol was then oxidized by cholesterol oxidase to generate cholestynone and hydrogen peroxide. In the presence of peroxidase, *p*-aminoantipyrine, and phenol, the hydrogen peroxide was oxidized to produce red-colored quinoneimine. In the assay procedure, specimen (10 μ L) was added to 1.0 mL cholesterol enzyme reagent (Bio-Technical Co) containing cholesterol esterase/cholesterol oxidase/horse-radish peroxidase/*p*-aminoantipyrine and incubated at 37°C for 10 minutes. The optical density of the colored product was measured at 505 nm against reagent blank using a double-beam scanning UV-VIS spectrophotometer (PharmaSpec Model UV-1700, Shimadzu Corporation). Concentrations of the cholesterol were determined from a calibration curve constructed from different cholesterol concentrations. Amounts of cardiac cholesterol were calculated and expressed as milligram per gram tissue dry weight.

2.5. Statistical analyses

Three outcome factors were set as fish consumption habits (≤ 2 fish-meal/wk vs > 2 fish-meal/wk), history of heart disease (with vs without), and causes of death (cardiac vs noncardiac cause). The level of omega-3 and omega-6 fatty acids, triglyceride, and cholesterol in each outcome factor category was compared by 2-sample Wilcoxon rank sum test. Fisher exact and χ^2 tests were used to analyze the difference in gender between the 2 groups. Student *t* test and 2-sample Wilcoxon rank sum tests were used for analyzing the difference in age between the 2 groups [48]. All analyses were performed using Intercooled Stata (version 8.0, Stata Corp, College station, Tex). All measurements were considered to be statistically significant when the *P* < .05.

3. Results

3.1. Omega-3 and omega-6 fatty acids in the heart and fish consumption habits

The age and gender were not different between the 2 groups. The total measured omega fatty acids in the heart tissues taken from cadavers who consumed more than 2 fish-meal/wk were significantly higher than those who ate 2 or less fish-meal/wk (Table 1). The levels of both omega-3 (ie, EPA + DHA) and omega-6 (AA + LA) fatty acids in the heart were also significantly higher in the group that consumed fish more frequently. For omega-3 fatty acid

Table 1
Contents of fatty acids in heart tissues and the fish consumption habits

Fatty acids	≤ 2 Fish-meal/wk ^a	> 2 Fish-meal/wk ^b	<i>P</i>
Age			.556 ^c
Mean (SD)	68 (14)	70 (11)	
Median (range)	70 (26-104)	71 (49-97)	
Gender, n (%)			.155 ^d
Male	37 (69)	21 (46)	
Female	17 (31)	25 (54)	
Total measured omega fatty acids ^e			.007 ^c
Mean (SD)	525.3 (322.8)	745.9 (404.0)	
Median (range)	404.8 (57.4-1503.1)	700.5 (148.0-1385.3)	
Omega-3 (EPA + DHA)			.008 ^c
Mean (SD)	96.0 (62.3)	138.7 (84.3)	
Median (range)	84.5 (7.5-330.7)	119.6 (23.9-381.2)	
Omega-6 (AA + LA)			.009 ^c
Mean (SD)	429.3 (279.7)	607.2 (335.2)	
Median (range)	322.3 (49.9-1172.4)	612.3 (35.8-1168.2)	
EPA			.127 ^c
Mean (SD)	4.5 (10.3)	2.5 (6.0)	
Median (range)	0 (0-69.0)	0 (0-32.00)	
DHA			.006 ^c
Mean (SD)	91.6 (62.8)	136.2 (84.0)	
Median (range)	84.5 (5.8-330.7)	115.8 (20.5-349.2)	
AA			.004 ^c
Mean (SD)	240.3 (193.5)	383.9 (245.2)	
Median (range)	168.7 (8.9-824.5)	376.15 (32.3-784.6)	
LA			.178 ^c
Mean (SD)	189.0 (107.7)	223.3 (122.6)	
Median (range)	178.1 (23.0-505.4)	235.6 (0-477.3)	
Triglyceride			.950 ^c
Mean (SD)	1500.8 (186.3)	1495.4 (167.7)	
Median (range)	1466.8 (1134.8-2049.6)	1497 (1193.4-1947.4)	
Cholesterol			.986 ^c
Mean (SD)	1196.4 (233.0)	1188.8 (232.6)	
Median (range)	1136.1 (830.2-1717.0)	1153.0 (688.3-1996.8)	

Parameters with *P* < .05 indicate significant difference between the 2 groups.

^a n = 54.

^b n = 46.

^c Two-sample Wilcoxon rank sum test.

^d Fisher exact test.

^e Total measured omega fatty acids: EPA + DHA + AA + LA.

levels, the DHA level was significantly higher in those who consumed more than 2 fish-meal/wk. The EPA level, however, was not significantly different between the 2 groups. For omega-6 fatty acid levels, the AA level was significantly higher in those who consumed more than 2 fish-meal/wk. The LA level was not significantly different between the 2 groups. The levels of triglyceride and cholesterol in the heart tissues were not different between the 2 groups. No gender differences were found for the levels of omega-3 and omega-6 fatty acids, as well as the cholesterol and triglyceride levels in heart tissue of cadavers (data not shown).

3.2. Omega-3 and omega-6 fatty acid contents in heart tissues and history of CHD

To seek the relationship between the levels of omega-3 and omega-6 fatty acids in the heart tissues and the history of CHD, cadavers were classified into 2 groups: one with a history of CHD and the other without a history of CHD (Table 2). Age and gender were not different between the 2 groups. Neither omega-3 nor omega-6 levels measured in this study were different between cadavers with a history of CHD and those without CHD. Similarly, the total measured

omega fatty acids, triglyceride, and cholesterol were not significantly different between the 2 groups.

3.3. Omega fatty acid contents in heart tissues and cardiac mortality

The association between omega fatty acid levels in heart tissues and cardiac mortality was investigated by dividing cadavers into 2 groups based on the cause of mortality, (ie, cardiac and noncardiac causes; Table 3). Both age and gender were not significantly different between the 2 groups.

Table 2
Fatty acids contents in heart tissues classified by the history of CHD

	No history of CHD ^a	With history of CHD ^b	P
Age			.418 ^c
Mean (SD)	68 (14)	71 (11)	
Median (range)	71 (26-104)	74 (51-97)	
Gender, n (%)			.671 ^d
Male	48 (63)	14 (58)	
Female	28 (37)	10 (42)	
Total measured omega fatty acids ^e			.448 ^f
Mean (SD)	616.6 (385.4)	659.0 (355.2)	
Median (range)	575.2 (99.0-1503.1)	671.9 (57.4-1283.7)	
Omega-3 (EPA + DHA)			.122 ^f
Mean (SD)	111.31 (79.0)	129.4 (64.9)	
Median (range)	97.3 (13.6-318.2)	135.55 (7.5-234.2)	
Omega-6 (AA + LA)			.675 ^f
Mean (SD)	505.3 (321.5)	529.6 (311.2)	
Median (range)	478.6 (35.8-1172.4)	532.0 (49.9-1065.1)	
EPA			.707 ^f
Mean (SD)	3.9 (9.4)	2.5 (5.3)	
Median (range)	0 (0-69.1)	0 (0-18.6)	
DHA			.120 ^f
Mean (SD)	107.5 (79.2)	126.8 (65.3)	
Median (range)	94.7 (10.9-349.2)	131.7 (5.8-232.2)	
AA			.859 ^f
Mean (SD)	305.6 (233.3)	308.6 (220.6)	
Median (range)	259.4 (32.3-824.5)	246.0 (8.9-714.5)	
LA			.272 ^f
Mean (SD)	199.7 (177.0)	203.0 (111.3)	
Median (range)	205.5 (0-505.4)	234.5 (23.0-422.9)	
Triglyceride			.465 ^f
Mean (SD)	1507.4 (189.1)	1469.5 (131.3)	
Median (range)	1483.5 (1134.8-2049.6)	1460.9 (1225.4-1761.8)	
Cholesterol			.397 ^f
Mean (SD)	1178.1 (211.8)	1239.9 (285.9)	
Median (range)	1135.5 (688.3-1717.0)	1210.0 (830.2-1996.8)	

Parameters with $P < .05$ indicate significant difference between the 2 groups. None of the determined parameters was significantly different between the 2 groups as indicated by $P > .05$.

^a n = 76.

^b n = 24.

^c Student *t* test.

^d χ^2 test.

^e Total measured omega fatty acids: EPA + DHA + AA + LA.

^f Two-sample Wilcoxon rank sum test.

Table 3
Fatty acids contents in heart tissues classified by causes of death

	Noncardiac mortality ^a	Cardiac mortality ^b	P
Age			.926 ^c
Mean (SD)	69 (13)	69 (13)	
Median (range)	71 (26-104)	67 (51-97)	
Gender, n (%)			.526 ^d
Male	54 (61)	8 (73)	
Female	35 (39)	3 (27)	
Total measured omega fatty acids ^e			.381 ^c
Mean (SD)	640.9 (382.7)	512.9 (320.9)	
Median (range)	608.1 (99.0-1503.1)	436.8 (57.4-1097.7)	
Omega-3 (EPA+DHA)			.415 ^c
Mean (SD)	118.0 (76.5)	96.6 (71.3)	
Median (range)	104.8 (13.6-381.2)	71.0 (7.5-234.2)	
Omega-6 (AA + LA)			.293 ^c
Mean (SD)	522.9 (319.6)	416.3 (298.2)	
Median (range)	501.4 (35.8-1172.4)	325.5 (49.9-920.0)	
EPA			.551 ^c
Mean (SD)	3.6 (8.9)	3.4 (6.4)	
Median (range)	0 (0-69.1)	0 (0-18.6)	
DHA			.447 ^c
Mean (SD)	114.4 (77.1)	93.2 (69.1)	
Median (range)	102.9 (10.9-349.2)	71.0 (5.8-215.6)	
AA			.067 ^c
Mean (SD)	320.8 (233.8)	189.3 (149.1)	
Median (range)	272.7 (32.3-824.5)	167.2 (8.9-442.7)	
LA			.620 ^c
Mean (SD)	202.1 (110.7)	227.1 (153.7)	
Median (range)	216.4 (0-505.4)	180.3 (23.0-477.3)	
Triglyceride			.197 ^c
Mean (SD)	1506.0 (182.4)	1436.5 (114.2)	
Median (range)	1503.8 (1134.8-2049.6)	1421.0 (1288.2-1676.7)	
Cholesterol			.250 ^c
Mean (SD)	1182.2 (225.0)	1279.7 (276.9)	
Median (range)	1147.3 (688.3-1996.8)	1424.7 (849.6-1602.3)	

Parameters with $P < .05$ indicate significant difference between the 2 groups. None of the determined parameters was significantly different between the 2 groups as indicated by $P > .05$.

^a n = 89.

^b n = 11.

^c Two-sample Wilcoxon rank sum test.

^d Fisher exact test.

^e Total measured omega fatty acids: EPA + DHA + AA + LA.

Both omega-3 and omega-6 fatty acids measured in the heart tissues were not different between cadavers with cardiac mortality and those with noncardiac mortality. The total measured omega fatty acid levels, triglyceride, and cholesterol were also not significantly different between the 2 groups.

3.4. Omega fatty acid contents in heart tissues and the cause of death in cadavers with a history of CHD

In cadavers with a history of heart disease, we sought to determine the association between omega-3 and omega-6 fatty acid contents in the hearts and cardiac mortality in this group (Table 4). Cadavers with a history of heart disease were classified into 2 groups by cause of death (ie, cardiac vs noncardiac mortality). Age and gender were not significantly different between the 2 groups. The total measured omega fatty acid levels in the heart were significantly lower in cadavers in the cardiac mortality group than in the noncardiac mortality group. The levels of omega-3 (EPA + DHA) and omega-6 (AA + LA) fatty acids were also significantly lower in the group with cardiac mortality. Although the EPA level was not different between the 2 groups, the DHA level was significantly lower in cadavers with cardiac mortality. Similar to the DHA level, the AA level in the heart was also significantly lower in the cardiac mortality group. Both triglyceride and cholesterol levels in the heart tissues were not different between the 2 groups.

4. Discussion

The major findings of this study are as follows: (1) both omega-3 (EPA + DHA) and omega-6 (AA + LA) fatty acids levels in the heart tissues, especially the DHA and AA, are associated with fish consumption and (2) in heart tissues taken from cadavers with CHD, low levels of both omega-3 (EPA + DHA) and omega-6 (AA + LA) in the heart, specifically the DHA and AA, are associated with cardiac mortality.

