รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของ SB203580 ต่อการทำงานทาสรีรวิทยาและชีวเคมีของหัวใจ เมื่ออยู่ในภาวะกล้ามเนื้อหัวใจขาดเลือด

Effect of p38 inhibitor, SB203580, on cardiac physiological and biochemical functions during myocardial ischemia/reperfusion injury

โดย ผู้ช่วยศาสตราจารย์ ดร.สราวุธ คำปวน และคณะ

มิถุนายน 2556

สัญญา

เลขที่.....

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มหาวิทยาลัยนเรศวร

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บทคัดย่อ

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ชื่อนักวิจัย และสถาบัน

ผู้ช่วยศาสตราจารย์ ดร. สราวุธ คำปวน
ภาควิชาเทคนิคการแพทย์ คณะสหเวชศาสตร์
มหาวิทยาลัยนเรศวร

อีเมล์: sarawutk@nu.ac.th / swthek@gmail.com

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บทคัดย่อ:

ไมโตเจน-แอคทิเวทเตทโปรตีนไคเนส (MAPKs) มีบทบาทสำคัญในการบาดเจ็บเนื่องจากภาวะกล้ามเนื้อหัวใจขาดเลือดหรือการทำให้เลือดกลับมาเลี้ยงหัวใจอีกครั้ง โดยจากการศึกษาที่ผ่านมาการยับยั้งการกระตุ้น p38 MAPK ก่อนการเกิดภาวะหัวใจขาดเลือด หรือ ภาวะเลือดกลับมาเลี้ยงหัวใจอีกครั้ง สามารถป้องกันอันตรายที่เกิดกับหัวใจได้ อย่างไรก็ตามการยับยั้ง p38 MAPK ในระหว่างกล้ามเนื้อหัวใจขาดเลือด หรือในระหว่างการทำให้เลือดกลับมาเลี้ยงหัวใจอีกครั้งยังไม่เป็นที่ทราบแน่ชัด ในการศึกษานี้จึงได้ทดสอบสมมติฐานว่าการยับยั้ง p38 MAPK ในเวลาที่แตกต่างกันระหว่างการเกิดภาวะกล้ามเนื้อหัวใจขาดเลือด สามารถป้องกันภาวะหัวใจเต้นผิดจังหวะ, ลดกล้ามเนื้อหัวใจตาย, ลดความผิดปกติของหัวใจห้องล่างและการทำงานของไมโตคอนเดรียได้ การทดลองทำโดยนำหนูขาวเพศผู้สายพันธุ์วิสตาร์มาทำการมัดเส้นเลือดที่ไปเลี้ยงกล้ามเนื้อหัวใจห้องซ้ายด้านหน้าเป็นเวลา 30 นาที และทำให้เลือดกลับมาเลี้ยงกล้ามเนื้อหัวใจอีกครั้งเป็นเวลา 120 นาที โดยมีการให้ตัวยับยั้ง p38 MAPK คือ SB203580 ก่อนภาวะกล้ามเนื้อหัวใจขาดเลือด, ระหว่างกล้ามเนื้อหัวใจขาดเลือด หรือจุดเริ่มต้นของภาวะเลือดกลับมาเลี้ยงหัวใจอีกครั้ง ผลการทดลองพบว่าการยับยั้งการทำงานของ p38 MAPK ด้วย SB203580 โดยการให้ก่อนหรือระหว่างภาวะกล้ามเนื้อหัวใจขาดเลือด สามารถลดภาวะหัวใจเต้นผิดปกติและลดการเกิดภาวะหัวใจห้องล่างเต้นเร็วหรือผิดจังหวะชนิดร้ายแรงได้ แต่ไม่มีผลเมื่อให้ตัวยับยั้ง ณ ขณะเริ่มต้นของภาวะเลือดกลับมาเลี้ยงหัวใจอีกครั้ง นอกจากนั้นการให้ SB203580 ก่อนหรือระหว่างภาวะกล้ามเนื้อหัวใจขาดเลือดสามารถลดการกระตุ้น HSP27 และเพิ่มการกระตุ้น Cx43 อย่างมีนัยสำคัญ นอกจากนี้พบว่าบริเวณของกล้ามเนื้อหัวใจตายและระดับของ cytochrome *c* ลดลงอย่างมีนัยสำคัญในทุกกลุ่มที่ได้รับ

SB203580 ในทุกช่วงเวลาของการให้ SB203580 การศึกษาผลของ SB203580 ต่อการทำงานของไมโตคอนเดรียที่แยกจากกล้ามเนื้อหัวใจพบว่า การได้รับ SB203580 สามารถลดการบวมของไมโตคอนเดรียและลดการสร้างอนุมูลอิสระจากไมโตคอนเดรียได้ โดยเฉพาะอย่างยิ่งในหนูกลุ่มที่ได้รับยาก่อนภาวะกล้ามเนื้อหัวใจขาดเลือด อย่างไรก็ตามการให้ SB203580 ก่อนและระหว่างการทำให้กล้ามเนื้อหัวใจขาดเลือดเท่านั้นที่สามารถลดการเปลี่ยนแปลงของความต่างศักย์ไฟฟ้าที่ผนังเซลล์ของไมโตคอนเดรียได้อย่างมีนัยสำคัญ การได้รับ SB203580 พบว่าสามารถลดความเสียสภาพของโครงสร้างภายในไมโตคอนเดรียได้ จากการศึกษาพบว่าการให้ SB203580 ก่อนภาวะกล้ามเนื้อหัวใจขาดเลือดสามารถลดระดับ phosphorylated-p38 MAPK, HSP27, p53, CREB, และ α-B-crystalline ลดระดับการแสดงออกของโปรตีนที่ควบคุมกระบวนการ apoptosis ได้แก่ Bax แต่ไม่พบการเปลี่ยนแปลงการแสดงออกของ Bcl-2 ลดระดับของ cytochrome c และ caspase 3 อย่างไรก็ตาม การได้รับ SB203580 ณ ขณะเริ่มต้นของภาวะเลือดกลับมาเลี้ยงหัวใจอีกครั้ง สามารถยับยั้งการกระตุ้น CREB และ α-B-crystalline ได้แต่ไม่มีผลต่อการแสดงออกของโปรตีนที่ควบคุมกระบวนการ apoptosis จากการศึกษานี้แสดงให้เห็นว่าระยะเวลาของการยับยั้ง p38 MAPK ในภาวะกล้ามเนื้อหัวใจขาดเลือดมีความสำคัญต่อประสิทธิภาพในการป้องกันการเสียหายของหัวใจในภาวะกล้ามเนื้อหัวใจขาดเลือด และ ภาวะเลือดกลับมาเลี้ยงหัวใจอีกครั้ง

คำหลัก : p38 MAPK, SB203580, ภาวะกล้ามเนื้อหัวใจขาดเลือด, ภาวะหัวใจเต้นผิดปกติ, การทำงานของไมโตคอนเดรีย

Abstract

Project Code : MRG5480017

Project Title : Effect of p38 inhibitor, SB203580, on cardiac physiological and biochemical functions during myocardial ischemia/reperfusion injury.

Investigator : Assistant Professor Dr. Sarawut Kumphune

Department of Medical Technology

Faculty of Allied Health Sciences, Naresuan University

E-mail Address : sarawutk@nu.ac.th / swthek@gmail.com

Project Period : 24 months

Abstract:

The mitogen-activated protein kinases (MAPKs) play an important role in ischemia/reperfusion (I/R) injury. Previous evidence suggests that p38 MAPK inhibition before ischemia is cardioprotective. However, whether p38 MAPK inhibition during ischemia or reperfusion provides cardioprotection is not well known. We tested the hypothesis that p38MAPK inhibition at different times during I/R protects the heart from arrhythmias, reduces the infarct size, attenuates ventricular dysfunction, and reduced I/R induced cardiac mitochondrial dysfunction. Adult Wistar rats were subject to a 30-minute left anterior descending coronary artery occlusion, followed by a 120-minute reperfusion. A p38 MAPK inhibitor, SB203580, was given intravenously before left anterior descending coronary artery occlusion, during ischemia, or at the onset of reperfusion. The results showed that SB203580 given either before or during ischemia, but not at the onset of reperfusion, decreased the ventricular tachycardia/ventricular fibrillation (VT/VF) incidence and heat shock protein 27 phosphorylation, and increased connexin 43 phosphorylation. The infarct size and cytochrome c level was decreased in all SB203580-treated rats. The ventricular function was improved only in SB203580-pretreated rats. Administration of SB203580 at any time point of I/R injury significantly attenuated the ROS generation and cardiac mitochondrial swelling. However, SB203580 given at the onset of reperfusion failed to improve mitochondrial membrane

potential change. Moreover, administration of SB203580 at any time point protected I/R injury induced mitochondrial ultrastructure rupture. In addition, pre-treatment of SB203580 significantly reduced the phosphorylation of p38 MAPK, HSP27, p53, CREB, and α-B-crystalline, decreased Bax expression without any changes in Bcl2 expression, and reduced cytochrome c and cleaved caspase 3 levels. However, SB203580 given at the onset of reperfusion could only inhibit the phosphorylation of CREB and α-B-crystalline, without an effect on apoptotic regulatory proteins. These findings suggest that timing of p38 MAPK inhibition with respect to onset of ischemia is an important determinant of therapeutic efficacy and cardiac mitochondria protection.

Keywords : p38 MAPK, SB203580, ischemia/reperfusion injury, arrhythmias, mitochondrial functions

Executive summary

Ischemic Heart Disease is predicted to be the major cause of death among the population around the world in 2030. This phenomenon seems to be happened everywhere in the world, not only in developed countries. Some recent studies showed that ischemic heart disease has become one of the major causes of death in Thai population.

Myocardial ischemia therefore exists when the reduction of coronary flow is so severe that the supply of oxygen to the myocardium is inadequate for the oxygen demands of the tissue. This results in the accumulation of metabolites in the ischemic region. Overwhelming severe and prolonged ischemia finally results in cellular necrosis. It has been known that myocardial ischemia is a good stimulant of 38kDa mitogen activated protein kinase (p38 MAPK). Activation of this p38-MAPK resulted in many cellular responses, which predominantly cause cellular necrosis. Moreover, it has been demonstrated that ischemia followed by reperfusion results in more profound p38-MAPK activation and also plays a more significant role in subsequent myocardial injury. Therefore, it has been thought that inhibition of p38-MAPK in myocardial ischemia/reperfusion could have therapeutic potential. This idea is supported by the evidence that inhibition of p38-MAPK activation and its activity by pharmacological inhibitor, such as SB203580, could reduce the infarct size and improve cardiac function. Although the knowledge that p38 activation is aggravated by myocardial ischemia/reperfusion injury, there are some evidence lighted up the contrary data suggested that activation of p38 is beneficial and protect the heart from ischemic damage. However, the advantage of p38 activation seems to less convincing according to the lesser number of publications that supported the idea. Finally, there is an attempt to use the p38 MAPK inhibitor as therapeutic drug, which is now entering the pre-clinical study for acute coronary syndrome.

The information that demonstrated beneficial outcome of p38 inhibition using pharmacological inhibitors was mostly come from the experiment with pre-ischemic treatment of inhibitor. Nevertheless, the role of p38-MAPK activation and the consequences of its inhibition in postischemic and reperfusion period, especially in an *in vivo* model, have not been intensively determined. While having insufficient information, it must be aware that the inhibitor drug is not safe enough to use in real patients. Therefore, it will be more clinically useful to know if inhibition of p38-MAPK activation at postischemic state such as at reperfusion period will provide cardioprotective effect. Intensive studies in an *in vivo* model will provide some useful information closely related to the actual physiological events occur in the body.