Epidemiologic studies have reported that there is an association between consumption of fish that is rich in omega-3 fatty acids and cardiovascular disease [1,14,15,49,50]. Basic and clinical studies have also demonstrated the correlation between omega-3 supplements and an increase in omega-3 contents in both plasma and cardiac tissues [17–19]. In this study, the total measured omega fatty acids and the levels of omega-3 (ie, EPA + DHA) and omega-6 (ie, AA + LA) fatty acids in the heart tissues of cadavers are associated with fish consumption habits. However, for the omega-3 fatty acids, only the DHA level in the heart is associated with fish consumption habits. This association is not observed for EPA level in the heart tissues. For omega-6 fatty acids, only the AA level in heart tissues is associated with fish consumption habits. The LA levels are not different between the 2 groups.

Table 4

Fatty acids contents in heart tissues in cadavers with heart disease classified by the causes of death

	Cardiac cause ^a	Noncardiac cause ^b	P
Age			.591 ^c
Mean (SD)	69 (14)	71 (8)	
Median (range)	67 (51–97)	74 (57–82)	
Gender, n (%)			.678 ^d
Male	6 (67)	8 (53)	
Female	3 (33)	7 (47)	
Total measured omega fatty acids ^c			.016 ^c
Mean (SD)	435.1 (278.3)	793.3 (333.9)	
Median (range)	396.5 (57.4–885.7)	829.1 (265.9–1283.7)	
Omega-3 (EPA + DHA)			.040 ^c
Mean (SD)	92.01 (72.9)	151.8 (49.5)	
Median (range)	71.0 (7.5–234.2)	155.3 (55.3–232.2)	
Omega-6 (AA + LA)			.022 ^c
Mean (SD)	343.0 (265.2)	641.5 (288.2)	
Median (range)	319.0 (49.9–845.8)	656.8 (210.6–1065.1)	
EPA			.070 ^c
Mean (SD)	4.2 (6.9)	1.5 (4.1)	
Median (range)	0.6 (0–18.6)	0 (0–13.4)	
DHA			.034 ^c
Mean (SD)	87.8 (69.9)	150.1 (51.5)	
Median (range)	71.0 (5.8–215.6)	155.3 (46.0–232.2)	
AA			.005 ^c
Mean (SD)	154.6 (134.1)	401.0 (212.8)	
Median (range)	145.2 (8.9–422.9)	400.8 (74.0–714.5)	
LA			.245 ^c
Mean (SD)	188.4 (136.5)	240.5 (92.7)	
Median (range)	151.9 (23.0–422.9)	272.6 (88.1–360.2)	
Triglyceride			.456 ^c
Mean (SD)	1446.9 (124.0)	1483.1 (137.9)	
Median (range)	1435.5 (1288.2–1676.7)	1503.8 (1225.4–1676.7)	
Cholesterol			.355 ^c
Mean (SD)	1283.9 (282.0)	1213.5 (294.7)	
Median (range)	1424.7 (849.6–1602.3)	1189.5 (830.2–1996.8)	

Parameters with $P < .05$ indicate significant difference between the 2 groups.

^a n = 9.

^b n = 15.

^c Two-sample Wilcoxon rank sum test.

^d Fisher exact test.

^e Total measured omega fatty acids: EPA + DHA + AA + LA.

A number of studies have demonstrated the significance of omega-3 fatty acid supplements in reducing mortality of omega-3 fatty acid supplements in reducing mortality [1,8,11]. Epidemiologic studies also support these findings [50,51]. For omega-6 fatty acids, there are reports that suggest reducing their intake [22–24,29], reports indicating no significant effects [25–28], and recommending increasing their intake to reduce the risk of CHD [52–55]. Despite this growing body of evidence and controversy, direct association between omega-3 fatty acids content in the heart tissues and cardiac mortality in the general population without food supplements has never been tested.

This study is the first to investigate this relationship. When the cadavers were classified into 2 groups based on the history of CHD, the levels of omega-3 and omega-6 fatty acids in heart tissues were not different between the groups with and without the history of CHD. Similarly, when the cause of mortality was used to classify the sources of heart tissues, no significant difference was found for the omega-3 and omega-6 contents in the hearts between cardiac and noncardiac mortality groups. However, only when cadavers with a history of CHD were categorized into 2 groups using the causes of death, we found that the group with cardiac mortality had lower levels of omega-3 (EPA + DHA) and omega-6 (AA + LA) fatty acids as well as total measured omega fatty acids in the hearts. More importantly, we found that the DHA and AA levels in heart tissues were significantly lower in the cardiac mortality group. All of these findings suggest that both omega-3 and omega-6 contents in the heart may not protect against all-cause mortality. However, the cardiac mortality prevention of omega-3 and omega-6 fatty acids, particularly the DHA and AA levels in the heart, is notably effective only in those who already have pathological hearts.

Raith et al [56] previously reported that omega-3 supplements in patients with implantable cardioverter defibrillator and with a history of ventricular tachycardia (VT) or ventricular fibrillation do not reduce the risk of VT or ventricular fibrillation, compared with a placebo. In addition, they found that fish oil may be pro-arrhythmic in implantable cardioverter defibrillator patients with a history of VT prior to receiving this supplement. Their findings indicated that the type of arrhythmia is important in determining the beneficial effects of omega-3 fatty acids in the heart [56].

In this study, our findings also indicate that omega-3 fatty acids may be beneficial only in those with underlying heart disease. These findings also suggest that the underlying pathology of the heart is an important parameter in determining whether an omega-3 supplement is beneficial. This hypothesis has recently been elucidated by Ruijter et al, who proposed that omega-3 fatty acids could be either pro- or anti-arrhythmic, depending on the underlying mechanisms of arrhythmias in each individual [57].

In addition to the importance of DHA, our study also indicates a similar importance of AA, an omega-6 fatty acid, in the heart. The association between its low levels and cardiac mortality in cadavers with a history of heart disease suggests that it could play an equally important role to DHA in cardioprotective effects. A recent American Heart Association Science Advisory Statement clearly indicates the cardioprotective benefits of omega-6 fatty acids in reducing the risk of cardiovascular disease [30] and supports our findings.

In the past decades, a number of studies have emphasized the clinical significance of omega-3 fatty acids in preventing arrhythmias and heart disease, as well as reducing mortality [1,14,15,49,50]. The results of this study demonstrate the

possibly equal importance of AA, an omega-6 fatty acid, in reducing cardiac mortality. Further studies with a large population are needed to verify the clinical significance of the findings presented in this study.

There are several limitations in this study. First, the population in this study is small, particularly in those with a history of CHD. Second, detailed demographic data such as history of drugs taken, underlying diseases, and history of arrhythmias are not included. In addition, our study was designed to study omega-3 fatty acids, particularly DHA and EPA, and omega-6 fatty acids (ie, AA and LA) in the heart tissues of cadavers. Therefore, other fatty acids were not investigated in this study.

The levels of omega-3 (EPA + DHA) and omega-6 (AA + LA) in the heart are associated with the amount of fish consumption. In cadavers with a history of CHD, these levels are associated with cardiac mortality. These findings indicate that both omega-3 and omega-6 fatty acids, particularly DHA and AA, could be useful in reducing cardiac mortality in those with a history of CHD.

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Basic nutritional investigation

Effects of curcuminoid supplement on cardiac autonomic status in high-fat–induced obese rats

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Abstract

Objective: Sudden cardiac death in obesity is frequently associated with sympathetic activation due to an elevated plasma free-fatty acid (FFA) level. Curcuminoids, the phenolic yellowish pigments of turmeric, display antioxidative and lipid-lowering activities. We hypothesized that curcuminoids ameliorate cardiac sympathovagal disturbance in high-fat–induced obese rats.

Methods: Male Wistar rats were divided into five groups. A normal-diet control (NDC) group received a normal-fat diet (12% calories as fat) and a high-fat–diet control (HDC) group received a high-fat diet (60% calories as fat) for 12 wk. Three other groups received high-fat diets with curcuminoid supplement at concentrations of 30 mg (HD₃₀), 60 mg (HD₆₀), and 90 mg (HD₉₀) per kilogram of body weight every day for 12 wk. Heart rate variability was determined to assess cardiac autonomic status at weeks 0 and 12.

Results: Body weight, visceral fat mass, plasma FFA, and glucose levels increased significantly in the HDC group compared with the NDC group. Low frequency power in normalized units (LFnu) and the ratio of LF to high-frequency power (HF) in the HDC group were significantly higher, whereas HFnu in the HDC group was significantly lower than in the NDC group. Plasma FFA levels correlated significantly with LFnu and LF/HF ratio. Compared with the HDC group, plasma FFA, glucose levels, LFnu, and LF/HF ratio were significantly decreased in the HF₃₀, HF₆₀, and HF₉₀ groups.

Conclusion: Elevated plasma FFA in high-fat–induced obese rats is associated with an increased LF/HF ratio, an expression of sympathovagal disturbance. Curcuminoid supplementation ameliorates cardiac autonomic imbalance in high-fat–fed rats, probably due to its lipid-lowering effect. © 2009 Elsevier Inc. All rights reserved.

Keywords:

Curcuminoids; Obesity; Heart rate variability; Sympathovagal disturbance

Introduction

Risk of acute coronary syndrome and other cardiovascular complications has increased along with a growing obesity epidemic. Premature ventricular contractions causing arrhythmia and sudden death also frequently occur in obese people

[1]. Although the exact mechanism remains unknown, reports have indicated that obesity raises the risk of cardiovascular diseases partly through its effects on vascular risk factors such as hypertension, dyslipidemia, insulin resistance, glucose intolerance, and type 2 diabetes [2,3].

Hyperlipidemia, particularly increased plasma free fatty acid (FFA) levels, is common in obese subjects [4]. Elevated plasma FFAs are associated with a larger number of ventricular premature complexes in normal subjects and non-ischemic diabetic patients [5]. Although several risk factors may contribute to these events, sympathetic nervous system activation has been proposed as responsible for arrhythmia in obesity [5–7]. Previous studies have demonstrated that portal

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fatty acid infusion can stimulate sympathetic nervous system activity [8]. Furthermore, Verwaerde et al. [9] demonstrated that tachycardia induced by acute elevation of plasma FFA concentrations in dogs was mainly caused by impairment of parasympathetic control. These findings indicate the important role of fatty acids in autonomic control.

Currently, analysis of heart rate variability (HRV) is widely used to assess the autonomic nervous system and provides information on risk of cardiovascular diseases and mortality [10,11]. Decreased HRV, an expression of an exaggerated cardiac sympathetic nervous system, is associated with increased cardiovascular mortality [11–13]. Previous studies have demonstrated that elevated plasma FFA levels stimulate cardiac sympathetic nervous activity in healthy subjects [14] and in non-insulin-dependent diabetic (NIDDM) patients [7]. Blood pressure variability and HRV study also show that increased plasma FFAs in rats significantly increases low-frequency power in normalized units (LFnu) and decreases high-frequency power in normalized units (HFnu), causing a significant increase in the LF/HF ratio [15]. This finding indicates that an elevated level of plasma FFA is strongly linked to a reduced vagal component of the cardiac baroreflex and inversely correlated with baroreflex sensitivity.

Curcuminoids, a group of phenolic compounds isolated from the rhizome of the plant *Curcuma longa* Linn., have been reported as an efficient antioxidant, anticarcinogenic, anti-inflammatory, and hypoglycemic agent [16–24]. Curcuminoids exert hypocholesterolemic actions on lipid metabolism [25,26]. Furthermore, dietary curcuminoids have been shown to prevent liver triacylglycerol accumulation and epididymal adipose tissue weight gain and decrease plasma very low-density lipoprotein/triacylglycerol in rats fed a high-fat diet [27]. However, the role of curcuminoids on plasma FFA is not known.

We tested the hypotheses that 1) curcuminoids decrease plasma FFA concentrations of high-fat-induced obese rats and 2) curcuminoids ameliorate cardiac sympathovagal disturbance in obese rats and that this effect is associated with a lipid-lowering action. HRV measurement was employed to assess cardiac autonomic status.

Materials and methods

Animals

Male Wistar rats (100–120 g) from the National Animal Center, Salaya Campus, Mahidol University, Thailand, were housed in the animal facility, where the temperature was maintained at approximately 24–25 °C with 12-h dark/light cycle. The experimental protocol adhered to the Guide for the Care and Use of Animals in compliance with the National Institutes of Health guideline for the care and treatment of animals and followed Faculty of Medicine, Chiang Mai University, standard operating procedures for animal care and research.

Study protocol

Sixty rats were randomly selected and divided into five groups ($n = 12$ each). The normal-diet control (NDC) group received standard rat chow (C.P. Mice Feed Food no. 082; Bangkok, Thailand; energy content 3.8 kcal/g) containing fat for 12% of total energy. The high-fat-diet control (HDC) group consumed a high-fat diet comprised of fat for 60% of total energy with energy content calculated at 5.6 kcal/g [28] (Table 1). The curcuminoid-supplemented groups received a high-fat diet and 30, 60, or 90 mg of curcuminoids per kilogram of body weight (HF₃₀, HF₆₀, HF₉₀). Curcuminoids (Sigma Chemical, St. Louis, MO, USA) were dissolved in a 0.5% tragacanth (Fluka Chemical, Milwaukee, WI, USA) suspension, and a 2% curcuminoid solution was administered daily at different doses (30, 60, or 90 mg/kg body weight) for 12 wk by oral gavage. The NDC and HFC groups were given the same dose of vehicle (0.5% tragacanth suspension). Animals had free access to food and water. Body weight and food intake were recorded daily. Spectral analysis of HRV was performed to determine sympathetic activity before treatment with curcuminoids or vehicle and at week 12. At the end of week 12, animals were fasted overnight and anesthetized deeply. The abdominal cavity was opened following abdominal median line. Blood samples were collected from the right atrium. Samples for assay of glucose were kept on ice in tubes precoated with sodium fluoride. Samples for insulin, FFA, and triacylglycerol assays were taken in tubes with ethylenediaminetetra-acetic acid. Plasma was separated and stored at –70 °C for subsequent biochemical analyses. Visceral fats, including retroperitoneal, epididymal, and perirenal fat pad [29], were removed and weighed.

Measurement of HRV

Cardiac autonomic activity was assessed by spectral analysis of R-R interval variability. Rats were anesthetized with ether for lead insertion and then left conscious for at least 5 min before recording an electrocardiogram [30]. Lead II on the electrocardiogram was recorded continuously for 10 min by Power Lab with chart 5.0 as described previously [31]. During electrocardiographic recording, animals were calm and in a familiar environment without

Table 1
Composition of experiment diets*

	Normal diet		High-fat diet	
	g	%kcal	g	%kcal
Carbohydrate	495.30	51.99	365.00	13.96
Fat	83.70	19.76	310.00	59.16
Protein	269.00	28.23	250.00	26.88
Vitamins–minerals	65.40	0.00	70.00	0.00
Fiber	34.30	0.00	30.00	0.00

* Diet ingredients and nutrient analyses were modified from Srinivasan et al. [28]. Energy per gram (kilocalories per gram): carbohydrate 4, fat 9, and protein 4.

unnecessary noise and movement. Electrocardiographic data were analyzed using MATLAB [31]. R-R interval was determined using peaks of the QRS complex from the electrocardiogram and stored as an interval tachogram. The time domain variables measured were the standard deviation of all R-R intervals and the root mean square of successive difference. Frequency domain analysis, i.e., the power spectra of R-R interval variability, was obtained using the fast Fourier transform algorithm. High frequency (HF ~ 0.6 – 3 Hz and varying with respiration), low frequency (LF ~ 0.2 – 0.6 Hz), and very LF (≤ 0.2 Hz) were determined [30,32]. The LF and HF components were expressed in normalized units. Values in normalized unit for LF (LFnu) or HF (HFnu) were obtained by dividing the component by total power minus very LF. The power of the HF component provides an index of cardiac vagal activity [33], whereas the LF component is determined by sympathetic activity with vagal modulation [34]. The LF/HF ratio is considered an index of cardiac sympathetic–parasympathetic tone balance [10].

Determination of insulin resistance (homeostasis model assessment index)

Insulin resistance was assessed by homeostasis model assessment (HOMA) [35], a mathematical model describing the degree of insulin resistance starting from fasting plasma insulin and glucose concentrations. The HOMA index is calculated as follows:

$$\text{HOMA} = \frac{[\text{fasting insulin level}(\mu\text{U/mL}) \times \text{fasting glucose level}(\text{mmol/L})]}{22.5}$$

Analytical procedures

Plasma glucose concentration was determined using a colorimetric assay based on H_2O_2 using a commercially available kit (Biotech). Plasma triacylglycerol and FFA levels were assayed with commercially available kits (Biocode Hycl and Wako). Plasma insulin level was measured by sandwich enzyme-linked immunosorbent assay using a kit (LINCO Research).

Statistical analysis

Data are presented as mean $\pm$ standard error. Statistical analyses were carried out using one-way analysis of variance and Fisher's least significant difference test for post hoc testing to identify specific mean differences. Simple correlation analysis was used to determine the relation among plasma parameters and visceral and HRV measurements. Statistical significance was accepted at $P < 0.05$.

Results

Animal characteristics

Initial animal body weight was not different among experimental groups. Body weights in all animals increased significantly after 12 wk. Twelve weeks of high-fat feeding resulted in a significant increase of body weight, accompanied by accumulation of visceral fat in the HDC group compared with the NDC group (Table 2). Analysis of dietary records showed that average daily energy intake over 12 weeks in the HDC group was significantly higher than in the NDC group. These results suggest that high-fat-induced obesity characterized by an increase of body weight with substantial accumulation of visceral fat results from increased energy intake. No significant differences in body weight or visceral fat mass were found among the HDC and HD₃₀, HD₆₀, and HD₉₀ groups. Average daily energy intake in the curcuminoid-supplemented groups was also comparable to that in the HDC group, although the value in the HD₉₀ group was significantly lower than in the HD₃₀ and HD₆₀ groups.

Fasting plasma parameters

Plasma FFA levels were significantly higher in the HDC than in the NDC group (Table 3). Furthermore, plasma FFA levels significantly correlated with visceral fat mass ($r = 0.546$; Fig. 1). However, there was no correlation between visceral fat and plasma FFA acid levels after curcuminoid supplementation ($r = 0.020$). Compared with the HDC

group, plasma FFA levels were significantly lower in all curcuminoid-supplemented groups. However, decreased plasma FFA levels associated with curcuminoids were not dose dependent. A trend of an increase of plasma triacylglycerol levels was found in the HDC group. A curcuminoid supplement of 90 mg/kg of body weight was associated with a decrease in plasma triacylglycerol levels. Plasma triacylglycerol level was significantly lower in the HD₉₀ group than in the HDC, HD₃₀, and HD₆₀ groups.

Fasting plasma glucose level was significantly elevated in the HDC group compared with the NDC group. In contrast to fasting hyperglycemia, there was no significant difference between the HDC and NDC groups in fasting plasma insulin levels. Curcuminoid supplements at all doses (30, 60, and 90 mg/kg body weight) significantly lowered fasting plasma glucose concentrations compared with the HDC group but had no effect on plasma insulin levels. No significant differences in HOMA indexes were present among the HDC, NDC, and all curcuminoid-supplemented groups.

Table 2

Effects of curcuminoid supplementation on body weight, visceral fat mass, and energy intake in high-fat-induced obese rats*

	NDC	HDC	HD ₃₀	HD ₆₀	HD ₉₀
Body weight (g)					
Week 0	140.71 ± 8.20	140.71 ± 8.20	139.58 ± 4.94	140.00 ± 5.71	153.91 ± 5.58
Week 12	440.71 ± 10.66 [†]	510.00 ± 18.28 [†]	502.92 ± 17.6 [†]	517.67 ± 16.59 [†]	503.82 ± 10.91 [†]
Visceral fat (g)	26.37 ± 2.11	47.30 ± 3.89 [‡]	46.34 ± 3.52	46.52 ± 3.44	39.97 ± 2.09
Energy intake (kcal/d)	61.63 ± 1.93	102.99 ± 3.51 [‡]	108.89 ± 3.06	107.32 ± 3.73	97.85 ± 3.10 ^{§,}

HD₃₀, high-fat diet + curcuminoid supplementation at 30 mg/kg of body weight; HD₆₀, high-fat diet + curcuminoid supplement at 60 mg/kg of body weight; HD₉₀, high-fat diet + curcuminoid supplement at 90 mg/kg of body weight; HDC, high-fat–diet control; NDC, normal-diet control

* Each value is mean ± SE from 7–12 rats.

[†] Significantly different from week 0.

[‡] Significantly different from NDC group.

[§] Significantly different from HD₃₀ group.

^{||} Significantly different from HD₆₀ group ($P < 0.05$).

Heart rate variability

There were no differences in initial values of HRV variables among experimental groups (Table 4). At week 12, LFnu and the LF/HF ratio were significantly higher, whereas HFnu was significantly lower, in the HDC than in the NDC group (Table 5). Compared with the HDC group, LFnu and the LF/HF ratio were significantly lower, whereas HFnu was significantly higher, in the HD₃₀, HD₆₀, and HD₉₀ groups. In contrast, neither standard deviation of all R-R intervals nor root mean square of successive difference was affected by high-fat feeding alone or with curcuminoid supplementation. As presented in Table 6, LFnu and the LF/HF ratio significantly correlated with visceral fat mass ($r = 0.650$ and 0.616). Also, significant correlations were noted between plasma FFA levels and LFnu ($r = 0.589$) and the LF/HF ratio ($r = 0.580$), whereas inverse correlations were found between HFnu and visceral fat mass ($r = -0.650$) and plasma FFA levels ($r = -0.589$). However, there were no correlations between HRV with plasma triacylglycerol, plasma glucose, and plasma insulin concentrations.

There was also no correlation between HRV and visceral fat and plasma parameters in NDC rats or HD₃₀, HD₆₀, and HD₉₀ rats.

Discussion

The major findings of this study are as follows: 1) an increase of plasma FFA level causes cardiac autonomic disturbance in obesity induced by chronic high-fat feeding, and 2) curcuminoid supplementation effectively decreases plasma FFA levels and improves cardiac autonomic nervous system activity in obesity.

Effects of plasma FFA levels on plasma glucose and insulin in obese rats

The study demonstrates that a high-fat diet causes ventral obesity, as indicated by a significant increase of body weight with substantial accumulation of visceral fat. There is also a significant correlation between visceral fat mass and plasma FFA levels. It has been well recognized that visceral adipose tissue is less responsive to antilipolytic action of insulin but more responsive to lipolytic action of catecholamines compared with subcutaneous adipose tissue [36]. Thus, a significant increase in plasma FFA levels in high-fat-induced obese rats could be due to increased visceral adipocytes with a secondary exaggeration in lipolytic activity. This is consistent with a report showing that an elevated plasma FFA level is common in obese patients with hyperlipidemia [4].

Table 3

Effects of curcuminoid supplementation on fasting plasma parameters and HOMA index in high-fat-induced obese rats*

	NDC	HDC	HD ₃₀	HD ₆₀	HD ₉₀
FFA (mmol/L)	0.38 ± 0.05	0.51 ± 0.04 [†]	0.38 ± 0.03 [‡]	0.36 ± 0.03 [‡]	0.32 ± 0.01 ^{‡,§,}
TG (mg/dL)	85.14 ± 20.31	110.27 ± 17.37	109.51 ± 8.97	93.76 ± 9.29	56.10 ± 4.42 ^{‡,§,}
Glucose (mg/dL)	132.01 ± 4.62	153.54 ± 4.81 [†]	132.04 ± 5.60 [‡]	134.69 ± 7.12 [‡]	136.18 ± 4.08 [‡]
Insulin (ng/mL)	1.24 ± 0.13	1.35 ± 0.11	1.56 ± 0.61	1.51 ± 0.21	1.40 ± 0.25
HOMA index	11.13 ± 1.35	13.67 ± 1.57	14.41 ± 2.34	14.23 ± 1.67	11.97 ± 2.23

FFA, free fatty acid; HD₃₀, high-fat diet + curcuminoid supplementation at 30 mg/kg of body weight; HD₆₀, high-fat diet + curcuminoid supplement at 60 mg/kg of body weight; HD₉₀, high-fat diet + curcuminoid supplement at 90 mg/kg of body weight; HDC, high-fat–diet control; HOMA, homeostasis model assessment; NDC, normal-diet control; TG, triacylglycerol

* Each value is mean ± SE from 7–12 rats.

[†] Significantly different from NDC group.

[‡] Significantly different from HDC group.

[§] Significantly different from HD₃₀ group.

^{||} Significantly different from HD₆₀ group ($P < 0.05$).

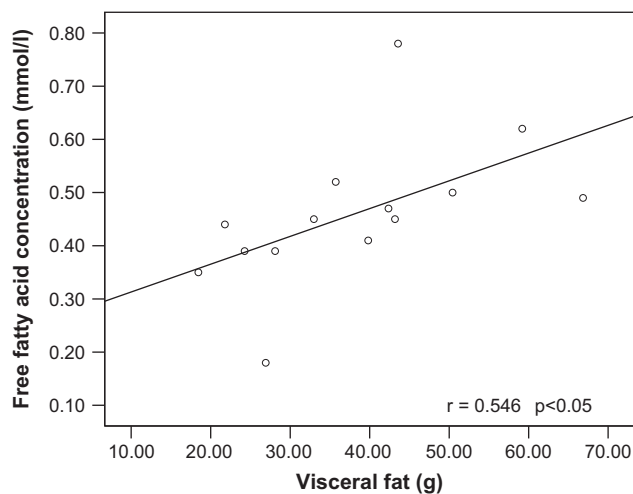


Fig. 1. Correlation between visceral fat and plasma free-fatty acid concentrations in rats receiving a normal diet or a high-fat diet for 12 wk.