Objectives

1. To determine the effect of SB203580 on cardiac physiology, functions, and sensitivity to infarction, in rat hearts subjected to *in vivo* ischemia/reperfusion.
2. To determine the effect of SB203580 on intracellular biochemical alterations in rat heart subjected to *in vivo* ischemia/reperfusion.
3. To determine the mechanism and protective effect of SB203580 on mitochondrial function after *in vivo* ischemia/reperfusion injury

Research Methodology

1. Preparation of rat myocardial ischemia/reperfusion (I/R)

Rats were anesthetized using an intramuscular injection of zolitil (50 mg/kg) and xylazine (0.15 ml/kg). To assess the adequacy of anesthesia, the parameters such as responsiveness, blood pressure and heart rate (HR) were monitored throughout the surgical procedures. Tracheostomy was performed, and the rat was ventilated with room air from a positive ventilator (Harvard Apparatus, model 683, Massachusetts, USA), which was started immediately with a tidal volume of 8-10% of body weight and ventilator rate of 70-110 breaths/min to maintain PCO_2, PO_2, and pH parameters under physiological condition.(1) The electrocardiogram (ECG) lead II was recorded throughout the experiment. The right carotid artery was cannulated for measuring the left ventricular (LV) pressure and volume using the pressure-volume (P-V) conductance catheter (SciSense, Ontario, Canada). The left femoral vein was cannulated for the administration of drug or vehicle. A left thoracotomy was performed via the fourth intercostal space to expose the heart, and the pericardium was opened. The left anterior descending coronary artery (LAD) was identified and ligated at approximately 2 mm from its origin by a 5–0 silk suture with a traumatic needle.(2) Both ends of the thread were passed through a small vinyl tube to form a snare.(2) Ischemia was confirmed by an ST elevation on the ECG and the change in color of the myocardial tissue of the ischemic area. After 30 min of ischemia, the ligature was loosened, and the ischemic myocardium was reperfused for 120 min. At the end of the protocol, the hearts were quickly excised when the animals were deeply anesthesized. Sham-operated animals received all of the above described surgical procedures, except that no LAD occlusion was performed.

2. Experimental group assignment

Seventy-six rats were used in this study. Rats were randomly allocated to one of seven groups (Figure 1). In a sham group (n=4), surgery was done without LAD occlusion. In the I/R group, rats were subject to a 30-min LAD occlusion followed by a 120-min reperfusion, and were divided into six subgroups (n=12/group) as illustrated in Figure 1A. In these six I/R

subgroups, rats were assigned to receive either vehicle (normal saline solution) or 2-mg/kg SB203580HCl (Tocris, Ellisville), a p38 MAPK inhibitor, intravenously at 3 different time points of I/R: 1) 15 min before ischemia (pretreatment), 2) 15 min after the LAD occlusion (during ischemia), or 3) at the onset of reperfusion.(Ref) Vehicle or drug of the same volume was administered intravenously via the femoral vein at 0.33 ml/min for 3 min.

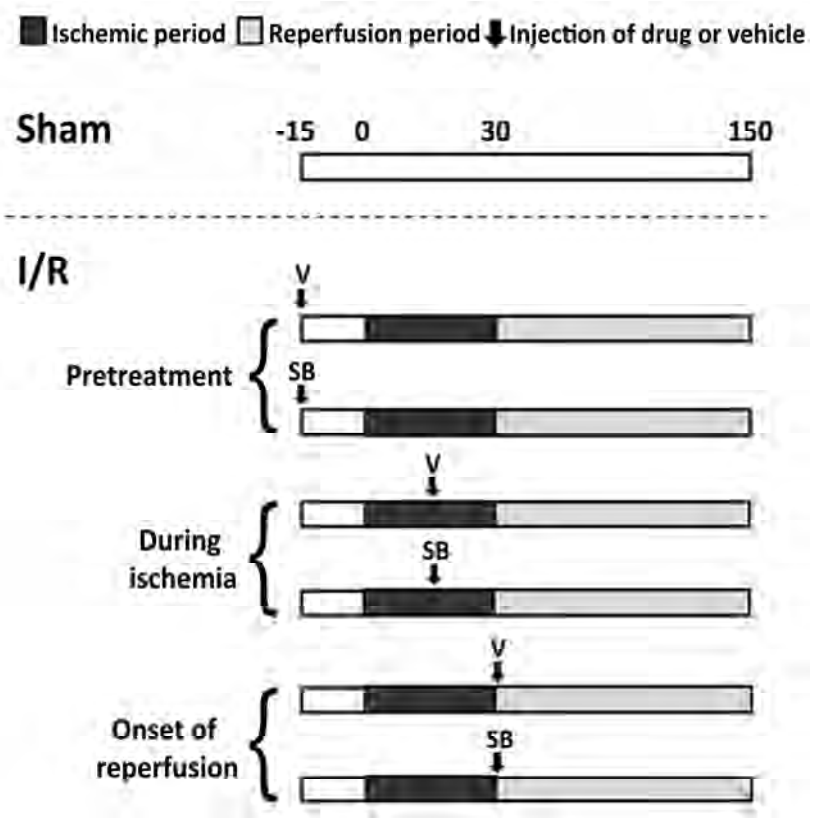

Figure 1: Study protocol and arrhythmia scores. Studyprotocol for experimental groups and timing of SB203580 (SB) or normal saline solution (vehicle, V) administration (A) and effect of SB203580 on the arrhythmia scores in ischemia/reperfusion (I/R) rats (B). *p<0.05 vs. vehicle group.

3. Arrhythmia determination

After surgical preparation, the ECG lead II was recorded using PowerLab 4/25T (ADInstruments, Inc.). Arrhythmias were characterized in accordance with the Lambeth Conventions,(3) and the scores were tabulated for the entire experimental period using a score described previously by Curtis and Walker. The score was based on the frequency and duration of arrhythmias detected (1 was the lowest and 5 was the highest arrhythmia incidence).(4) The criteria of arrhythmia score was as follows: 0: <50 ventricular premature beats; 1: 50-499 ventricular premature beats; 2: > 500 ventricular premature beats and/or one episode of spontaneously reverting ventricular tachycardia or ventricular fibrillation; 3:

more than one episode of spontaneously reverting ventricular tachycardia or fibrillation (<1 min total combined duration); 4: 1-2 min of total combined ventricular tachycardia or fibrillation; 5: >2 min of ventricular tachycardia or fibrillation.

4. Cardiac function determination

The heart rate (HR), end-systolic and end-diastolic pressure (ESP, EDP), maximum and minimum pressure (P_{max}, P_{min}), maximal (dP/dt_{max}) and minimum (dP/dt_{min}) slope of LV pressure waveform, stroke volume (SV), cardiac output (CO) and stroke work (SW) were measured and recorded using the PV loop system (SciSense, Ontario, Canada), and were assessed 15 min before the LAD occlusion (baseline), after 30 min of ischemia and at 90 min of the reperfusion period.

5. Infarct size determination

At the end of each experiment, the heart was excised and mounted on the modified Langendorff apparatus via the aorta. Cold saline solution was used to flush out the blood, after which the LAD was re-occluded and 1-ml Evans blue dye (0.5%) was injected to define the area at risk. Then, the hearts were frozen and sliced from apex to base into 7-8 transverse sections of approximately 1-mm thickness. Heart slices were incubated in 1% buffered 2,3,5-triphenyltetrazolium chloride (TTC) (pH 7.4) at 37°C for at least 15 min to define the necrotic myocardium, followed by placement in 10% formalin (15-20 hours) to enhance the contrast between the stained and unstained TTC tissue. The infarct area (TTC negative) and the non-ischemic area (Evans blue-stained area) were determined by the Image tool software version 3.0. The infarct size was calculated depending on the weight of each slice according to Reiss et al.'s formula.

6. Mitochondrial isolation

Mitochondria were freshly isolated from myocardial tissue by differential centrifugation as describe previously (5). Briefly, ventricular tissue were homogenized in ice-cold isolated buffer (300 mM sucrose, 0.2 mM EGTA, 5 mM TES, pH 7.2) and centrifuged at 800 x g, 4°C for 5 min. Then, the supernatant were collected and re-centrifuged at 8,800 x g, 4°C for 5 min. The mitochondrial pellet was washed by resuspending in ice-cold isolation buffer and re-centrifuged at 8,800 x g, 4°C for 5 min. Mitochondrial protein concentration was determined by the Bicinchoninic Acid (BCA) method, using bovine serum albumin (BSA) as a standard. The isolated cardiac mitochondria that harvested from I/R rat hearts with different time point of SB203580 administration was used to examine mitochondrial swelling, mitochondrial ROS production, and alteration of mitochondrial membrane potential ($\Delta\Psi_m$).

7. Determination of mitochondrial swelling

To determine the mitochondrial sensitivity to mitochondrial membrane permeability transition (mPT), the mitochondrial swelling was measured. The isolated cardiac mitochondria was re-suspended in respiration buffer that consisting of 100 mM KCl, 50 mM sucrose, 10 mM HEPES, 5 mM KH_2PO_4. The permeability transition-induced swelling of mitochondria was measured by rapid loss of the absorbance at λ540 nm by spectrophotometric method. The isolated cardiac mitochondria (0.4 mg/ml) were incubated with 1.5 ml of respiration buffer, and then measured the decreasing in the absorbance for 30 min at room temperature. The data were represented in arbitrary units of absorbance.

8. Determination of mitochondrial ROS production

It has been known that mitochondrial ROS production increased in many pathological conditions such as myocardial ischemia/reperfusion and aging (6, 7). Generation of mitochondrial ROS caused oxidative damage to the cell and finally resulting in cell death. We tested the hypothesis that SB203580 could protect mitochondrial functions by reducing the mitochondrial ROS generation. The mitochondrial ROS production was assessed by measuring the intensity of the fluorescent signal of fluorescent 2′,7′-dichlorohydrofluorescein (DCF), which is converted from non-fluorescent 2',7' - dichlorofluorescein – diacetate (DCFH-DA) in the presence of ROS(8). Isolated cardiac mitochondria (0.4 mg/ml) were incubated with 2 μM DCFH-DA for 30 min at room temperature. The fluorescence intensity was determined by fluorescence microplate ready with the excitation at λ485 nm and emission at λ530 nm. The ROS level was expressed in arbitrary units of fluorescence intensity of DCF.

9. Determination of mitochondrial membrane potential changes

One of the molecular responses of mitochondria occurring in myocardial ischemia/reperfusion injury is an opening of the mitochondrial permeability transition pore (MPTP), which consequently results in the change of mitochondrial membrane potential ($\Delta\Psi$m), and finally leading to cell death. We tested the hypothesis that treatment of SB203580 can protect the loss of $\Delta\Psi$m. The JC-1 or 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide is a lipophilic cationic dye that capable of entering the mitochondrial membrane. Monomeric form of JC-1 could fluoresce in green. Increasing of mitochondrial membrane potential changes ($\Delta\Psi$m) causes aggregation of the dye, which appeared in red fluorescein. Alteration of $\Delta\Psi$m causes changing in red: green ratio. Isolated cardiac mitochondria (0.4 mg/ml) were incubated with 5 μM JC-1 at 37°C for 30 min. The fluorescence intensity for monomeric green fluorescein was determined by fluorescence microplate ready with the excitation at λ485 nm and emission at λ530 nm, while the aggregate red fluorescein was determined by fluorescence microplate ready with the excitation at λ485 nm and emission at λ590 nm. The ratio of red/green fluorescence

intensity ratio was determined. The decreased red/green fluorescent intensity ratio indicated mitochondrial membrane depolarization.

10. Identification of cardiac mitochondrial ultrastructure

The mitochondrial pellet was fixed in 2.5% glutaraldehyde at 4°C overnight. Then, the pellet was rinsed in 0.1 M phosphate buffer (PO_4) for 15 min twice and post-fixed in 1% cacodylate-buffer osmium tetroxide for 2 hr at room temperature. The mitochondrial pellet was rinsed in 0.1M phosphate buffer (PO_4) 5 min twice and was dehydrated in a graded series of ethanol, 50% ethanol for 5 min twice, 70% ethanol for 5 min twice, 85% ethanol for 5 min twice, 95% ethanol for 5 min twice and 100% ethanol for 5 min twice, respectively. After that, the pellet was infiltrated with propylene oxide (PO) for 10 min twice, followed by the cocktail between resin and PO in 1:2 ratio for 30 min, resin and PO in 1:1 ratio for 60 min and resin for overnight, respectively. On the next day, the pellet was embedded in EM-bed 812 resin (9). Ultrathin sections were cut with diamond knife, placed in copper grids and stained with uranyl acetate and lead citrate. The cardiac mitochondria were identified with transmission electron microscope (TEM).

11. Western blot analysis

At the end of each experiment, the heart was rapidly excised, and then the whole ventricular tissue was collected, quickly frozen in liquid nitrogen and stored at -80°C until analysis. Heart proteins were lysed with extraction buffer (20 mmol/L Tris HCl, 1 mmol/L Na_3VO_4, 5 mmol/L NaF) and separated by electrophoresis on 10% or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred onto a polyvinylidene difluoride (PVDF) membranes. After immunoblots were blocked for 1 h with 5% non-fat dry milk in Tris-buffer saline (pH 7.4) containing 0.1% Tween 20, they were probed overnight at 4°C with the primary antibodies that recognize total-p38, phospho-p38, and phospho-HSP27 (Ser82); a downstream substrate of p38 MAPK, connexin 43 (Cx43) and phospho-Cx43 (Ser368); a marker of intercellular electrical communication, Bcl-2, cytochrome *c* (Cell Signaling Technology, Danvers, MA, USA) and Bax (Santa Cruz Biotechnology, Inc, California, USA); a marker of apoptosis, and actin (Sigma-Aldrich, Tokyo, Japan); a loading control, phospho-HSP27 (Cell Signaling Technology, Danvers, MA, USA), total-p53, phospho-p53, total-CREB, phosphor-CREB, total-alpha B-crystalline, phospho-alpha B-crystalline (Santa Cruz Biotechnology, Inc, California, USA). followed by 1-h of incubation at room temperature with the horseradish peroxidase-conjugated secondary antibody. The fold phosphorylation of Cx43 at Ser368 per total Cx43 was measured. The Cx43 antibody detected the total Cx43 protein at the molecular weight that ranged from 39 to 44 kDa and phosphorylated Cx43 (S368) at molecular weight ranging from 42 to 46 kDa. The blots were visualized by ECL reagent. The film images of the western blots were scanned and were analyzed using Image J (NIH image) analysis

software. For quantitation of the proteins of interest, phosphorylated proteins were normalized to total protein expression.

12. Statistical Analysis

All data are expressed as mean ± standard error of mean, and statistics were calculated using SPSS (Statistical Package for Social Sciences, Chicago, IL, USA). Drug-induced changes in arrhythmia scores, time to VT/VF onset, infarct size, as well as hemodynamic parameters at baseline, during ischemia, and reperfusion periods were analyzed using one way ANOVA and followed by a Fisher's Least Significant Difference (LSD) test. Alterations of protein expression were analyzed using Mann-Whitney U test. Comparisons between groups for the VT/VF incidence and mortality rate were performed using a Chi-square test. A p-value < 0.05 was accepted as statistically significant.

Results

Occurrence of arrhythmias during I/R

There was no arrhythmia incidence in sham rats. In vehicle-treated I/R rats, before ischemia, during ischemia or at the onset of reperfusion, arrhythmias were detected and defined as baseline arrhythmia score (4.2 ± 0.3, 4.4 ± 0.3 and 3.8 ± 0.3, respectively). After treatment with SB203580 either before LAD occlusion or during ischemia, the arrhythmia scores were significantly (p<0.05) decreased, compared with the vehicle-treated rats of each group (Figure 2). However, SB203580 administration at the onset of reperfusion did not reduce the arrhythmia scores (Figure 1B). There were no significant differences in arrhythmia scores among all vehicle control groups. The occurrence of VT/VF was observed in all of the vehicle-treated groups (Figure 3A). Treatment of SB203580 either before LAD occlusion or during ischemia significantly reduced the VT/VF incidence during the whole period of I/R (38% and 33% reduction, respectively) (Figure 2B). However, SB203580 treatment did not alter the occurrence of VT/VF, when given at the onset of reperfusion (Figure 3A). The time to VT/VF onset after LAD occlusion was not different when vehicle or SB203580 was administered before ischemia, during ischemia or at the onset of reperfusion (vehicle groups; 380 ± 17 s, 367 ± 19 s, 391 ± 18 s, SB203580 groups; 400 ± 15 s, 368 ± 31 s, 358 ± 22 s, respectively) (Figure 3B).

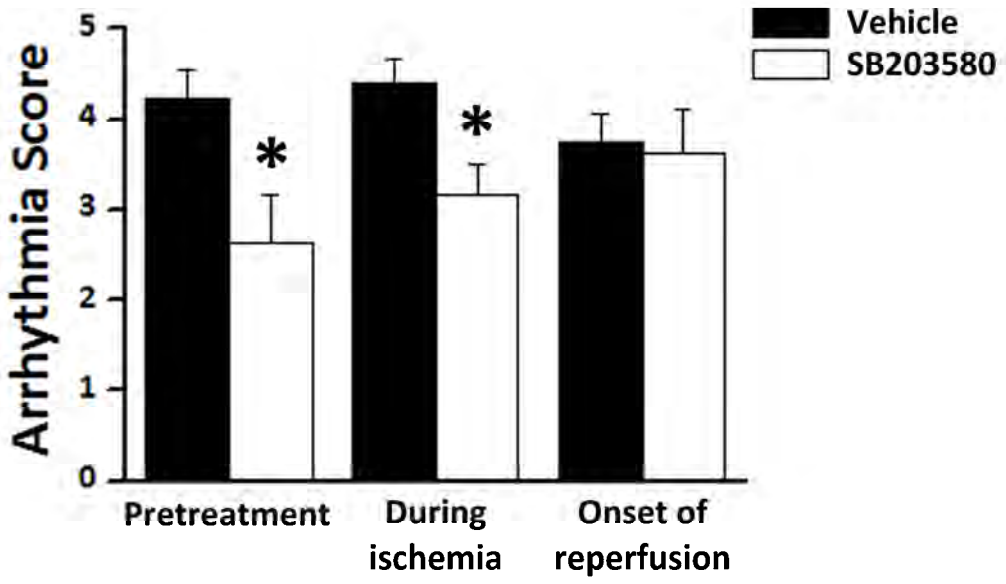

Figure 2: The arrhythmia scores. The effect of SB203580 on the arrhythmia scores in ischemia/reperfusion (I/R) rats (B). *p<0.05 vs. vehicle group.

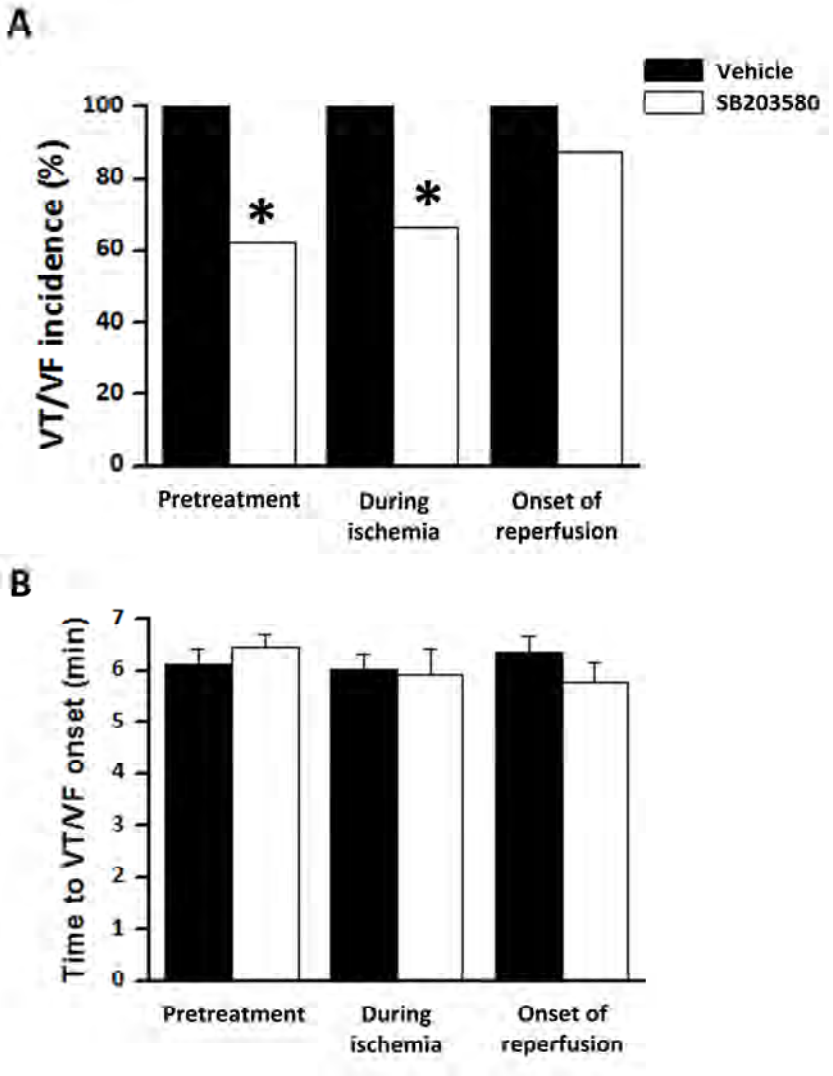

Figure 3: Arrhythmia determination. The incidence of ventricular tachycardia or ventricular fibrillation (VT/VF) (A) and time to ventricular tachycardia or ventricular fibrillation (VT/VF) onset (B) in ischemia/reperfusion rats. *p<0.05 vs. vehicle group.

SB203580 inhibited p38 MAPK activity

Assessment of the effectiveness of SB203580 to inhibit p38-MAPK activity was performed by measuring the phosphorylation of downstream substrate HSP27. In I/R rats, the phosphorylation of HSP27 was significantly (p<0.05) increased in all groups, compared with the sham group (Figure 4A). Administration of SB203580 significantly inhibited p38 MAPK activity, which in turn attenuated the phosphorylation of its downstream substrate HSP27 when given before and during LAD occlusion, compared with vehicle-treated groups (Figure 4A). However, SB203580 did not decrease the level of HSP27 phosphorylation when given at the onset of reperfusion (Figure 4A).

SB203580 reduced incidence of arrhythmia by increasing connexin 43 phosphorylation

In myocardial ischemia, p38 MAPK activation could cause the dephosphorylation of Cx43, and induce the loss of cellular communication via gap function, resulting in cardiac arrhythmia. In the present study, we measured the effect of SB203580 on connexin 43 phosphorylation. The results showed that the phosphorylation of Cx43 was significantly (p<0.05) decreased in vehicle I/R groups, compared with the sham group (Figure 4B). However, SB203580 given before or during ischemia increased the phosphorylated Cx43, compared to the vehicle-treated group (Figure 4B). SB203580 given at the onset of reperfusion did not alter the phosphorylated Cx43 (Figure 4B).