In addition, fasting hyperglycemia was noted in high-fat-induced obese rats. An elevated level of plasma FFAs, particularly in obesity, has been shown to play an important role in the overproduction of glucose in the diabetic state [37]. Also, in type 2 diabetic patients, fasting hyperglycemia is caused by an inappropriate increase in hepatic glucose production [38,39]. It is most likely that an increase of portal FFAs leads to increased hepatic glucose output [3]. FFAs can also potentially influence hepatic glucose metabolism by several other metabolisms. FFAs have been shown to stimulate gluconeogenesis [40,41] and upregulate the glucose-6-phosphate system. Furthermore, most studies have reported that elevated plasma FFAs impair insulin-induced suppression of endogenous glucose production (i.e., cause hepatic insulin resistance) in normal control subjects or in patients with NIDDM [42,43]. This effect is due to an inhibition of normal insulin suppression of glycogenolysis by FFAs [42]. Moreover, the effect of FFAs in inhibition of insulin-stimulated glucose uptake in skeletal muscle [44,45] should be considered. This effect of FFAs is by the insulin-signaling cascade [46], resulting in inhibition of glucose transporter-4 (GLUT-4) translocation [47] and decreased muscle glycogen synthase activity [48].

Several studies have demonstrated that an increase in hepatic glucose production stimulated by acute elevated plasma FFA levels is antagonized by an FFA-mediated stimulation of insulin secretion [49,50]. Despite elevated plasma FFAs, our study demonstrated no change in fasting plasma insulin levels in high-fat-induced obese rats compared with normal-diet rats, suggesting that its effect on insulin response might be different from the effect caused by acute elevated plasma FFA levels. In this study, elevated plasma FFA levels in high-fat-induced obese rats occurred over months. It has been shown that obese individuals who are genetically predisposed to develop NIDDM may eventually lose their ability to increase insulin secretion in response to chronically elevated plasma FFA levels.

Roles of plasma FFA levels on HRV modulation

Heart rate variability analysis in the present study demonstrated that, in high-fat-induced obese rats, significant increases of LF and the LF/HF ratio were accompanied by significant HF decreases. It is generally considered that the HF component is a marker of the efferent vagal activity to the heart [51]. The interpretation of the LF component is more controversial. Although it was previously proposed as a marker of sympathetic modulation [52] other investigators have suggested that LF represents sympathetic and parasympathetic influences [53]. However, LF in absolute value seems to reflect baroreflex modulation, which is mainly of parasympathetic origin [54], whereas the LF/HF ratio and LFnu are markers of sympathovagal balance [10]. Our findings demonstrated that the impaired cardiac autonomic activity in high-fat-induced obese rats is associated with a reduced vagal contribution and an increased sympathetic contribution to cardiac autonomic control.

A correlation between HRV and visceral fat mass was also observed in this study. LF and the LF/HF ratio significantly correlated with visceral fat mass, whereas HF had a negative correlation with visceral fat mass. These findings indicate the links between central obesity and cardiac autonomic disturbance. An association between

Table 4
Heart rate variability at baseline*

	NDC	HDC	HD ₃₀	HD ₆₀	HD ₉₀
SDNN	2.59 ± 0.42	2.77 ± 0.67	2.89 ± 0.65	2.03 ± 0.43	3.77 ± 0.39
RMSSD	1.82 ± 0.27	2.85 ± 0.89	2.95 ± 1.10	2.60 ± 0.46	2.98 ± 0.48
LFnu	0.16 ± 0.00	0.16 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	0.17 ± 0.01
HFnu	0.84 ± 0.00	0.84 ± 0.01	0.82 ± 0.02	0.85 ± 0.01	0.83 ± 0.01
LF/HF ratio	0.19 ± 0.01	0.20 ± 0.01	0.21 ± 0.03	0.17 ± 0.01	0.20 ± 0.01

HD₃₀, high-fat diet + curcuminoid supplementation at 30 mg/kg of body weight; HD₆₀, high-fat diet + curcuminoid supplement at 60 mg/kg of body weight; HD₉₀, high-fat diet + curcuminoid supplement at 90 mg/kg of body weight; HDC, high-fat-diet control; HFnu, high-frequency power in normalized units; LFnu, low-frequency power in normalized units; NDC, normal-diet control; RMSSD, square root of the mean of the sum of the squares of differences between adjacent R-R intervals; SDNN, standard deviation of all R-R intervals

* Each value is mean ± SE from 7–12 rats.

Table 5

Effect of curcuminoid supplementation on heart rate variability indexes in high-fat-induced obese rats*

	NDC	HDC	HD ₃₀	HD ₆₀	HD ₉₀
SDNN	3.41 ± 0.49	3.91 ± 0.36	4.23 ± 0.62	3.87 ± 0.57	4.11 ± 0.36
RMSSD	2.42 ± 0.25	3.42 ± 0.61	4.11 ± 0.64	3.77 ± 0.82	3.82 ± 0.36
LFnu	0.17 ± 0.01	0.28 ± 0.01 [†]	0.19 ± 0.01 [‡]	0.17 ± 0.01 [‡]	0.18 ± 0.01 [‡]
HFnu	0.83 ± 0.01	0.72 ± 0.01 [†]	0.81 ± 0.01 [‡]	0.83 ± 0.01 [‡]	0.82 ± 0.01 [‡]
LF/HF ratio	0.20 ± 0.01	0.40 ± 0.03 [†]	0.24 ± 0.01 [‡]	0.20 ± 0.01 [‡]	0.21 ± 0.01 [‡]

HD₃₀, high-fat diet + curcuminoid supplementation at 30 mg/kg of body weight; HD₆₀, high-fat diet + curcuminoid supplement at 60 mg/kg of body weight; HD₉₀, high-fat diet + curcuminoid supplement at 90 mg/kg of body weight; HDC, high-fat–diet control; HFnu, high-frequency power in normalized units; LFnu, low-frequency power in normalized units; NDC, normal-diet control; RMSSD, square root of the mean of the sum of the squares of differences between adjacent R-R intervals; SDNN, standard deviation of all R-R intervals

* Each value is mean ± SE from 7–12 rats.

[†] Significantly different from NDC group.

[‡] Significantly different from HDC group ($P < 0.05$).

visceral obesity and impaired cardiac autonomic activity has been previously reported, but findings were inconsistent. A previous HRV study showed that, in postmenopausal obese women, impaired cardiac autonomic activity varied depending on regional body fat distribution [55]. A significant increase of cardiac sympathetic and parasympathetic activity was pronounced in women with combined upper body obesity and visceral obesity. Conversely, Peterson et al. [56] reported depression of sympathetic and parasympathetic activity in healthy men with an increasing percentage of body fat. Significant inverse correlations were found between the percentage of body fat and variation in R-R interval after β -adrenergic blockade, heart rate, and plasma catecholamine concentrations. These inconsistent results were probably due to differences in the subjects and methods used in those studies. However, a report by Zahorska-Markiewicz et al. [57] using 24-h continuous electrocardiographic and Ewing testing in obese women demonstrated that cardiac autonomic disturbance with high-fat-induced obesity is characterized by overactivity of the cardiac sympathetic nervous system with a relative reduction of parasympathetic nervous system activity. These findings are consistent with the results of our study.

Table 6

Correlation between HRV and visceral fat and plasma parameters in rats that consumed normal diets or high-fat diets for 12 wk

	HRV		
	LFnu	HFnu	LF/HF ratio
Visceral fat (g)	0.650 [†]	−0.650 [†]	0.616*
Plasma free fatty acid (mmol/L)	0.589*	−0.589*	0.580*
Plasma triacylglycerol (mg/dL)	−0.168	0.168	−0.173
Plasma glucose (mg/dL)	0.423	−0.423	0.389
Plasma insulin (ng/mL)	−0.423	0.423	−0.442

HFnu, high-frequency power in normalized units; HRV, heart rate variability; LFnu, low-frequency power in normalized units

* $P < 0.05$

[†] $P < 0.01$.

Effects of curcuminoids on plasma FFA, glucose, and insulin

Curcuminoids, the phenolic yellowish pigment of turmeric, are known to benefit human health [16]. The present results demonstrate that the increased plasma FFA levels that developed in high-fat-induced obese rats were significantly countered by administration of curcuminoids. Although the exact mechanism by which curcuminoids decrease lipid levels was not investigated, it has been previously shown that curcumin, the active component of curcuminoids, modulates several important molecular targets, including transcription factors such as peroxisome proliferator-activated receptor- γ (PPAR- γ) [58]. PPARs are known to regulate the gene involved in fatty acid uptake and storage and in inflammatory and glucose homeostasis [59]. Curcumin has been reported to activate PPAR- γ in rat hepatic stellate cells activated by oxidative stress [60], to exhibit PPAR- γ ligand-binding activity in the GAL4-PPAR- γ chimera assay, and to accelerate triacylglycerol accumulation in adipocytes [21]. Curcumin has been shown to dramatically induce expression of the PPAR- γ gene and activate PPAR- γ -inactivated hepatic stellate cells [60]. Therefore, an activation of PPAR- γ could be responsible for a decrease of plasma FFA levels after curcuminoid supplementation in high-fat-fed rats in this study.

The present study also shows that curcuminoid supplementation suppressed an increase in fasting blood glucose in high-fat-induced obese rats. This antihyperglycemic effect of curcuminoids could be an indirect action resulting from a decreased influx of plasma FFAs into the liver, thus lowering hepatic glucose production. However, a previous study using alloxan-induced diabetic rats demonstrated that curcuminoids were effective in controlling blood glucose level and enzymes of glucose metabolism [61]. A study using genetically altered type 2 diabetic mice also indicated that one mechanism responsible for the antihyperglycemic effect of curcuminoids was mediated by PPAR- γ activation. In addition to its effect on lipid metabolism, activation of PPAR- γ has been reported to increase the expression and translocation of the glucose transporters GLUT-1 and -4, thus increasing

glucose uptake into liver and skeletal muscle cells and decreasing plasma glucose levels [62].

Effects of curcuminoids on cardiac autonomic nervous system activity

The novel findings in the present study were that curcuminoid supplementation improved cardiac autonomic disturbance in high-fat-induced obese rats. Although the mechanism responsible for cardiac autonomic activity disturbance in high-fat-induced obesity remains unresolved, the results of a significant correlation between plasma FFA levels and HRV could indicate a strong link between FFAs and cardiac autonomic activity. Elevated plasma FFA levels have been associated with an increase of the ventricular premature complex in non-diabetic [63] and non-ischemic diabetic patients [64]. Also, elevated plasma FFA levels have been reported to stimulate cardiac sympathetic activity in healthy subjects [14] and in NIDDM patients [31]. Paolisso et al. [14] suggested that such a stimulatory effect of FFAs on cardiac sympathetic activity was indirect and mediated in part through an increase in plasma catecholamine concentrations, which in turn stimulated myocardial β_1 -receptors. The present results of a negative correlation between plasma FFA levels and the HF component are in agreement with findings demonstrating that elevated plasma FFA levels suppress myocardial vagal tone [15]. It has been suggested that a decrease of parasympathetic counteraction to sympathetic activation results in a shift toward sympathetic dominance [65]. At the cellular level, Shaltout and Abdel-Rahman [15] showed that elevated plasma FFA levels increase caveola sequestration of cardiac muscarinic cholinergic receptors (M_2 -mAChR), which result in muscarinic cholinergic receptor inactivation and attenuation of parasympathetic control on the heart. Based on these findings, the effects of curcuminoids were in part related to a decrease of plasma FFA levels, which in turn led to an improved cardiac autonomic disturbance in high-fat-induced obese rats.

A growing body of evidence has demonstrated that curcuminoids are a potent antioxidant [16,23,24,66]. Increased plasma FFA concentrations have been shown to be a pro-oxidant factor [63]. Manzella et al. [67] demonstrated that increased postprandial plasma FFA concentration is associated with an enhanced degree of oxidative stress and of the LF/HF ratio, an index of cardiac sympathovagal balance. It has also been reported that an impaired cardiac autonomic nervous system activity in type 2 diabetes is linked to elevated oxidative stress. This suggests that changes in cardiac sympathovagal balance correlate with the degree of oxidative stress [67]. Although oxidative stress was not determined in the present study, the additional antioxidant effect of curcuminoids cannot be excluded as a potential mechanism to improve cardiac autonomic nervous system activity in the present study. Future studies are needed to determine whether the effects of curcuminoids on cardiac autonomic activity are direct or indirect.