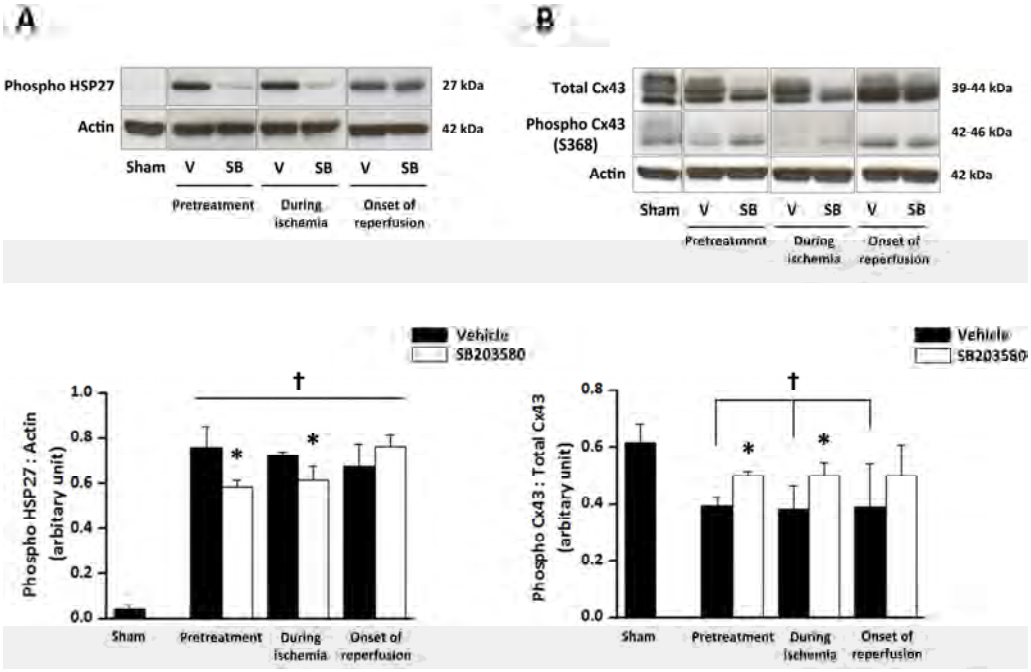

Figure 4: HSP27 and connexin 43 phosphorylation. Effects of SB203580 (SB) on myocardial HSP27 phosphorylation (A) and connexin 43 (Cx43) protein level (B) in heart tissue. **Upper panels** indicate representative immunoblots of myocardial rat ventricle tissue from each of the treatment regimes. **Lower panels** indicate quantitative data of phosphorylation of HSP27 and Cx43 normalized to total protein. Western blot analysis results were taken from the groups that were treated with vehicle (V) or SB before ischemia, during ischemia or at the onset of reperfusion in ischemia/reperfusion rats. †p <0.05 vs. sham group, *p<0.05 vs. vehicle group.

Infarct size

Administration of SB203580 at any times of I/R (i.e. pretreatment, during ischemia, or at the onset of reperfusion) significantly decreased the infarct size (30%, 31% and 27% reduction, respectively), compared with that in the vehicle treated-group (42 ± 4%, 46 ± 2% and 45 ± 3%, respectively) (Figure 5).

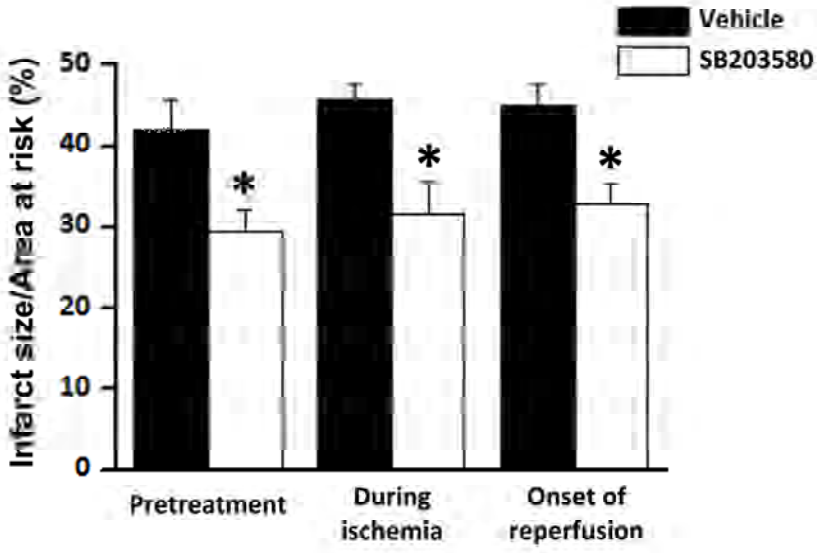

Figure 5: Infarct size determination. Effects of SB203580 on the infarct size in ischemia/reperfusion rats). (A) the percentage of Area at risk/left ventricular volume, (B) The percentage of infarction/AAR. *p<0.05 vs. vehicle group.

Bax, Bcl-2 and cytochrome *c* level

The expression of Bax to Bcl-2 and the level of cytochrome *c* were increased in the vehicle-treated and SB203580-treated I/R rats, compared to the sham group (Figure 6A and 6B). Although the infarct size was decreased in the SB203580-treated groups, administration of SB203580 at any time of I/R injury did not change the ratio of Bax and Bcl-2 expression, compared to the vehicle-treated rats of the same group (Figure 6A). However, SB203580 administration at any times of I/R injury, pretreatment, during ischemia, or onset of reperfusion significantly (p<0.05) decreased the mitochondrial cytochrome *c* release (6%, 2% and 8% reduction, respectively), compared to that in the vehicle-treated group (Figure 6B). Furthermore, there was no significant difference in mortality rate between vehicle and SB203580-treated group either before ischemia, during ischemia or at the onset of reperfusion (vehicle groups; 8%, 25%, 33% and SB203580 groups; 17%, 33%, 33%, respectively).

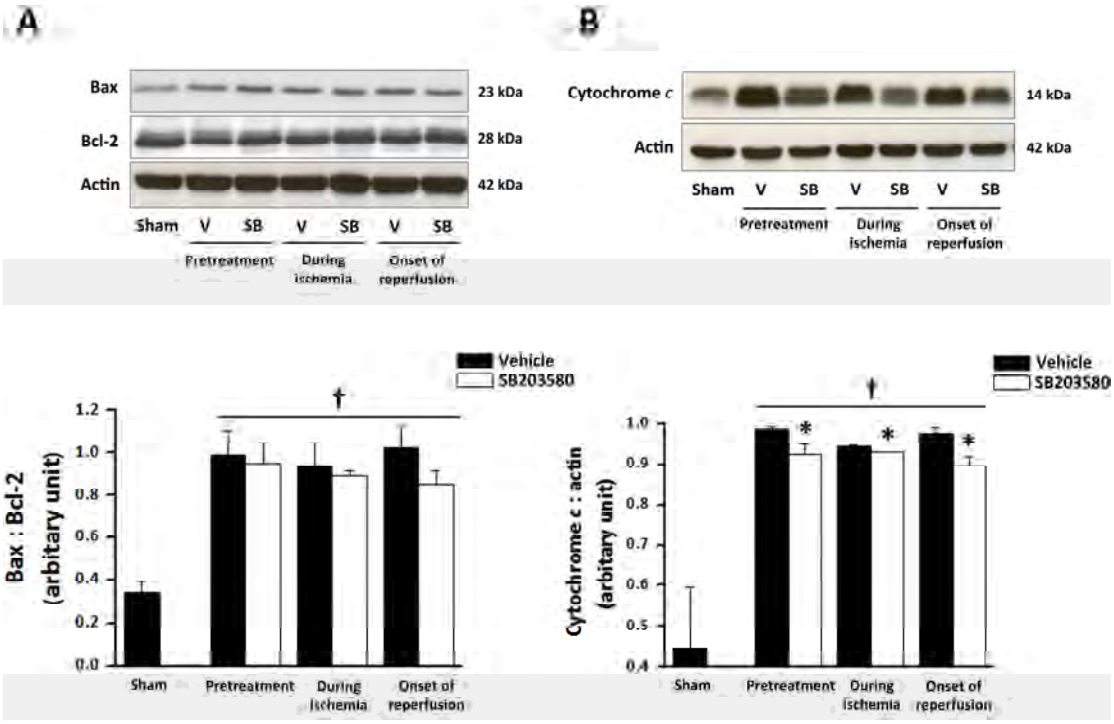

Figure 6: Bax, Bcl and cytochrome *c* expression. Immunoblots (upper panels) and quantitation (lower panels) of Bax and Bcl-2 expression (A) and the expression of mitochondrial cytochrome *c* release (B) in ventricle hearts treated with SB203580 (SB) before ischemia, during ischemia, or at the onset of reperfusion in ischemia/reperfusion rats, compared with the vehicle (V) control of each group †p<0.05 vs. sham group, *p<0.05 vs. vehicle group.

Effect of intervention on hemodynamic parameters

Changes in hemodynamics in the I/R group are summarized in Tables 1-3. At the baseline, no differences in hemodynamic parameters were found between groups (Table 1). Our results showed that the end systolic pressure (ESP), and dP/dt_{max} were attenuated by the ischemia and reperfusion. Moreover, the stroke volume and cardiac output were significantly decreased during ischemia. Pre-treatment of SB203580 partially improved ESP, P_{max}, P_{min}, dp/dt_{max} during ischemic and reperfusion periods. However, SB203580 administration during ischemia or at the onset of reperfusion period did not improve the hemodynamic parameters. Although SB203580 treatment could reduce the infarct size at any given study periods, the cardiac function could be improved only when SB203580 given prior to ischemia (Tables 2-3**).**

TABLE 1. Cardiac Function Parameters at Baseline in I/R Rats Treated With Vehicle or SB203580

	Pretreatment		During Ischemia		Onset of Reperfusion	
Hemodynamic Parameters	**V**	**SB**	**V**	**SB**	**V**	**SB**
HR (beats/min)	264 ± 14	271 ± 18	265 ± 20	274 ± 12	262 ± 10	266 ± 8
ESP (mm Hg)	148 ± 7	164 ± 14	144 ± 6	149 ± 12	141 ± 9	157 ± 8
EDP (mm Hg)	15 ± 4	13 ± 6	13 ± 7	13 ± 6	14 ± 6	14 ± 3
P_{max} (mm Hg)	148 ± 6	167 ± 13	150 ± 6	150 ± 12	146 ± 8	161 ± 7
P_{min} (mm Hg)	49 ± 4	51 ± 3	47 ± 4	44 ± 6	46 ± 5	44 ± 4
dP/dt_{max}(mm Hg/s)	9577 ± 752	10,316 ± 631	8947 ± 1134	8766 ± 1169	7948 ± 1576	8882 ± 777
dP/dt_{min}(mm Hg/s)	−4304 ± 399	−4572 ± 467	−4253 ± 359	−4267 ± 413	−4442 ± 290	−4978 ± 391
Stroke volume (mL)	0.17 ± 0.01	0.20 ± 0.02	0.21 ± 0.02	0.19 ± 0.03	0.18 ± 0.02	0.18 ± 0.01
Cardiac output (mL/min)	50 ± 4	55 ± 7	48 ± 7	50 ± 9	44 ± 4	49 ± 5
Stroke work (mm Hg/mL)	16 ± 2	21 ± 3	18 ± 2	15 ± 5	14 ± 2	18 ± 2

*$P < 0.05$ versus vehicle.

V, vehicle; SB, SB203580-p38 MAPK inhibitor; HR, heart rate; P_{max}, maximum pressure; P_{min}, minimum pressure; dP/dt_{max}, maximal slope of left ventricle pressure waveform; dP/dt_{min}, maximum slope of left ventricle pressure waveform.

TABLE 2. Cardiac Function Parameters at Ischemic Period in I/R Rats Treated With Vehicle or SB203580

	Pretreatment		During Ischemia		Onset of Reperfusion	
Hemodynamic Parameters	**V**	**SB**	**V**	**SB**	**V**	**SB**
HR (beats/min)	251 ± 15	274 ± 23	249 ± 14	280 ± 8	269 ± 15	290 ± 20
ESP (mm Hg)	124 ± 9	155 ± 6*	141 ± 10	126 ± 12	131 ± 10	133 ± 7
EDP (mm Hg)	15 ± 4	18 ± 4	15 ± 7	15 ± 9	15 ± 5	14 ± 4
P_{max} (mm Hg)	133 ± 7	159 ± 6*	145 ± 9	131 ± 11	134 ± 10	138 ± 8
P_{min} (mm Hg)	51 ± 4	61 ± 2*	51 ± 6	46 ± 7	50 ± 5	49 ± 4
dP/dt_{max} (mm Hg/s)	9580 ± 768	12,246 ± 414*	10,130 ± 1144	9281 ± 1366	10,063 ± 950	9822 ± 851
dP/dt_{min} (mm Hg/s)	−3611 ± 561	−4093 ± 320	−3875 ± 495	−3888 ± 503	−3430 ± 238	−3759 ± 444
Stroke volume (mL)	0.09 ± 0.01	0.11 ± 0.03	0.17 ± 0.02	0.12 ± 0.03	0.09 ± 0.02	0.10 ± 0.01
Cardiac output (mL/min)	25 ± 5	26 ± 4	39 ± 5	35 ± 8	24 ± 6	24 ± 3
Stroke work (mm Hg/mL)	7 ± 1	10 ± 2	11 ± 2	9 ± 3	8 ± 4	8 ± 2

*$P < 0.05$ versus vehicle.