Study limitations

This study demonstrated that curcuminoid supplementation improved cardiac autonomic disturbance in high-fat-induced obese rats and this was associated with decreased plasma FFA levels. One limitation of this study is that the definite cellular mechanism of curcuminoids lowering plasma FFAs was not investigated. Studies at the cellular level are the next step necessary to provide direct evidence to elucidate the mechanism by which curcuminoids decrease plasma FFAs and its clinical significance. Also, serum epinephrine or norepinephrine was not measured in this study. However, previous studies have indicated that plasma catecholamines are an indirect and rather insensitive index of sympathetic activity because most of the norepinephrine secreted from the sympathetic fibers is destroyed or taken up again, and only a minute fraction escapes from the neuroeffector junctions [68–70]. Moreover, previous human studies examining adrenergic activity in obesity by whole-body methods (i.e., urinary excretion or plasma levels/turnover of catecholamines) have demonstrated inconclusive results, with reports of normal, decreased, or increased sympathetic activity [56,71–75], suggesting that serum catecholamines may not be a reliable indicator for sympathetic activity.

Conclusion

The present study demonstrated that cardiac autonomic disturbance in obesity induced by high-fat feeding is associated with elevated plasma FFA levels. Long-term curcuminoid supplementation decreases plasma FFA levels, thus ameliorating cardiac autonomic disturbance in high-fat-induced obesity. Because elevated plasma FFA levels are an important variable affecting cardiac mortality risk, further studies are needed to clarify the nutritional and therapeutic benefits of curcuminoids.

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ROLES OF CURCUMIN IN PREVENTING PATHOGENESIS OF ALZHEIMER'S DISEASE

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ABSTRACT: *Curcumin is a yellow-orange powder derived from the rhizome of Curcuma longa Linn. In traditional medicine, curcumin has been used to treat several diseases. Much evidence has shown curcumin's anti-ulcer, anti-cancer, anti-hepatotoxic, anti-viral, accelerated wound healing, cardioprotective, hypoglycemic and anti-inflammatory properties. Recently, several in vitro and in vivo studies have shown that curcumin has various properties which help prevent the pathogenesis of Alzheimer's disease (AD). These effects include anti-amyloid, anti-inflammation, antioxidant, anti-apoptotic and cholesterol lowering properties. These findings suggest the possible benefits of curcumin in AD therapy. In this review, the pathogenesis of, as well as current therapeutic strategies for, AD are presented, and evidence of the role of curcumin in preventing the pathogenetic cascades which underlie AD are discussed.*

KEY WORDS: Alzheimer's disease, Anti-amyloid, Anti-apoptotic, Anti-inflammation, Antioxidant, Cholesterol lowering, Curcumin

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1. ALZHEIMER'S DISEASE

Alzheimer's disease (AD), the most common form of dementia in the elderly, is a progressive neurological disease resulting in irreversible neuronal loss, particularly in the brain regions of the cortex and hippocampus. The clinical hallmarks of AD are progressive impairment in memory, judgment, decision making, orientation to surroundings and language (Brion, 1996). Diagnosis of AD is based on clinical neurological examination. A definite diagnosis can be made only by autopsy, in which the pathological hallmarks of AD are neuronal loss, extracellular senile plaques containing the peptide amyloid- β (A β), and neurofibrillary tangles, a hyperphosphorylated form of the microtubular proteins named tau in neurons (Brion, 1996).

Clinical diagnosis of AD is based on criteria developed by the

National Institute of Neurologic and Communicative Disorder and Stroke Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). Clinical AD can be divided into definite AD with a clinical diagnosis with histological confirmation, probable AD with the typical clinical syndrome without histological confirmation, and possible AD with atypical clinical features but no alternative diagnostic appearance and no histological confirmation. The typical symptoms of AD are memory impairment, deterioration of language, and visuospatial deficits. Motor and sensory abnormalities, gait disturbances and seizures are uncommon symptoms, but they can be seen in the late phases of AD (McKhann et al., 1984).

The prevalence of AD in the United State (US) is approximately 1% among people 65 to 69 years old and this prevalence increases to 40-45% among people aged 95 years and over (Hy and Keller, 2000). In 2000, there were 4.5 million people with AD in the US (Hebert et al., 2003). The number of patients with AD in the US is expected to increase almost threefold, to approximately 13.2 million people, by the year 2050 because of the rapid growth of the older population and the longer lifespan of people (Hebert et al., 2003). The AD population will continue to rise unless new methods of AD prevention are discovered. Since the costs of caring for patients with AD are very high, searches for effective therapeutic interventions are necessary. Fillit and colleagues have shown that effective therapies in the early stages of AD can delay the progression of dementia and may offer economic benefits to patients' families, caregivers and society (Fillit and Hill, 2005).

The most common risk factors for AD are aging and a family history of dementia (Thomas and Bruce, 2005). A family history of dementia suggests that genetics is one factor in the pathogenesis of AD. It has been proposed that AD is associated with gender, level of education and numerous environmental factors (Flirski and Sobow, 2005; Thomas and Bruce, 2005). Moreover, some studies suggest that inflammation may have a significant role in the pathogenesis of AD (Flirski and Sobow, 2005; Brion, 1996; in't Veld et al., 2001). It has been shown that the use of non-steroid anti-inflammatory drugs (NSAIDs) is correlated with a decrease in the prevalence of AD symptoms (Giovannini et al., 2003).

Two possible aspects of the pathogenesis of AD are the genetic aspect and neurotransmitter abnormality in the brain (Bird, 2005; Martin, 1999). Several studies have shown that mutations in at least four genes could be involved in the pathogenesis of AD (Bird, 2005; Dufouil et al., 2005; Lendon et al., 1997). Those four genes are the presenilin 1 gene (PS-1), the presenilin 2 gene (PS-2), Amyloid- β protein precursor (APP) and Apolipoprotein $\epsilon 4$ (APOE). Mutations in PS-1 on chromosome 14, PS-2 on chromosome 1, and APP on chromosome 21 have been shown to be involved in the pathogenesis of most cases of familial early onset of AD (Bird, 2005). The genetics of late-onset AD (LOAD) are more mysterious and the only confirmed risk factor for LOAD remains APOE on chromosome 19 (Bird, 2005; Lendon et al., 1997). The APOE gene is involved in cholesterol transport and has three alleles: $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$. The $\epsilon 4$ allele of APOE shows a strong association with AD in the general population, including sporadic and late-onset familial cases of AD (Strittmatter et al., 1993). Approximately 40% of patients with AD have at least one $\epsilon 4$ allele in plasma whereas this allele appears in about 12% of people without AD (Reiss, 2005; Frisoni et al., 1998). Patients diagnosed with clinical AD have significantly higher expression of the $\epsilon 4$ allele in plasma than control (Kuusisto et al., 1994). However, the absence of the $\epsilon 4$ allele does not eliminate the incidence of AD (Refolo et al., 2000; Wu et al., 2003). Dufouil and colleagues demonstrated that the interaction between the level of cholesterol and APOE genotype might influence the progression of AD (Dufouil et al., 2005).

Apart from the genetic etiology, the AD brain can also be affected by neuronal loss, or by the deposit of extracellular senile plaques containing the peptide A β and neurofibrillary tangles. The amyloid- β protein is a small protein produced by APP. The amyloid- β protein is cleaved from APP by enzymes named α , β , and δ -secretase. Normally, the APP is cleaved by α secretase into a 40 amino acid product, A β_{40} . This product is soluble and cleared from the brain. Mutation in the gene for the APP increased cleavage by β , and δ -secretase to form a longer 42 amino acid, A β_{42} (Selkoe, 2000). A β_{42} is a fibrillar amorphous form of amyloid- β protein that is toxic to neurons (Gruys, 2004). Diffuse deposits of A β_{42} cause the formation of amyloid plaques (Cummings, 2004). The chromosome 21 is duplicated in Down's syndrome (Sommer and Henrique-Silva, 2008); it has been proposed that excessive doses of the APP gene on chromosome 21 may be responsible for AD in adult Down's syndrome (Wolvetang et al., 2003).

In addition to the genetic etiology in AD pathogenesis, neurotransmitter abnormality, particularly acetylcholine (ACh), is another possible pathogenesis of AD. ACh is the major neurotransmitter required by the cholinergic system for some forms of learning and memory. ACh is produced by the synthetic enzyme cholineacetyltransferase (ChAT) which uses acetyl coenzyme A and choline as substrates for the formation of ACh. After ACh is released into the synaptic cleft, it is metabolized into choline and acetate by two cholinesterase enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Ellis, 2005). In an AD brain, the amount of AChE is greater than the amount of BuChE (Ellis, 2005; Perry et al., 1978). However, in the late stages of AD,

BuChE activity can increase up to 165% of the normal physiological level (Perry et al., 1978). Up-regulation in levels of AChE and BuChE has been shown to be associated with amyloid plaque deposition (Guillozet et al., 1997). Some evidence suggests that BuChE may accelerate maturation of amyloid plaques which are associated with neuronal degeneration and the severity of AD symptoms (Guillozet et al., 1997). Several studies have also shown that the cognitive deterioration occurring in patients with probable AD is associated with progressive loss of cholinergic neurons and a consequent decline in levels of ACh and ChAT in the temporal lobe, parietal cortex and hippocampus (Guillozet et al., 1997; McKeith, 2002; Tiraboschi et al., 2000; Whitehouse et al., 1981). This evidence has led to the use of AChE inhibitors as a therapeutic drug of choice for AD. Blocking of cholinesterase activity can increase ACh concentration at the central synapses and enhance cholinergic function. Several clinical studies have demonstrated symptomatic improvements in patients treated with cholinesterase inhibitors (Ellis, 2005; Forchetti, 2005; Hebert et al., 2003; Heinrich and Lee, 2004; Lendon et al., 1997).

In addition to these two aspects of AD pathogenesis, there is evidence showing other possible causes of AD, including oxidation and lipid peroxidation, glutamatergic excitotoxicity, inflammation in neurons, and the activation of the apoptotic cell death cascade (Butterfield et al., 2002; Dickson, 2004). This evidence leads investigators to identify new agents for AD treatment including anti-apoptotic agents or glutamatergic N-Methyl-D-aspartate-receptor antagonists (Butterfield et al., 2002; Cummings, 2004).

2. TREATMENT OF ALZHEIMER'S DISEASE

At present, several regimens for AD therapy focus mainly on treatment at target sites along the pathogenetic cascades of AD. The details of the current therapeutic modalities of AD according to its pathogenesis are described below.

2.1 Anti-amyloid treatment for Alzheimer's disease

Vaccination against the amyloid- β protein is considered a new target therapy for AD. Schenk and colleagues reported that immunization with A β_{42} in transgenic mice, which express mutated human amyloid precursor protein under the control of platelet-derived growth factor promoter (PDAPP), reduced pre-aggregated amyloid plaques (Schenk et al., 1999), suggesting that A β_{42} immunization could be effective in the prevention and treatment of AD. Behavior testing in transgenic mice with a radial arm water maze demonstrated that immunization with A β_{42} improved memory (Janus et al., 2000; Morgan et al., 2000). Moreover, it has been shown that the memory improvement in those animals was correlated with the reduction of amyloid burden (Bard et al., 2000; DeMattos et al., 2001).

The first human neuropathological study after A β_{1-42} immunization (AN-1792) in patients with AD showed absent or sparse amyloid plaques in their brains, suggesting that immunization with AN-1792 resulted in the clearance of A β in patients with AD (Nicoll et al., 2003). However, Orgogozo and colleagues reported that 6% of patients receiving AN-1792 developed meningoencephalitis (Orgogozo et al., 2003). Further

studies are needed to evaluate the efficacy of vaccines and their side effects for safety in clinical use for patients with AD.

2.2 Cholinesterase inhibitor for Alzheimer's disease

Cholinergic innervation of the hippocampus is required for some forms of learning and memory. The degeneration of the cholinergic system is one hallmark of AD pathogenesis. Therefore, it is logical to presume that boosting the level of ACh by enhancing the activity of ChAT and/or inhibiting the activity of AChE may improve cognitive function (Poirier, 2005). Currently, the best-developed approach for cholinergic therapy has been the use of cholinesterase inhibitors, which is still one of the most promising therapies for AD.

It has been proposed that an intervention inhibiting both AChE and BuChE may decrease symptoms of dementia in patients with AD (Ballard, 2002). Several AChE inhibitors such as tacrine, donepezil, rivastigmine and galantamine are approved by the U.S. Food and Drug Administration (FDA) for AD treatment (Ballard, 2002). Galantamine and donepezil are selective and rapidly reversible AChE inhibitors, whereas rivastigmine is slowly reversible and inhibits both AChE and BuChE activity (Camps and Munoz-Torrero, 2002). A one-year clinical study indicated that the cholinesterase inhibitors decreased the rate of decline in cognitive test scores compared with subjects in a placebo group (Ellis, 2005). However, none of these cholinesterase inhibitors has been effective for the treatment of patients with AD in advanced stages (Ellis, 2005). Although cholinesterase inhibitors are safe, they can increase ACh levels throughout the body, resulting in cholinergic side effects, including diarrhea, bradycardia and muscle cramps (Kaduszkiewicz et al., 2005). Currently, the search for new AChE inhibitors with minimal side effects is of interest to many investigators (Chattipakorn et al., 2007; Chi et al., 2007; Ingkaninan et al., 2003; Oh et al., 2004; Wang et al., 2006).

2.3 N-methyl-D-aspartate (NMDA) receptor antagonist for Alzheimer's disease

Over stimulation of the N-methyl-D-aspartate (NMDA) receptor by glutamate, manifested as neuronal excitotoxicity, is implicated as one hallmark of AD (Reisberg et al., 2003). Doody and colleagues investigated the effect of memantine, a non-specific competitive antagonist at the glutamatergic NMDA receptor, in patients with AD and found that memantine monotherapy improved cognitive function in moderate stage patients with AD (Doody et al., 2004). Similarly, Forchetti showed that patients with AD, who received memantine together with routine donepezil therapy, have improved cognitive function and behaviors (Forchetti, 2005). These findings suggest that adjunctive memantine therapy with donepezil is effective for the treatment of moderate to severe AD. These regimens can improve cognition, activity in daily living, global function, behavior, and care dependence (Forchetti, 2005). Only one clinical trial in patients with mild stage AD demonstrated a slower progression of AD symptoms after a 28-week therapeutic course with memantine (Rossom et al., 2004). However, this regimen did not prevent global deterioration from AD (Rossom et al., 2004).

All of these findings suggest that memantine may have beneficial effects in patients with moderate to severe AD, but less effect in mild types of AD. Although the beneficial effects of memantine have been demonstrated, its adverse effects, such as dizziness, constipation, cataracts, nausea, dyspnea, confusion, headache, and urinary incontinence, have been documented (Raina et al., 2008; Rossom et al., 2004).

2.4 Hormone replacement therapy for Alzheimer's disease

Observational and longitudinal studies suggest that estrogen has several neuroprotective properties, such as lowering A β formation, enhancing cholinergic function and promoting synaptic plasticity and nerve growth processes (Cummings, 2004; Flirski and Sobow, 2005). These effects of estrogen may attenuate age-associated cognitive impairment or the development of Alzheimer's disease. However, this beneficial effect of estrogen has been demonstrated by only a few randomized clinical trials (Pinkerton and Henderson, 2005; Yoon et al., 2003).

Recently, a randomized open-labeled study showed that the daily life activities of patients with AD significantly improved after estrogen and progesterone replacement therapy and that the improvement was similar to that following tacrine therapy in those patients (Yoon et al., 2003). However, in a prospective placebo-controlled study of combined estrogen-progesterone therapy in asymptomatic postmenopausal women, an increase in the prevalence of dementia was reported (Pinkerton and Henderson, 2005). The discrepancy in results from these two studies may be due to differences in subjects included in each study. Due to this controversy, further investigations on the effects of hormone replacement therapy in patients with AD are warranted to evaluate its efficacy for clinical use.

2.5 Anti-inflammatory agents for Alzheimer's disease

Cultured brain cells are capable of generating inflammatory and immune complements such as inflammatory cytokines, acute phase reactants, and many proteases and protease inhibitors. These molecules appear in association with AD lesions (McGeer and McGeer, 1995). These findings suggest that the pathogenesis of AD is related to the inflammatory process. Therefore, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) may favorably influence the course of the AD.

This suggestion is confirmed by a clinical trial from in't Veld and colleagues in a three-year prospective study of 74 patients and 232 age- and sex-matched controls (in't Veld et al., 1998), demonstrating the correlation between the use of NSAIDs and the prevention of Alzheimer's disease. Similarly, a cohort study of 6989 subjects showed that long-term use of NSAIDs for 24 months might protect against AD (in't Veld et al., 2001). In addition, the administration of NSAIDs (triflusal) for 18 months could slow down the progression of Alzheimer's disease (Gomez-Isla et al., 2008).

In contrast, several studies showed that the use of NSAIDs did not prevent the progression of AD (ADAPT Research Group et al., 2007; Aisen et al., 2003; Arvanitakis et al., 2008; Beard et al., 1998; Kang et al., 2007). Investigators from the Mayo Clinic

found no correlation between the initiation of AD and the use of NSAIDs in the two years preceding the onset of dementia (Beard et al., 1998). Moreover, Aisen and colleagues found that patients with mild to moderate AD who routinely took a selective cyclooxygenase (COX) -2 inhibitor (rofecoxib) or low-dose NSAIDs (naproxen) for 1 year did not slow cognitive decline (Aisen et al., 2003). In addition, the administration of naproxen and celecoxib for 1 year did not prevent the progression of AD (ADAPT Research Group et al., 2007). The discrepancy in these results could be due to differences in types of NSAIDs used, times of exposure to NSAIDs, and study designs. Future studies are needed to define the role of NSAIDs in the treatment of AD.

2.6 Cholesterol lowering agents for Alzheimer's disease

A prospective trial surveying 10,000 people over 65 years of age in three big cities, Dijon, Bordeaux and Montpellier, demonstrated that high cholesterol levels in normal subjects were associated with the APOE genotype, which increased the risk of dementia, and that the use of lipid-lowering agents decreased the risk of dementia (Dufouil et al., 2005; Rodriguez et al., 2002). Wolozin and colleagues investigated the effect of cholesterol lowering agents, statins, on the prevalence of AD (Wolozin et al., 2000). Their study showed that patients who regularly took statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, had a lower incidence of AD than patients who took anti-hypertensive drugs (Wolozin et al., 2000). Recently, an *in vitro* study using primary neuron cultures derived from the Tg2576 mouse model of AD showed that anti-hypertensive drugs (propranolol-HCl, nicardipine-HCl and carvedilol) significantly reduce the accumulation of A β peptides (Wang et al., 2007). However, future *in vivo* studies are needed to confirm this finding.

2.7 Antioxidants for Alzheimer's disease

Oxidative stress may be a factor in the pathogenesis of AD. Antioxidants have been proposed as being beneficial in protecting the brain from oxidative stress (Prasad et al., 2002). Among those antioxidants, vitamin E, or α -tocopherol, has been shown to play an important role in the treatment of moderate to advanced AD (Sano et al., 1997). Dietary supplementation with vitamin E or C in patients with AD has been shown to slow the progression of AD (Grundman et al., 2002; Masaki et al., 2000; Martin, 2003; Pham and Plakogiannis, 2005). Although there are only a few studies confirming the beneficial effects of antioxidants in AD, vitamin E has been commonly recommended to patients with AD since it is inexpensive. Future large randomized clinical trials are needed to warrant its clinical use.

2.8 Herbal medicine for Alzheimer's disease

Although many drugs for AD therapy are available, the drug of choice has not yet been definitively established. Since current available medicines for AD are very expensive, the search for new drugs is of interest to many investigators. Several drugs commonly used today in the treatment of AD were developed from local and traditional medicine, such as galantamine, an AChE inhibitor which was developed from the alkaloids in "Snowdrop"

(Cummings, 2004). Oken and colleagues demonstrated that ginkgo biloba could significantly improve cognitive function in patients with AD, compared to a placebo (Oken et al., 1998). Furthermore, Kanowski and Hoerr showed that ginkgo biloba could reduce the rate of AD development and help to improve cognitive function in patients with AD as well as in patients with vascular dementia (Kanowski and Hoerr, 2003). However, recent findings showed that ginkgo has no beneficial effects for AD (Dekosky et al., 2008; Birks and Evan, 2009). Recently, Ingkaninan and colleagues have shown *in vitro* that ethanol extracts from *Tabernaemontana divaricata* root (TDE) at a concentration of 0.1 mg/ml inhibit more than 90% of AChE activity (Ingkaninan et al., 2003). We also have recently shown that TDE acts as a novel reversible neuronal AChE inhibitor in an animal model and could be beneficial in improving learning and memory (Chattipakorn et al., 2007). Currently, curcumin is another commonly used natural product that has been of interest since it has many beneficial effects, and some of those may provide therapeutic effects in AD prevention and treatment.

3. CURCUMIN

Curcumin is a natural product that has various properties, such as antioxidant, anti-inflammatory and cholesterol-lowering properties (Joe et al., 2004). All of these properties of curcumin may help to prevent AD or to prevent the progression of AD. In India, turmeric, a compound that contains a high level of curcumin, is commonly used in food preparation. Epidemiological study has found that India has one of the lowest prevalence of AD in the world (Chandra et al., 2001), suggesting that curcumin may have beneficial effects in AD therapy. The following paragraphs will describe all studies showing the possible role of curcumin in AD.

3.1 Biological and pharmacological properties of curcumin

Curcuma longa Linn., which belongs to the Zingiberaceae family, is a perennial herb that measures up to 1 m in height, with a short stem, and is distributed throughout tropical and subtropical regions of the world, including India and China (Araujo and Leon, 2001).

Curcuma longa Linn. is commonly known as turmeric. It is recognized by different names in different languages worldwide, such as haldi in Hindi, ukon in Japanese and kha min chan in Thai (Goel et al., 2008). Turmeric is widely consumed for a variety of purposes, including use as a dietary spice, known as curry, a dietary pigment and Indian folk medicine for the treatment of various illnesses. Furthermore, it has been used as an anti-inflammatory and anti-microbial agent for more than 1,000 years in South and Southeast Asia (Lodha and Bagga, 2000).

Turmeric is derived from the rhizome of *Curcuma longa* Linn. and contains polyphenolic yellow pigments known as curcuminoids. The curcuminoids contain 70-75% curcumin (diferuloylmethane), 15-20% demethoxycurcumin and 3% bisdemethoxycurcumin (Aggarwal et al., 2007) (Figure 1). Curcumin is a yellow-orange powder, insoluble in water, ether and aqueous solvents, but it is soluble in organic solvents such as ethanol, dimethylsulfoxide and acetone (Aggarwal et al., 2007). Curcumin was first discovered in 1815 and its chemical name is

1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) or diferuloylmethane (Aggarwal et al., 2007; Goel et al., 2008). The chemical formula of curcumin is $C_{21}H_{20}O_6$ and its molecular weight is 368.38 g/mol.

In traditional medicine, curcumin has been used for the treatment of several diseases such as dementia, cardiovascular diseases, cancer and arthritis (Lodha and Bagga, 2000). In Thailand, curcumin has been used to prevent and relieve rashes, itches, chafing and mosquito bites by topical application on the skin as a natural nonsteroidal anti-inflammatory drug (Aggarwal et al., 2007; Araujo and Leon, 2001; Lodha and Bagga, 2000). Several animal studies have demonstrated curcumin's anti-ulcer (Rafatullah et al., 1990; Swarnakar et al., 2005), anti-cancer (Chen et al., 1996; Cheng et al., 2001; Huang et al., 1994; Li et al., 2002; Lu et al., 1993; Ramachandran and You, 1999; Wu et al., 2000), anti-viral (Li et al., 1993; Mazumder et al., 1995), accelerated wound healing (Sidhu et al., 1998), cardioprotective (Nirmala and Puvanakrishnan, 1996b; Nirmala and Puvanakrishnan, 1996a), hypoglycemic (Arun and Nalini, 2002; Kuroda et al., 2005) and anti-inflammatory effects (Ambegaokar et al., 2003; Joe and Lokesh, 1997; Lal et al., 1999; Lal et al., 2000; Lim et al., 2001). A summary of curcumin's properties is shown in Table 1.

FIGURE 1. The composition of turmeric from the rhizome of *Curcuma longa* Linn.