V, vehicle; SB, SB203580-p38 MAPK inhibitor; HR, heart rate; P_{max}, maximum pressure; P_{min}, minimum pressure; dP/dt_{max}, maximal slope of left ventricle pressure waveform; dP/dt_{min}, maximum slope of left ventricle pressure waveform.

TABLE 3. Cardiac Function Parameters at Reperfusion Period in I/R Rats Treated With Vehicle or SB203580

	Pretreatment		During Ischemia		Onset of Reperfusion	
Hemodynamic Parameters	**V**	**SB**	**V**	**SB**	**V**	**SB**
HR (beats/min)	261 ± 16	263 ± 15	290 ± 8	253 ± 24	252 ± 13	281 ± 14
ESP (mm Hg)	141 ± 14	162 ± 11	167 ± 11	150 ± 13	129 ± 9	135 ± 16
EDP (mm Hg)	12 ± 3	17 ± 2*	14 ± 4	12 ± 10	13 ± 5	13 ± 8
P_{max} (mm Hg)	146 ± 10	163 ± 11	170 ± 10	154 ± 13	132 ± 9	140 ± 15
P_{min} (mm Hg)	42 ± 3	58 ± 2*	52 ± 6	46 ± 6	44 ± 5	44 ± 7
dP/dt_{max} (mm Hg/s)	7951 ± 782	11,553 ± 473*	10,446 ± 1229	9295 ± 1230	8788 ± 944	8805 ± 1372
dP/dt_{min} (mm Hg/s)	−4666 ± 749	−4558 ± 980	−5239 ± 574	−4477 ± 897	−4010 ± 730	−4530 ± 903
Stroke volume (mL)	0.09 ± 0.02	0.08 ± 0.02	0.14 ± 0.02	0.08 ± 0.03	0.08 ± 0.02	0.11 ± 0.01
Cardiac output (mL/min)	25 ± 5	20 ± 6	37 ± 3	24 ± 10	19 ± 4	29 ± 2
Stroke work (mm Hg/mL)	9 ± 2	7 ± 2	14 ± 1	8 ± 4	5 ± 2	8 ± 2

*$P < 0.05$ versus vehicle.

V, vehicle; SB, SB203580-p38 MAPK inhibitor; HR, heart rate; P_{max}, maximum pressure; P_{min}, minimum pressure; dP/dt_{max}, maximal slope of left ventricle pressure waveform; dP/dt_{min}, maximum slope of left ventricle pressure waveform.

p38 MAPK inhibition by SB203580 improved cardiac mitochondrial function

I/R injury caused cardiac mitochondrial swelling by decreasing the absorbance at 540 nm, increasing ROS production, and mitochondrial membrane depolarization (Figure 7). Administration of SB203580 at any time points of I/R protocol significantly attenuated cardiac mitochondrial swelling. However, pretreatment of SB203580 was found to be the most effective timing to prevent cardiac mitochondrial swelling, compared to drug treatment during ischemia or at the onset of reperfusion (Figure 7A). Administration of SB203580 at any time points in I/R could also significantly reduce ROS production in cardiac mitochondria caused by I/R injury, compared to the vehicle control group (Figure 7B). For cardiac mitochondrial membrane potential alteration, we found that administration of SB203580 prior to ischemia or during ischemia significantly prevented the change of $\Delta\Psi_m$ caused by I/R injury, when compared to the vehicle control group (Figure 7C). However, SB203580 administered at the onset of reperfusion failed to prevent mitochondrial depolarization caused by I/R (Figure 7C).

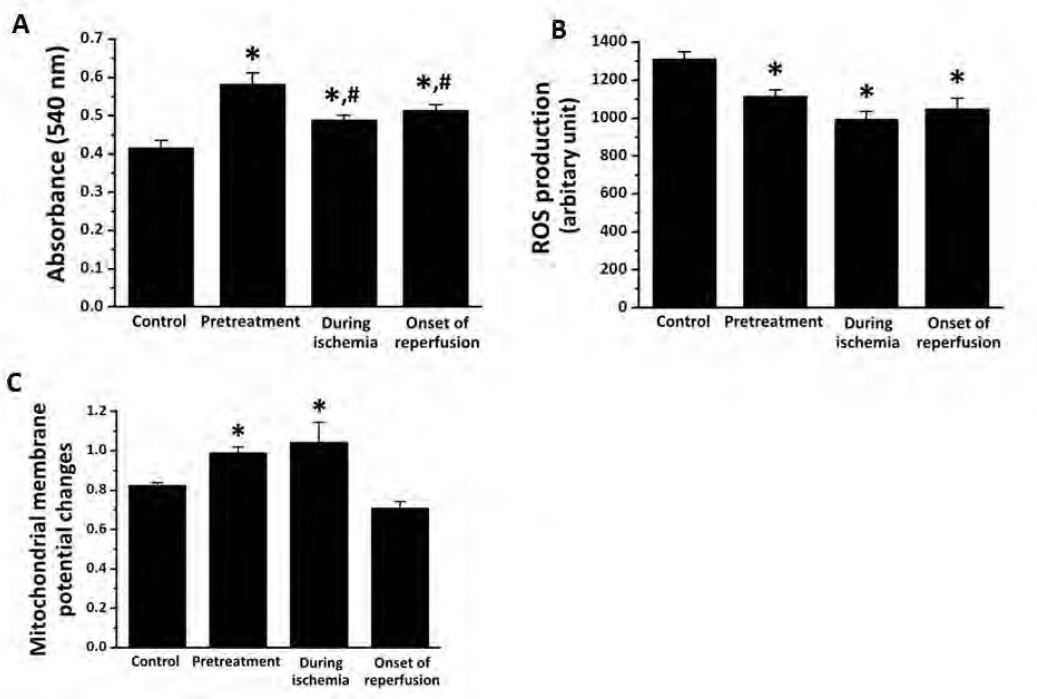

Figure 7: The effect of SB203580 on cardiac mitochondrial functions. (A) mitochondrial swelling (B) mitochondrial ROS production, (C) mitochondrial membrane potential, when administration before, during ischemia, or at the onset of reperfusion in ischemia/reperfusion rats (n=4-7 animals/group). *$p<0.05$ vs. vehicle group, $^{\#}p<0.05$ vs. pretreatment group.

Inhibition of p38 MAPK protects I/R-induced cardiac mitochondrial ultrastructure disruption.

Our results demonstrated that I/R injury not only caused cardiac mitochondrial dysfunction, but also distorted the cardiac mitochondrial ultrastructure by increasing matrix space and disorganization of cristae (Figure 8A). Administration of SB203580 at any time points could preserve the cardiac mitochondrial ultrastructure from the disruption caused by I/R (Figure 8B-C). Interestingly, administration of SB203580 prior to ischemia gave the most effective treatment to protect I/R induced-cardiac mitochondria ultrastructure rupture (Figure 8D).

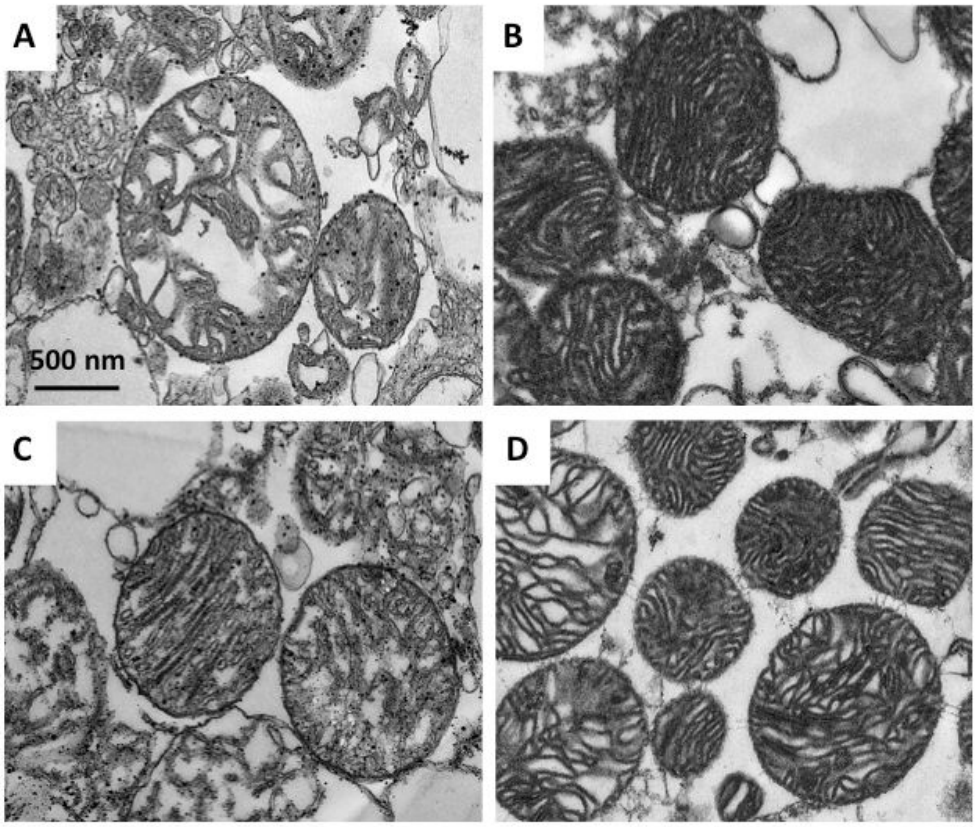

Figure 8: **Effect of SB203580 on mitochondrial ultrastructure**. The cardiac mitochondria were isolated from ischemia/reperfusion rats in the presence of SB203580 before (B), during ischemia (C), or at the onset of reperfusion (D) (n=4-5 animals/group). *$p<0.05$ vs. vehicle group.

p38 MAPK inhibitor, SB203580, protect cardiac mitochondria by attenuation of apoptotic regulatory molecules activation

Myocardial I/R have been shown to cause activation of p38 MAPK and its activities by phosphorylation of downstream signaling molecules HSP27. Administration of p38 MAPK inhibitorSB203580, prior to or during ischemia, significantly reduced p38 MAPK phosphorylation as well as its activity to phosphorylate downstream substrate HSP27 (Figure 9A-C). However, SB203580 given at the onset of reperfusion could not reduce the p38 MAPK phosphorylation as well as the level of phosphorylated HSP27 (Figure 9A-C). Moreover, we found that pretreatment of SB203580 significantly inhibited the phosphorylation of p53 and CREB, while SB203580 given during ischemia failed to inhibit the activation of these two downstream molecules (Figure 9). In addition, SB203580 given at the onset of reperfusion could significantly inhibit phosphorylation of CREB, but not p53. In addition, the α-B-crystalline phosphorylation was also significantly inhibited at any time points of SB203580 administration.

Since mitochondria are the key machinery driven cell death, especially the necro-apoptosis in I/R injury, the effects of SB203580 on apoptotic regulatory molecules such as Bax, Bcl2, caspase 3, and cytochrome c were also determined. Our results showed that administration of SB203580 prior to or during ischemia, but not at the onset of reperfusion, significantly decreased Bax expression without any changes in Bcl2 expression level (Figure 10). Administration of SB203580 significantly reduced the cytochrome c level only when it was given prior to the onset of ischemia, but failed to change the cytochrome c level when treated during ischemia or at the onset of reperfusion (Figure 10). Moreover, pretreatment of SB203580 as well as given during ischemia significantly reduced the level of cleaved caspase 3. However, SB203580 given at the onset of reperfusion failed to prevent caspase 3 cleavages (Figure 10).