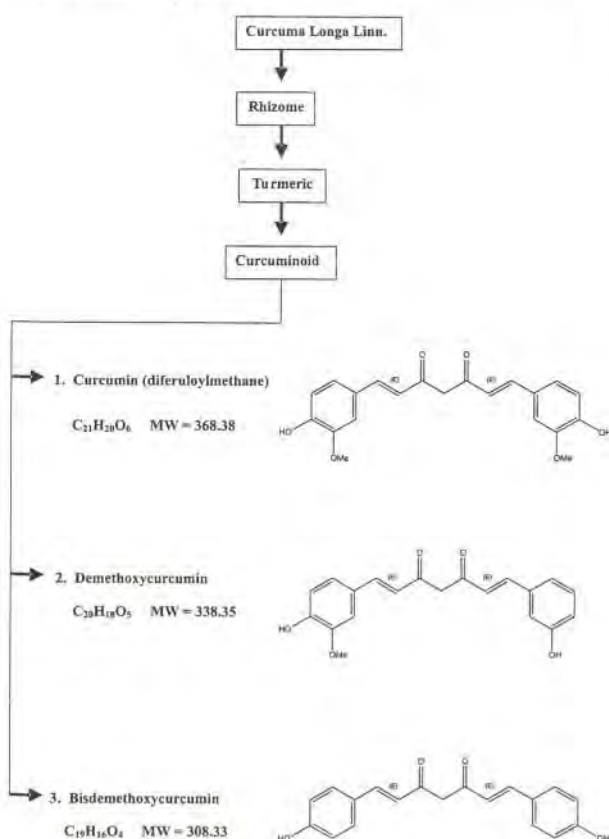


TABLE 1. Summary of curcumin properties

Properties	Studied models / Effective dosage
Anti-ulcer	Rats / 50 mg/kg, p.o. (Rafatullah et al., 1999); 40 mg/kg, i.p. (Swarnakar et al., 2005)
Anti-cancer	Cell culture / 25 μ M (Wu et al., 2000); 60 μ M (Chen et al., 1996); 20 & 40 μ M (Ramachandran and You, 1999) Hamsters / 10 mmol, topical (Li et al., 2002) Mice / 0.5-4.0%, p.o. (Huang et al., 1994); 1, 3 & 10 μ M, topical (Lu et al., 1993) Humans / 500-8000 mg/day, p.o. (Cheng et al., 2001)
Anti-viral	Cell culture / 2, 5 & 10 μ M (Li et al., 1993); 40 μ M (Mazumder et al., 1995)
Accelerated wound healing	Rats and Guinea pigs / 40 mg/kg, p.o. (Sidhu et al., 1998)
Cardioprotective	Rats / 200 mg/kg, p.o. (Nirmala and Puvanakrishnan, 1996a; 1996b)
Hypoglycemic	Mice / 200 & 1000 mg / 100 g diet (Kuroda et al., 2005) Rats / 80 mg/kg, p.o. (Arun and Nalini, 2002)
Anti-inflammatory	Cell culture / 4, 5, 10, 15 & 20 μ M (Ambegaokar et al., 2003; Joe and Lokesh, 1997) Mice 160 & 5000 ppm, p.o. (Lim et al., 2001), Humans / 375 mg / 3 times/day, p.o. (Lal et al., 1999; Lal et al., 2000)
Anti-amyloid	Cell culture / 0.1-1 μ M (Ono et al., 2004) Mice / 160, 500 & 5000 ppm, p.o. (Lim et al., 2001, Yang et al., 2005); 7.5 mg/kg, i.v. (Garcia-Alloza et al., 2007)
Cholesterol-lowering	Mice / 4g/kg, p.o. (Kamal-Eldin et al., 2000)
Antioxidant	Cell culture / 75 & 250 μ M (Reddy and Lokesh, 1996), Rats / 30 mg/kg (Reddy and Lokesh, 1996); 1g/kg (Wahlstrom and Blennow, 1978); 0.1% (Kaul and Krishnakantha, 1997); 2%, p.o. (Sharma et al., 2001)
Anti-apoptotic	Cell culture / 2.5, 50, 100 & 200 μ M (Zhu et al., 2004); 20 & 60 μ M (Moos et al., 2004)

3.2. Possible roles of curcumin in Alzheimer's disease

Strong evidence from several studies suggests that amyloid plaques, oxidative stress, neuronal inflammation, and neuronal apoptosis are involved in the pathogenesis of AD (Cummings, 2004; Kermer and Liman, 2004). Recently, several studies *in vitro* and *in vivo* showed that curcumin has various properties which may possibly prevent the pathogenesis of AD, such as anti-amyloid, anti-inflammation, cholesterol-lowering, antioxidant, and anti-apoptotic properties (Aggarwal et al., 2007; Goel et al., 2008; Joe et al., 2004). The details of these benefits are described in the following sections and summarized in Figure 2.

3.2.1 Anti-amyloid properties of curcumin

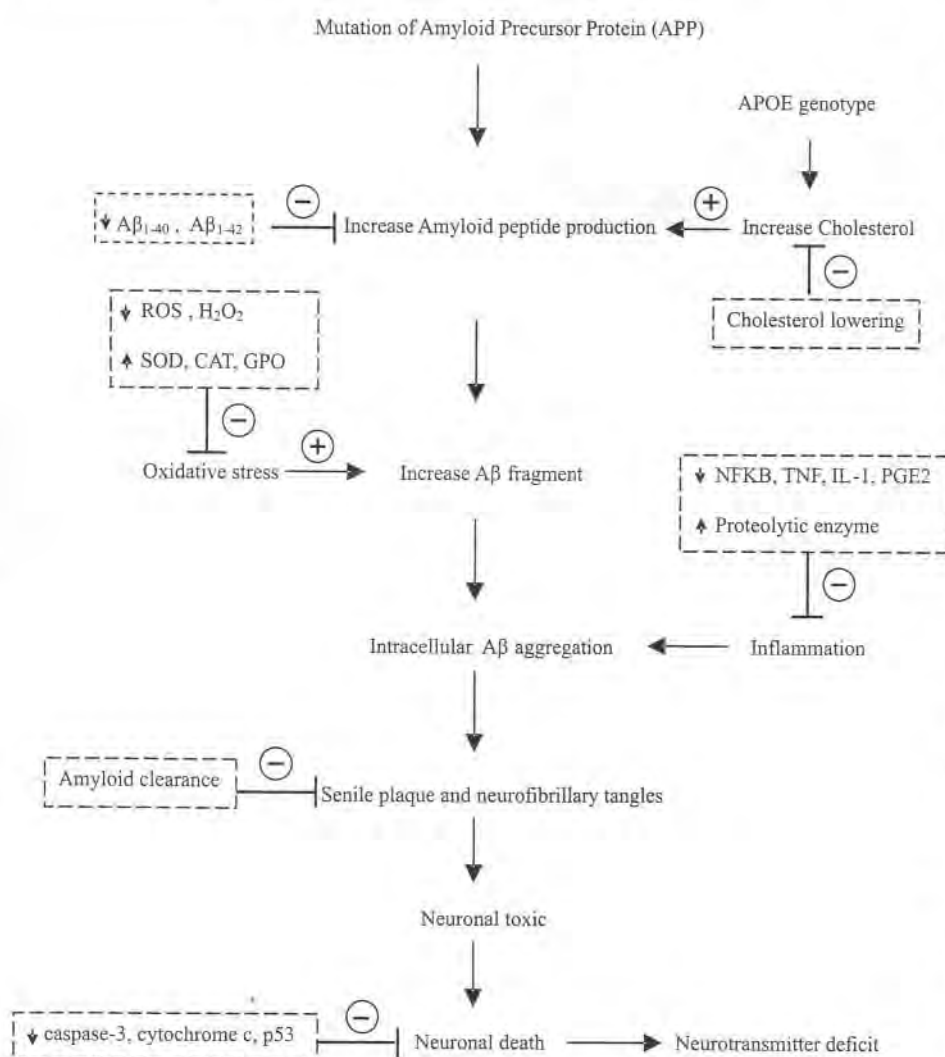
Since the accumulation of A β is one hallmark of the pathogenesis of AD, the destabilization of A β would be an attractive therapeutic target for AD treatment. Evidence of curcumin's anti-amyloid properties has been shown in both *in vivo* and *in vitro* studies.

Using fluorescence spectroscopic analysis with thioflavin T and an electron microscope in an *in vitro* study, Ono and colleagues demonstrated that curcumin inhibited A β formation, extension and destabilization from A β_{1-40} and A β_{1-42} in a dose-dependent manner (Ono et al., 2004). This effect of curcumin has been shown to be due to the molecular structure of curcumin, which is potent in A β binding (Ringman et al., 2005). Curcumin was

shown to inhibit aggregation as well as disaggregated fibrillar $A\beta_{40}$, indicating that it has favorable stoichiometry for $A\beta$ inhibition (Yang et al., 2005).

Another possible mechanism in the inhibition of $A\beta$ aggregation is related to macrophages. An *in vitro* study showed that AD patients have decreased clearance of amyloid plaques and this defect is related to the decreased phagocytosis of $A\beta_{1-42}$ by the innate immune cells, monocyte or macrophages (Zhang et al., 2006). Zhang and colleagues demonstrated that the $A\beta$ uptake by macrophages was significantly increased after macrophages were treated with curcuminoids, suggesting that curcuminoids may improve immune clearance of amyloid in the AD-affected brain (Zhang et al., 2006).

FIGURE 2. Possible roles of curcumin in Alzheimer's disease. The pathogenic cascade of Alzheimer's disease is multifactorial, including genetics, oxidative stress, inflammation, and infection. The possible effect of curcumin, as indicated in the dashed boxes, may interrupt the developing Alzheimer's disease in multiple steps. ⊕: Stimulating process, ⊖: Inhibiting process, NFκB: Nuclear factor-κB, TNF: Tumor necrosis factor, IL-1: Interleukin-1, PGE-2: Prostaglandin 2, ROS: Reactive oxygen species, SOD: Superoxide dismutase, CAT: Catalase, GPO: Glutathione peroxidase



The effect of curcumin on anti-amyloid formation was also investigated in several animal models of AD, such as the APPswe/PS1dE9 mouse model and the APPSw (Tg2576) mouse model (Garcia-Alloza et al., 2007; Lim et al., 2001; Yang et al., 2005). The study in aged Tg 2576 mice which have $A\beta$ accumulation showed that the administration of curcumin reduced amyloid levels and plaque burden by directly binding to small $A\beta$ species (Lim et al., 2001; Yang et al., 2005). In addition, low dose consumption of dietary curcumin (160 part per million (ppm)) reduced soluble and insoluble $A\beta$ and plaque burden in aged TG 2576 mice (Lim et al., 2001; Ringman et al., 2005; Yang et al., 2005). Recently, a study using multiphoton microscopy and longitudinal imaging to evaluate the real-time effect of systemic curcumin administration on existing $A\beta$ deposits in aged APPswe/PS1dE9 transgenic mice found that curcumin (7.5 mg/kg) reduced plaques and partially restored the altered neurite structure near, and away from, plaques (Garcia-Alloza et al., 2007). These findings suggest that curcumin has beneficial effects in reducing the $A\beta$ accumulation and neurotoxicity along the pathogenic cascade of AD in transgenic mice. Future clinical studies are warranted to evaluate the clinical significance of curcumin.

Apolipoprotein E (APOE) is a plasma protein involved in cholesterol transport (Mahley, 1988). The APOE-knockout mice showed a reduction of amyloid plaques (Bales et al., 1997). Evidence suggests that APOE is one factor involved in the formation of the pathologic form of amyloid plaque in AD because APOE is bound to extracellular senile plaques and to intracellular neurofibrillary tangles (Namba et al., 1991). However, Koistinaho and colleagues showed that APOE could down-regulate APP-induced ischemic susceptibility (Koistinaho et al., 2002). Thus, it is not yet known if APOE is a beneficial or harmful factor in AD. Furthermore, the secretion of APOE requires protein prenylation. It has been shown that monoterpene, curcumin derivative, inhibits the protein prenylation (Chen et al., 1997), which suggest that curcumin should have beneficial effect for AD. However, Cheung and colleagues used dot-blotting and immunoblotting to analyze the effect of curcumin on the level of APOE in a mouse microglial cell line (Cheung et al., 2007). They found that curcumin at physiologically attainable concentrations in the submicromolar to low micromolar ranges can increase the secretion

of APOE. On the other hand, at concentrations over 6.25 μ M, which have been demonstrated to cause cell toxicity, curcumin reduced APOE secretion (Cheung et al., 2007). All of these findings indicate that further investigations regarding the effects of curcumin are needed to warrant its possible role in AD treatment.

3.2.2 Anti-inflammatory properties of curcumin

Several studies have suggested that curcumin has an anti-inflammatory effect similar to that of NSAIDs. Since inflammatory processes, including increased concentrations of cytokines, have been found in the AD-affected brain (Brion, 1996), it is logical to assume that the use of anti-inflammatory drugs, such as NSAIDs, may decrease the risk of AD.

The effect of curcumin on inflammation has been investigated in several cell types such as cancer cells and bone marrow cells (Chan et al., 1998; Lev-Ari et al., 2008). However, its effect on microglia, neuroglia, astrocytes and oligodendrocytes is little known. By using C-6 rat glioma, 2B-clone cells and a mixed colony of both neuroglial cells, Ambegaokar and colleagues found that curcumin inhibits the proliferation of microglia and astrocyte gliosis (Ambegaokar et al., 2003). In addition, Xu and colleagues found that a low dose of curcumin inhibited AP-1 and nuclear factor-kB (NFkB)-mediated transcription of inflammatory cytokines and cyclo-oxygenase-2 (Xu et al., 1997). These findings suggest that curcumin can inhibit inflammation at several levels of the inflammatory process.