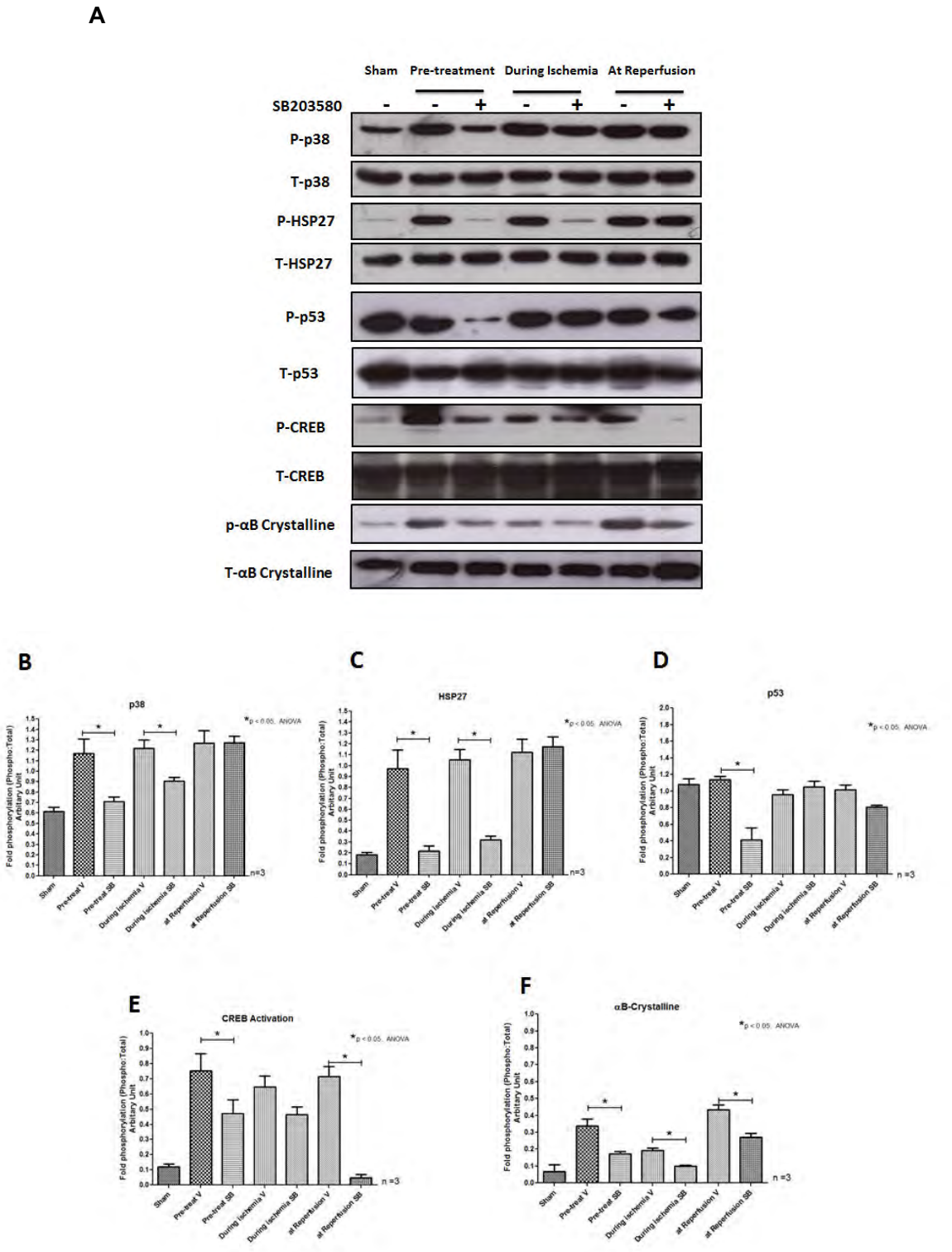

Figure 9: **The effect of SB203580 on p38 MAPK activation and downstream substrates, HSP27, p53, CREB, and α-B-crystalline**. The heart homogenate were collected and subjected to Western blot analysis detecting the activation of interested proteins (A). The quantitation of fold phosphorylation were represented in B-F(n=3 animals/group).

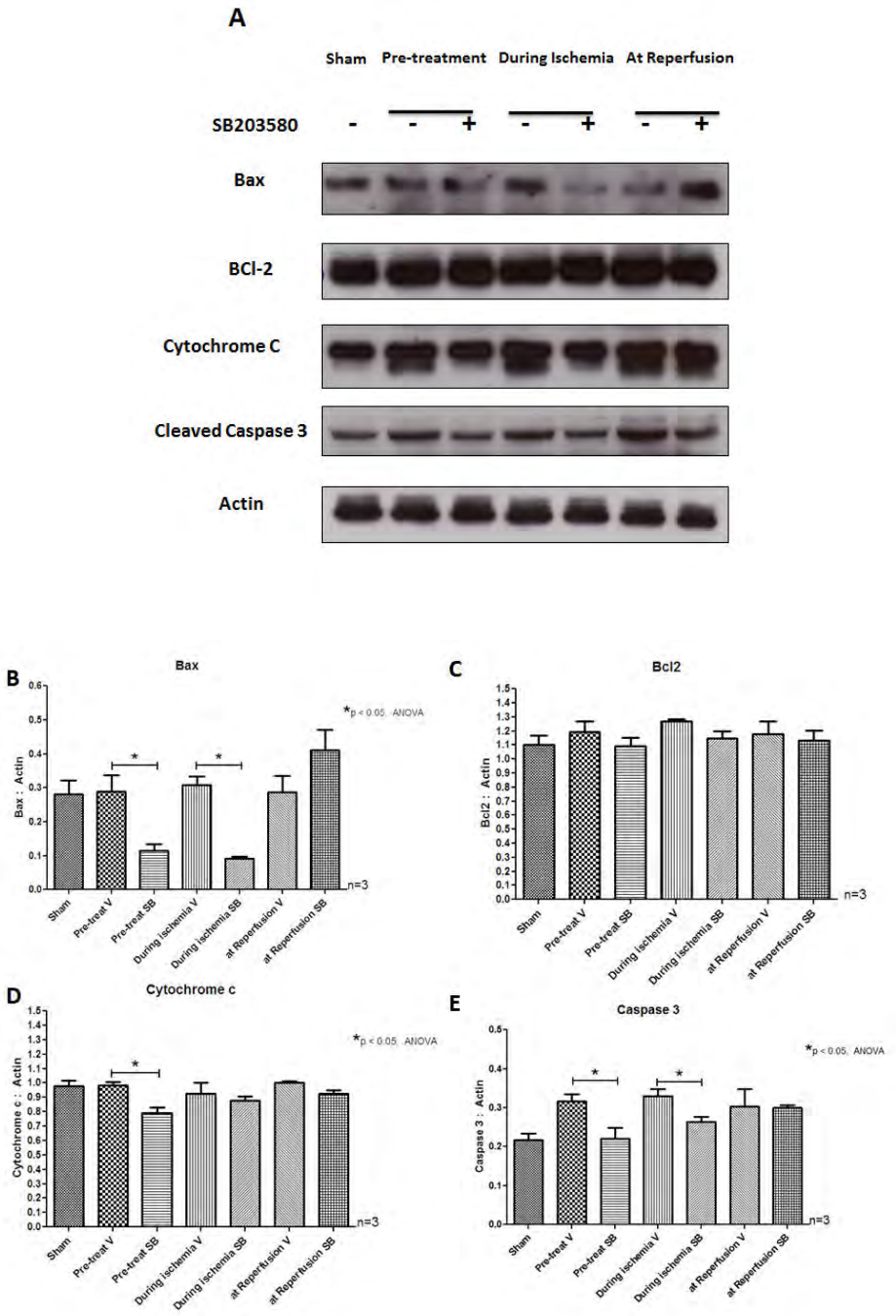

Figure 10: **The effect of SB203580 on apoptotic regulatory proteins.** The heart homogenate were collected and subjected to Western blot analysis detecting the expression of Bax, Bcl2, Cytochrome c, and cleaved caspase-3 (A). The quantitation of fold phosphorylation were represented in B-D (n=3 animals/group).

Discussion and conclusion

The major findings of this study are that in the setting of I/R injury, administration of SB203580 before or during ischemia decreased the arrhythmia scores, the incidence of VT/VF, the phosphorylation of HSP27 and increase the Cx43 phosphorylation, whereas SB203580 given at the onset of reperfusion did not provide those beneficial effects. However, SB203580 decreased the infarct size in all I/R groups. Furthermore, SB203580 reduced cytochrome *c* release in all I/R groups with the decreasing in total Bax without changes in Bcl-2 expression. Left ventricular function was improved only when SB203580 was administered prior to ischemia. In addition, administration of SB203580 before or during ischemia attenuated cardiac mitochondrial dysfunction caused by I/R as indicated by preventing mitochondrial swelling, reducing mitochondrial ROS generation, and attenuating mitochondrial membrane potential depolarization. However, given SB203580 at the onset of reperfusion could attenuate only mitochondrial swelling and ROS production, but failed to prevent the loss of mitochondrial membrane potential. In addition, inhibition of p38 MAPK activation as well as its activity reduces activation of p53 and CREB, reduced apoptotic regulatory protein Bax, cytochrome c release, and caspase 3.

p38 MAPK has been shown to be involved in myocardial injury and cell death, and the inhibition of p38 MAPK activation in the ischemic heart has been shown to reduce cell death, infarct size, and attenuate the degradation of LV function.(10-15) Despite these potential cardioprotective benefits, the effects of p38 MAPK inhibitor given during myocardial I/R on post-ischemic fatal arrhythmias has never been elucidated. Ischemic-induced fatal arrhythmias are known to be responsible for high mortality in AMI patients.(16-18) Several studies demonstrated the beneficial effects of pharmacological interventions on cardiac electrophysiological alterations, such as decreased VF incidence and prolonged time to the first occurrence of VF during I/R.(19, 20) However, this is the first study to demonstrate the effect of p38 MAPK inhibitor, SB203580, on cardiac arrhythmias during myocardial I/R in an in vivo rat model. In I/R rats, SB203580 given before or during ischemia decreased the incidence of VT/VF and the arrhythmia scores, whereas SB203580 given at the onset of reperfusion did not protect the heart from arrhythmia during I/R. The possible underlying mechanism of p38 MAPK inhibition in a reduction of arrhythmia could be due to the improvement in cellular communication via Cx43, which is the major gap junction protein found in adult mammalian hearts.(21)

During I/R, dephosphorylation of Cx43 occurred, leading to the loss of intercellular electrical communication via gap junctions in the ischemic heart, results in conduction abnormalities and reentrant arrhythmias.(22, 23) A previous study also demonstrated that in heterozygous Cx43 knockout mice subjected to acute ischemia, a higher incidence of arrhythmogenesis including an increase in the frequency of premature ventricular beats, spontaneous VT, and rapid onset of the first run of VT was observed.(24) Cx43 has been shown to be regulated by p38 MAPK under ischemia,(25) ischemic preconditioning,(25) and α-adrenergic stimulation.(26, 27) Therefore, under ischemic condition, p38 MAPK activation could lead to dephosphorylation of Cx43,(25) resulting in decreased gap junction communication and increased occurrence of arrhythmias. However, the actual link between p38 MAPK, Cx43, and arrhythmogenesis in response to I/R has never been investigated.

The present study demonstrates that SB203580 given only before or during ischemia in I/R rats increased the phosphorylation of Cx43 at Ser368, which was associated with decreased arrhythmia scores and VT/VF incidence in the SB203580-treated rats. Since increased Cx43 phosphorylation at the Ser368 site has been shown to enhance myocardial resistance to ischemic injury,(28) the increased phosphorylated Cx43 by p38 MAPK inhibition could be responsible for arrhythmia reduction found in this study. Furthermore, phosphorylation of Cx43 at this site has been shown to limit the infarct size via the gap junction dependent mechanisms.(29) Therefore, this together with decreased cytochrome *c* release could be responsible for infarct size reduction found in this study.

A prior study demonstrated that the activation of p38 MAPK reduced cardiac contractility in cultured rat cardiomyocytes, and that inhibition of p38 MAPK reversibly enhanced this effect by increasing the myofilament response to calcium.(30) We observed that timing of p38 MAPK inhibition with respect to onset of ischemia importantly modulated the effectiveness of the drug; attenuation of left ventricular dysfunction was only observed when SB203580 was administered prior to onset of ischemia, despite the fact that infarct size was decreased in all given periods of SB203580. This effect could be due to the protective effect of SB203580 on preventing the p38 MAPK activation which was initiated during ischemia. Therefore, inhibition of p38 MAPK after ischemia, in which p38 MAPK was already activated, could be too late and thus did not attenuate cardiac dysfunction.

SB203580 action on p38 MAPK activation was confirmed by our findings that SB203580 significantly inhibited HSP27 phosphorylation, i.e. a downstream substrate of p38 MAPK activity, when given before or during myocardial I/R. However, its administration at the onset of reperfusion did not inhibit HSP27 phosphorylation. In myocardial I/R, it has been shown previously that p38 MAPK was activated during ischemia and was reactivated during reperfusion.(11) Our results that SB203580 effectively decreased HSP27 phosphorylation when given before ischemia as well as during ischemia but not at reperfusion, indicated that activation of HSP27 during ischemia occurred late after an LAD occlusion, whereas HSP27 reactivation occurred immediately after reperfusion. Although SB203580 did not reduce HSP27 phosphorylation when administered at the onset of reperfusion, it decreased the infarct size and the level of the cytochrome *c* release. This finding implied that the effect of SB203580 on the infarct size reduction may occur by means of not only the decreased HSP27 phosphorylation, but also the other downstream transcription factors such as p53,(31) STAT1 (signal transducer and activator transcription 1),(32) and CHOP (C/EBP-Homologous Protein)(33) which have been shown to involve in cellular apoptosis. However, the actual mechanism regarding the inhibition of p38 MAPK during reperfusion will need further investigation in the future.

Previous studies demonstrated that SB203580 reduced the infarct size when it was given prior to ischemia in both the *in vivo*,(13, 15) and *ex vivo* rabbit I/R model.(34) In the present study, our results demonstrated further the beneficial effect of infarct size reduction by SB203580 given even during ischemia or at the onset of reperfusion. This beneficial effect was similar to when SB203580 was given before myocardial ischemia. These findings suggest that myocardial insult leading to myocardial cell death occurred later during reperfusion(35) as confirmed by equal infarct size reduction by p38 MAPK inhibitor given before or during ischemia and at the onset of reperfusion. This is also consistent with a previous report that myocardial ischemia alone only provoked myocardial damage, and that reperfusion activated apoptosis and increased the myocardial injury.(36) In apoptosis, Bax and Bcl-2 are proteins that play an important role in mitochondrial outer membrane permeabilisation

pore regulation. Bax promote pore formation on mitochondrial outer membrane, while Bcl-2 prevents this phenomenon. The releasing of cytochrome *c* from mitochondria also occurs as process in apoptosis and regulates cellular morphological alteration in apoptosis. Therefore, Bax, Bcl-2, and cytochrome c have been used as marker proteins for apoptotic. In the present study, we showed *for the first time* the direct effect of p38 MAPK inhibitor, SB203580, on cardiac mitochondrial function, and providing molecular mechanistic insights of cardiac mitochondrial protection by p38 MAPK inhibition through p53→Bax→ cytochrome c →caspase 3 cascades. Moreover, our findings suggest the significance of the timing of p38 MAPK inhibition to achieve the effective therapeutic outcomes.

During I/R injury, cardiac mitochondria loss its function and could contribute to electrical and contractile dysfunction of the cardiac cell and the whole heart. The excessive formation of ROS during I/R injury has been shown to induce prolonged-opening of MPTP, dissipates the proton electrochemical gradient or $\Delta\Psi_m$, consequently cause ATP insufficiency, leading to further ROS generation, loss of intact cardiac mitochondrial ultrastructure, and finally resulting in cardiac mitochondrial swelling and rupture. This process then triggers the apoptotic program due to the leakage of pro-apoptotic molecules from ruptured mitochondria. Therefore prevention or attenuation the degree of mitochondrial dysfunction caused by I/R injury is one of the fascinating clinical targets.

In the present study, our data show that inhibition of p38 MAPK by pharmacological inhibitor, SB203580, could inhibit p38 MAPK activation and its activity. Moreover, SB203580 could reduce mitochondrial dysfunction, damage, and ROS production, and attenuate the mitochondrial stress triggering necro-apoptosis in the heart. Moreover, our data also suggest the significance of timing of drug administration on prevention of cardiac mitochondrial dysfunction caused by I/R. Pretreatment of the inhibitor significantly protect I/R induced cardiac mitochondrial dysfunction, ROS production, ultrastructure damaging, and also attenuate the activation of apoptotic regulatory molecules. However, treatment of the inhibitor during ischemia or at the onset of reperfusion demonstrated a partial effect and be in the way that the quicker treatment, the better of protective outcomes. Our present data show that treatment of SB203580 during ischemia seems to have more benefit to cardiac mitochondria than given at the onset of reperfusion. This is similar to our previous findings about the effect of different SB203580 administration on infarct size and the incidence of fatal arrhythmia (37). In fact, given drug before onset of ischemia seem to be impractical in real life because ischemia is an unpredictable episode. Post-ischemic period is more likely to be the most effective timing for treatment. Therefore, drug administration soon after myocardial ischemia should give the most clinical benefits and patient outcomes.

In response to ROS and I/R stress, p53 protein has been known to accumulate in the mitochondrial matrix and directly mediate mitochondrial outer membrane permeabilization (MOMP) and resulting in the release of cytochrome c, which subsequently activating caspase 3(38). This activation cascade caused programmed cell death or apoptosis. Recently, a novel role of p53 in mitochondria has been reported. p53 accumulation during I/R injury caused MPTP opening, leading to the influx of ions, which resulting in $\Delta\Psi_m$ dissipation, attenuation of the oxidative phosphorylation, and ATP depletion(38). The mitochondrial swelling and rupture further caused sequestered cell death factor releasing, which then orchestrated the cell death. Therefore, p53 activation in I/R is considered as a key molecule to trigger cellular necro-apoptosis(39). It has been reported that p53 could be

activated by p38 MAPK(40-42), and mediated cell death(43). In $p38^{-/-}$ cells, phosphorylation of p53 at ser15 was decreased and contributed to down regulation of Bax protein level in cardiomyocytes (44), suggesting the role of p38 MAPK in p53-Bax regulation. Moreover, phosphorylation of p53 by p38 MAPK has been shown to stabilize p53 from Mouse double minute 2 homolog (Mdm2) association and trigger p53 degradation by ubiquitin system(45). Therefore, based on our data, inhibition of p38 MAPK by SB203580 reduced p53 activation, and Bax expression level, which in turn attenuated the release of cytochrome c level to trigger the activity of caspase 3 mediated apoptosis. These findings suggest the therapeutic effect of p38 MAPK inhibitor on cardiac mitochondria protection.

The impairment of mitochondrial activity has been found to activate cAMP-responsive element-binding protein (CREB) phosphorylation at Ser133(46), which in turn activated p53 in transcriptional dependent manner. Down regulation of p53 could also affect the p53 mediate mitochondrial dysfunction and apoptosis. Therefore, inhibition of CREB phosphorylation could be a p53 regulatory target. It has been shown that, during ischemia, CREB is activated as a downstream event of p38 MAPK activation(47). Our findings are consistent with this report. Inhibition of p38 MAPK by SB203580 could reduce I/R induced CREB phosphorylation. However, we found that the protein expression level of p53 was not significantly different. This could possibly due to the study protocol, which aimed to study the acute effect of p38 MAPK inhibitor. The duration of inhibitor treatment as well as I/R protocol may not be sufficient to initiate the transcriptional processes of p53. Therefore, our findings suggest that administration of SB203580 could inhibit p53 activation in a transcriptional independent manner.

From our study, p38 MAPK is phosphorylated as a result of myocardial I/R injury and lead to cardiac mitochondrial dysfunction and ultrastructure rupture, which consequently result in cell death. Inhibition of p38 MAPK activation and its activity by pharmacological inhibitor SB203580 protect cardiac mitochondria from I/R injury by reducing the phosphorylated p53 in transcription independent manner (Figure 11). Non-phosphorylated p53 is degraded by ubiquitin proteolytic system, which is insufficient to mediate mitochondrial membrane permeabilization and reduce myocardial I/R induced cardiac mitochondrial dysfunction (Figure 11). On the other hands, SB203580 reduced phosphorylated p53 level could also influence Bax expression, which reduces mitochondrial membrane permeability and cytochrome c level, and reduce caspase 3 level (Figure 11). These mechanistic insight of SB203580 on cardiac mitochondria explain the cardioprotective effect of p38 MAPK inhibition in myocardial I/R. Another possible explanation concerning the cardiac mitochondria protective effect of SB203580 is the attenuation of the voltage dependent anion channel (VDAC) phosphorylation, which is a porin protein involved in mitochondrial regulator of cell survival(48-51). Phosphorylation of VDAC-1 facilitates other protein binding in MPTP and mediates mitochondrial damage. Schwertz et al reported that VDAC-1 was a downstream substrate of p38 MAPK during I/R injury (52). Inhibition of p38 MAPK by PD169316 significantly reduced phosphorylation of VDAC-1, and reduced cardiac cell injury (52). However, the effect of SB203580 on VDAC-1 phosphorylation in cardiac mitochondrial from myocardial I/R model need to be further investigated.