The effect of curcumin on the early events in the course of inflammation was investigated in a human monocytic macrophage cell line. It has been demonstrated that curcumin inhibits lipopolysaccharide-induced production of tumor necrosis factor (TNF), interleukin (IL)-1, cyclo-oxygenase, lipoxigenases, prostaglandin E2 and leukotriene in cell lines (Chan, 1995; Kohli and Ali, 2005). Curcumin could also induce the release of proteolytic enzymes such as collagenase, elastase, and hyaluronidase from activated macrophages (Joe and Lokesh, 1997). In Tg2576 mice (Lim et al., 2001), low and high concentrations of curcumin significantly decreased oxidized proteins and interleukin-1 β , a pro-inflammatory cytokine which is normally elevated in the brains of mice with AD (Lim et al., 2001).

These anti-inflammatory effects of curcumin, as summarized in Table 2, indicate that curcumin exhibits an anti-inflammatory effect similar to that of NSAIDs. The advantage of curcumin over NSAIDs is that it has fewer or no known side effects. As a result, curcumin may be beneficial for the treatment of inflammatory disease, as well as in AD, in which inflammation is an important part of its pathogenetic cascades.

3.2.3 Cholesterol lowering properties of curcumin

It is known that the level of cholesterol is associated with amyloid- β deposits in the brain (Sparks, 1997). In an *in vivo* study, Refolo and

colleagues demonstrated that the administration of a cholesterol-lowering drug (BM15.766) in mice for five weeks reduced plasma cholesterol, $A\beta_{1-40}$ and $A\beta_{1-42}$ accumulation in the brain (Refolo et al., 2001). This study suggests that lowering cholesterol by pharmacological treatment may be an effective approach for reducing the risk of developing AD. Similarly, the consumption of curcumin (4 g/kg/day) in mice for four weeks significantly reduced low density lipoprotein and very low density lipoprotein levels in plasma and the total cholesterol level in the liver (Kamal-Eldin et al., 2000). Furthermore, curcumin also inhibited the formation and extension of $A\beta$ and destabilization of $A\beta_{1-40}$ and $A\beta_{1-42}$ in a dose-dependent manner (Ono et al., 2004). All of these findings suggest that curcumin may possibly reduce $A\beta$ -formation similar to BM15.766. However, studies directly investigating the effects of curcumin on cholesterol and $A\beta$ reduction are warranted to evaluate its clinical significance.

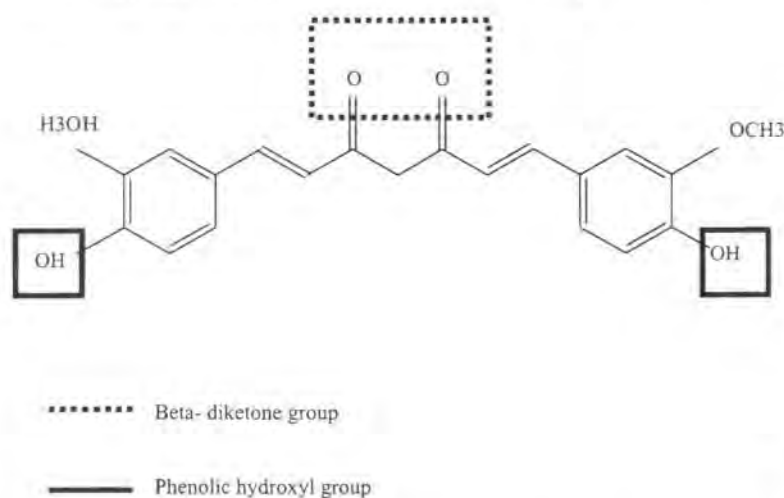
3.2.4 Antioxidant properties of curcumin

Oxidative stress plays an important role in the pathogenesis of AD (Brion, 1996; Cummings, 2004). Epidemiological studies demonstrated that consumption of antioxidant agents, such as curcumin, could reduce the risk of AD (Dunsmore et al., 2001; Goel et al., 2008; Lim et al., 2001). The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and an enol form of β -diketone, and these structures show typical radical-trapping ability as a chain-breaking antioxidant agent (Dunsmore et al., 2001; Goel et al., 2008; Ringman et al., 2005) (Figure 3). It is known that the generation of reactive oxygen species (ROS), H_2O_2 and nitrite radicals can activate macrophages to initiate inflammation (Marriott et al., 2008; Tasaka et al., 2008). Curcumin has been shown to significantly inhibit the generation of these free radicals (Joe and Lokesh, 1997). Dunsmore and colleagues have demonstrated that the antioxidant effect of curcumin could be due to its induction of heat shock protein (Dunsmore et al., 2001), proteins serving as molecular chaperones involved in the protection of cells from various forms of stress (Calabrese et al., 2003).

TABLE 2 . Summary of anti - inflammatory effect of curcumin.

Anti-inflammatory effects	Studied models
Inhibiting nuclear factor -kB (NFkB)	Bone marrow stromal cells (Xu et al., 1997)
Inhibiting tumor necrosis factor (TNF), interleukins (IL)-1, cyclo -oxy genase, lipoxigenases, prostaglandin E2 and leukotriene	Human monocytic macrophage cell lines (Chan, 1995; Kohli and Ali, 2005)
Releasing of proteolytic enzymes such as collagenase, elastase, and hyaluronidase	Rat peritoneal macrophage cells (Joe and Lokesh, 1997)

FIGURE 3. Chemical structural formula of curcumin, which includes two phenolic hydroxyl and beta-diketone groups, these structures show typical radical-trapping ability as a chain-breaking antioxidant agent.



In an *in vivo* study Reddy and Lokesh (Reddy and Lokesh, 1996) demonstrated that the oral administration of 30 mg/kg/day curcumin for 10 days in rats could decrease iron-induced hepatic damage by lowering lipid peroxidation and protecting renal and neural glial cells from oxidative stress. Rats who consumed 0.1% curcumin for three weeks showed the lowering of lipid peroxidation by 12.5–22.6% in the liver, 23.7–24.1% in the kidney, 14.4–18.0% in the spleen, and 16.0–31.4% in the brain (Kaul and Krishnakantha, 1997). Moreover, phenolic substances in curcumin also enhance antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPO) (Sharma et al., 2001; Wahlstrom and Blennow, 1978).

Metals can induce A β aggregation and cell toxicity, and are concentrated in the AD-affected brain (Tougu et al., 2008). It has been reported that metal chelation may reduce A β aggregation or oxidative neurotoxicity. Desferrioxamine and clioquinol, well-known metal chelators, have been shown to exhibit anti-AD effects (Smith et al., 2007). Baum and Ng found that curcumin has a metal chelating property (Baum and Ng, 2004). They found that curcumin has an affinity for copper, zinc, and iron ions, and binds the redox-active metals iron and copper rather than redox-inactive zinc. Therefore, curcumin might exert a net protective effect against A β aggregation via antioxidant activity and metal chelation (Baum and Ng, 2004). Future studies are needed to validate this hypothesis.

All of these antioxidant effects of curcumin, including radical-trapping structure (β -diketone), increased production of heat shock protein, and increased metal chelator properties, indicate that this natural product may be a good anti-oxidant agent that might be beneficial in AD treatment.

3.2.5 Anti-apoptotic properties of curcumin

Apoptosis is a type of cell death process and is involved in both physiological and pathological processes of AD (Flirski and Sobow, 2005). Reactive oxygen species (ROS) or other stimuli can lead to

a release of cytochrome C from mitochondria, which can play a key role in caspases' activation of apoptotic signals (Annunziato et al., 2003; Bobba et al., 2004).

Zhu and colleagues investigated the effect of curcumin on apoptosis in primary cortical rat culture and showed that curcumin increases cell viability and decreases cell apoptosis (Zhu et al., 2004). The mechanisms of reduced apoptosis by curcumin include blocking caspase-3, altering the expression of Bcl-2 family protein, lowering reactive oxygen species (ROS), lowering cytochrome C release (Chan, 1995) and disrupting the conformation of p53 (Moos et al., 2004). Since apoptosis is involved in the pathophysiologic process of AD, curcumin may be useful in AD therapy through its anti-apoptotic activity.

4. CLINICAL STUDIES OF CURCUMIN AND AD

Although many *in vivo* and *in vitro* studies confirm the effectiveness of curcumin against AD pathology via multiple pathophysiological cascades, there are currently only a few clinical studies investigating the effects of curcumin in AD.

A controlled randomized clinical trial investigating the effects of curcumin on the lipid profile biomarkers of AD and the cognitive scale in elderly subjects with a six-month follow-up reported that curcumin consumption does not affect serum isoprostane, amyloid- β protein, lipid profile or cognitive scale (Baum et al., 2007; Baum et al., 2008). However, the serum A β tended to increase, which might suggest that curcumin disrupts A β deposits in brain and the released A β then leaves the brain into the serum, where it is detected (Baum et al., 2008). Furthermore, the level of vitamin E was raised in this study, indicating that curcumin possibly slows down AD progression by an antioxidant mechanism (Baum et al., 2008). Currently, there are ongoing clinical trials in patients with mild to moderate AD, investigating the effects of curcumin on changes in the levels of biological markers such as cholesterol level, isoprostanes, alpha-1-anti-chymotrypsin, C-reactive protein, tau, A β_{1-40} and A β_{1-42} , and in cognitive scale after 2 or 4 g/day of curcumin consumption for 6–12 months (Kelley and Knopman, 2008; Ringman et al., 2005). This clinical trial is still ongoing; therefore, the results are not yet known. The safety and tolerability of these two different doses of curcumin are also being investigated in these trials (Kelley and Knopman, 2008; Ringman et al., 2005).

In nondemented subjects (Ng et al., 2006), Ng and colleagues reported that subjects who consumed curry occasionally (once or more in six months but less often than once a month), often (once or more a month but less than once a week), or very often (once a week or more, or daily), had significantly better scores on the Mini-Mental State Examination (MMSE), a widely used instrument that provides a global measure of cognitive function, than subjects who never or rarely (never or less than once in 6 months) consumed curry (Ng et al., 2006). These findings suggest that curry consumption may improve cognitive performance in nondemented subjects.

5. SAFETY AND TOXICITY OF CURCUMIN

Studies in animal models have shown that administration of

maximal doses of curcumin (5g/kg) does not have toxic effects (Wahlstrom and Blennow, 1978). Similarly, no toxicity of curcumin has been observed in rats fed 1.2g/kg curcumin for 14 days (Sharma et al., 2001). Also, the safety of curcumin has been confirmed in a clinical study (Chainani-Wu, 2003). Curcumin has been shown to be safe in six clinical trials, in which one trial used 8 g of curcumin per day for three months and the other five trials used 1.125-2.5 g per day (Chainani-Wu, 2003). In addition, curcumin is currently listed as a coloring and flavoring agent in food by the United States Food and Drug Administration (FDA) with only mild adverse side effects, such as gastric irritation and nausea (Abebe, 2002).

Although many studies have shown that curcumin has an anti-cancer property, Frank and colleagues' study did not support this finding. Their study demonstrated that consumption of 0.5% curcumin in Long-Evans Cinnamon (LEC) rats, a model for human Wilson's disease, in which chronic hepatitis and liver tumors develop, did not alter the rate of acute liver failure or the incidence of liver or kidney tumors. The lack of an anti-cancer property of curcumin may be related to enhanced oxidative stress by excess copper in this model (Frank et al., 2003). In addition, Sakano and Kawanishi demonstrated that curcumin could induce DNA fragmentation and base damage in the presence of copper and isozymes of cytochrome p450(CYP) (Sakano and Kawanishi, 2002). This finding indicated that 1-20 mM curcumin may exhibit carcinogenic potential through oxidative DNA damage by its metabolite. Furthermore, curcumin could induce the accumulation of tumor-inducing DNA damage in the colon (Moos et al., 2004). This damage was mediated by inhibiting p53 function, a critical protein in the protection against genomic stress (Moos et al., 2004). Moreover, Yoshino and colleagues demonstrated that 10-20 mM curcumin could act as a pro-oxidant agent in the cell with the presence of transitional metals and may induce DNA damage and apoptotic cell death (Yoshino et al., 2004). In humans, a few studies have shown that curcumin induces allergic dermatitis and urticaria (Hata et al., 1997; Liddle et al., 2006; Swierczynska and Krecisz, 1998). Further *in vivo* and clinical studies are necessary to examine these adverse effects of curcumin.

CONCLUSIONS

Various benefits of curcumin such as anti-amyloid, anti-inflammatory, antioxidant, anti-apoptotic and cholesterol-lowering properties may help to prevent the pathogenesis of AD. Furthermore, due to its low cost with very low or no side effects, curcumin could be a key agent for the development of a new drug for AD. Further clinical trials are required to evaluate its clinical significance in AD treatment.

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