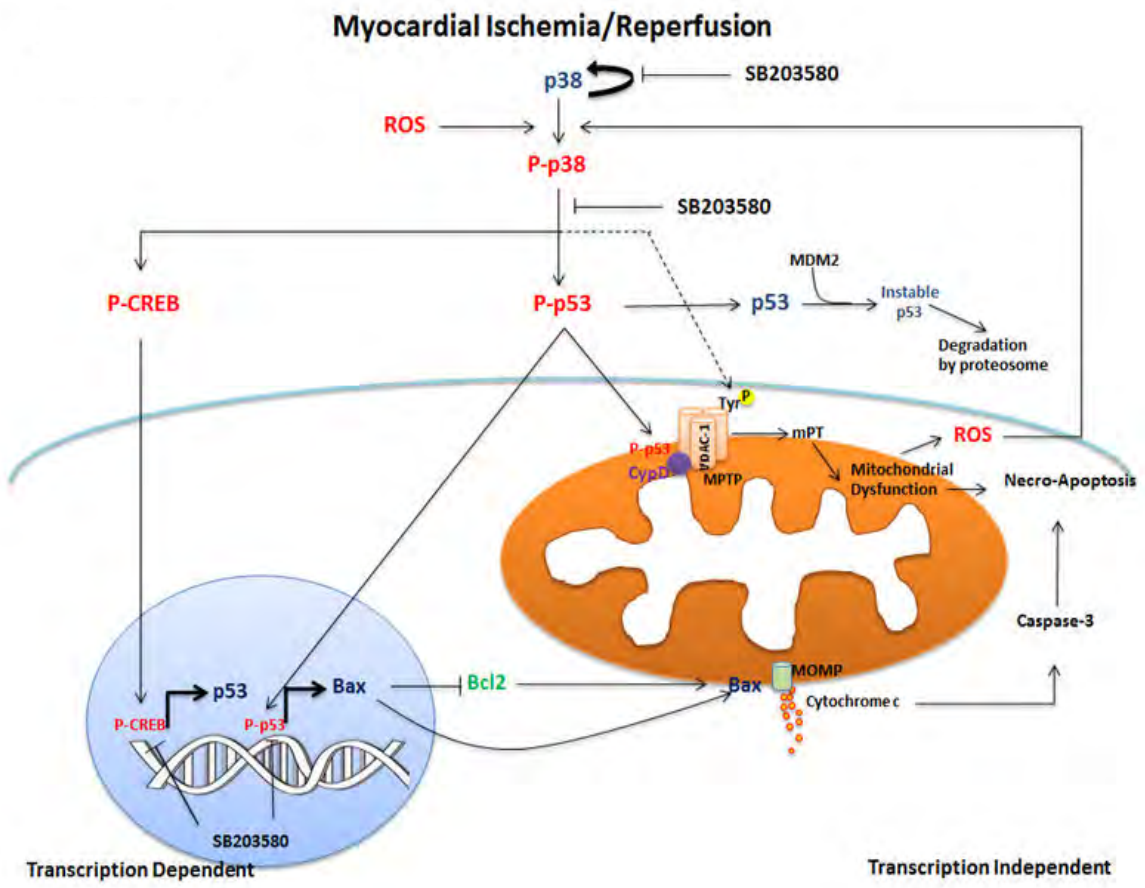

Figure 6: **The mechanistic pathway of I/R activate p38 MAPK and downstream activation involving mitochondrial trigger cell death**. Myocardial I /R injury cause p38 MAPK activation, which are consequently, activate p53 phosphorylation. Phosphorylated p53 stabilized and accumulated in mitochondrial matrix during I/R injury and mediate MPTP opening. On the other hand, activation of p53 could activate Bax expression, which regulates cytochrome c release, and activation of caspase 3. P38 MAPK can also phosphorylate CREB, which in turn regulate p53 pathway in transcription dependent manner. Inhibition of p38 MAPK by SB203580 could reduce p53 phosphorylation, CREB phosphorylation and then protect mitochondria from injury and cell death.

Conclusion

In conclusion, this is the first report to demonstrate that p38 MAPK inhibition by SB203580 could reduce cardiac mitochondrial dysfunction caused by I/R injury through the attenuation of p53-mediate mitochondrial trigger cell death. Moreover, our data suggest that the therapeutic potential of SB203580 to protect cardiac mitochondria from I/R injury could provide more clinical benefit when given prior to reperfusion.

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Outputs

Peer Review Articles

1. **Kumphune S.**, Surinkaew S., Chattipakorn S.C., Chattipakorn N. Inhibition of p38 MAPK activation protects cardiac mitochondria from ischemia/reperfusion injury. *Journal of Molecular and Cellular Cardiology*. 2013 (*Manuscript in preparation*)

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Role of p38 inhibition in cardiac ischemia/ reperfusion injury

Sarawut Kumphune, Siriporn Chattipakorn & Nipon Chattipakorn

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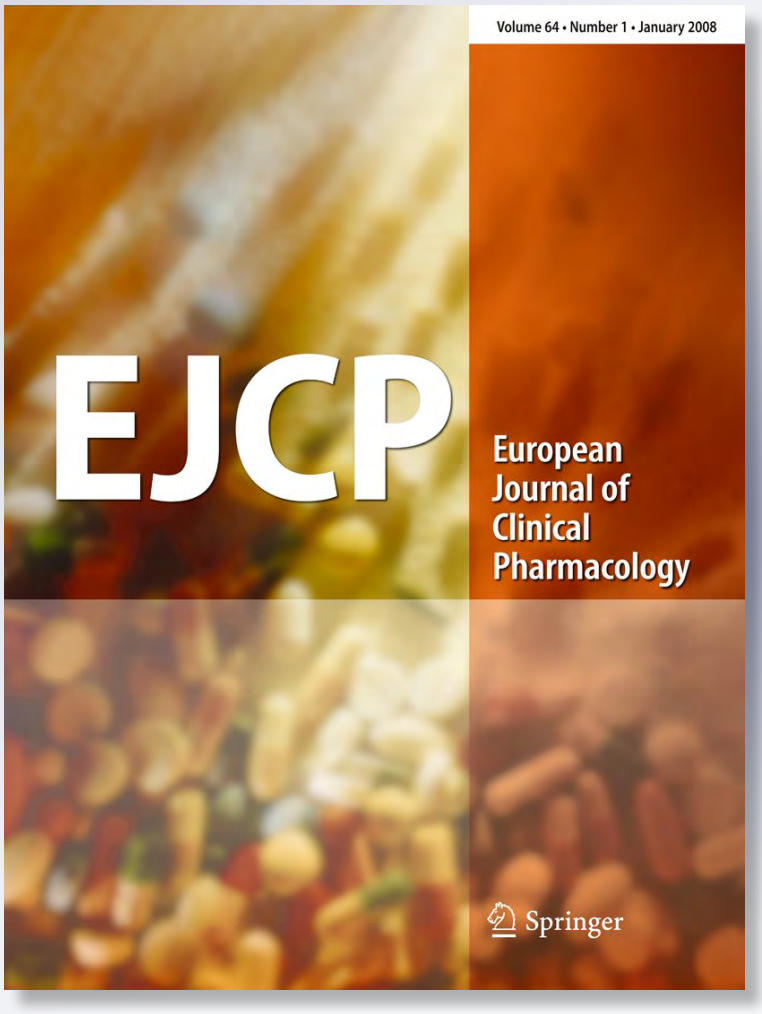

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REVIEW ARTICLE

Role of p38 inhibition in cardiac ischemia/reperfusion injury

Sarawut Kumphune · Siriporn Chattipakorn · Nipon Chattipakorn

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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

S. Kumphune
Department of Medical Technology,
Faculty of Allied Health Sciences, Naresuan University,
Phitsanulok 65000, Thailand

S. Kumphune · S. Chattipakorn · N. Chattipakorn (✉)
Cardiac Electrophysiology Research and Training Center,
Faculty of Medicine, Chiang Mai University,
Chiang Mai 50200, Thailand
e-mail: nchattip@gmail.com

S. Chattipakorn
Department of Oral Biology and Diagnostic Science,
Faculty of Dentistry,
Chiang Mai University,
Chiang Mai 50200, Thailand

N. Chattipakorn
Cardiac Electrophysiology Unit, Department of Physiology,
Faculty of Medicine, Chiang Mai University,
Chiang Mai 50200, Thailand

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-alpha (TNFα), 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 Thr^{180}-Gly^{181}-Tyr^{182} 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, p38α and β are highly homologous [26] and sensitive to pyridinyl imidazole molecules, such as SB203580 [27], but they have only 60% homology with p38γ and δ, which are resistant to SB203580 [27]. p38α 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 TNFα, 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 p38α 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 μM	1 hour before and during simulated ischemia	Cell viability by MTT assay	SB203580 increased cell viability	[50]
		1 μM	30 min before and maintain during 7 hr sI	Cell viability by MTT assay	SB203580 increased cell viability	[36]
	HL-1 cardiac muscle cell line	10 μM	SB before 45-min hypoxia SB before reoxygenation	Mitochondrial ROS, Apoptosis by propidium iodide (PI) staining	SB203580 decreased mitochondrial ROS and apoptosis	[63]
		10 μM	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 μM	During 2-h sI	LDH release, and cell viability by MTT assay	SB203580 increased cell viability, reduced LDH release	[61]
		1 μM	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]
	Isolate neonatal rat cardiac myocytes	1 or 10 μM	1 μM SB30 min before 2 h of hypoxia 1-or 10 μM 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 μM	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 μM	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 μM 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 μM	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]
	Isolate neonatal rat cardiac myocytes (NRCMs)	500 nM–1 μM RWJ	30 min before exposed to H_2O_2 for 24 h	TUNEL	RWJ reduced apoptosis	[33]
	Isolate adult rat ventricular myocytes (ARVMs)	1 and 10 μM	30 min before and during 4-h sI	LDH release	SB203580 reduced LDH release	[36]
	Isolate adult rat cardiac ribroblasts (ARCFs)	10 μM	20 min before exposured to H_2O_2	apoptosis by Annexin V-FITC	SB203580 reduced apoptosis	[55]
		10 μM	Throughout experiment of sI	Cell viability by MTS assay	SB203580 reduced apoptosis	[56]
Dog	Isolate cardiac cells	10 μM	During 3-h hypoxia	TUNEL	SB203580 reduced apoptosis	[62]
Rat	H9c2 Rat cardiac myoblast cell line	15 μM	1 hour before and during preconditioning and wash away after preconditioning	Cell viability by MTT	SB203580 blocked protective effect of IPC	[50]
Rabbit	Isolate ventricular myocytes	10 μM	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 μM SB203580	10 min before global ischemia	Infarct size	SB203580 reduced infarct size	[43]
	120 min of low-flow or moderate flow ischemia	1 μM 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 μM 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 p38α drug-resistant mice	- 1 or 10 μM SB203580 - 1 μM 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 p38α drug resistant (DR) mice - BIRB 796 reduced infarct size in both wildtype and p38αDR mice	[24]
Rat	Heart was perfused with 300 mM H_2O_2 for 80 min	1 mM SB203580	Perfused 5 min before exposure to H_2O_2	Developed pressure coronary flow, end-diastolic pressure, and creatine kinase in effluent	SB203580 reduced cell death and myocardial dysfunction	[47]
	30-min global ischemia/ 120 min reperfusion	10 μM 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 μM 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	Apoptosis by TUNEL, CK activity in effluent, Left ventricular function	FR 167653 reduced apoptosis, CK leakage, and improved cardiac contractile function during reperfusion	[44]
	25 min global ischemia/ 40 min reperfusion	20 μM 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 (10 min, 37°C) before normothermic reperfusion (30 min)	10 μM SB202190	during reperfusion	Left ventricular function	SB202190 improved Left ventricular function recovery	[45]
	30-min global ischemia/ 45-min reperfusion	10 μM 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 μM 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]
Rabbit	30-min focal ischemia/ 120-min reperfusion	1 μM SB203580	SB203580 5 min before I/R and 5 min after ischemia before reperfusion	Infarct size	SB203580 reduced infarct size	[38]
Mouse	30-min ischemia/120-min reperfusion in C57BL/6 or $mkk3^{-/-}$	1 μM 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 μM 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]
	4×4-min ischemia/6-min reperfusion followed by 30-min Ischemia/120-reperfusion in p38α or p38β DR mice	10 μM SB203580	During IPC	Infarct size	SB203580 abolished protective effect of IPC in wildtype and p38βDR but not p38αDR	[85]
Rat	30-min global ischemia/ 120-min reperfusion	10 μM 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 μM SB203580	5 min before first preconditioning cycle and wash out/5 min after preconditioning 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 SB203580 had no effect on infarct size	[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 μM 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, ±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	Cardiac output, LV pressure, End-systolic maximal elastance (EMax)	FR167653 improved heart-graft viability and cardiac function	[87]
	90-min occlusion/ 6-hr reperfusion	1.18 μg/kg/ min SB203580	during sustained ischemia	Infarct size	Continuous treatment of SB203580 during sustained ischemia reduced the infarct size	[52]
Mouse	Long-term treatment: regional ischemia/ reperfusion (LAD ligation) of MKK6bE transgenic mice	1200 ppm SB239068 in chow or food	SB239068 in drinking water for a total of 2–14 weeks (for hemodynamic and biochemical studies) or 12 weeks (for survival observations).	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	Long-term treatment with SC-409 for 2- to 14-weeks post-MI.	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).	-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	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 μM SB239063	Intramyocardial injection with SB239063 encapsulated in microsphere formulated from the polymer (PCADK) and inject (0.5 mg/ml corresponding to 2 μM 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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Inhibition of p38 MAPK During Ischemia, But Not Reperfusion, Effectively Attenuates Fatal Arrhythmia in Ischemia/Reperfusion Heart

Sirirat Surinkaew, BSc, Sarawut Kumphune, PhD,*† Siriporn Chattipakorn, DDS, PhD,*‡ and Nipon Chattipakorn, MD, PhD**

Abstract: The mitogen-activated protein kinases (MAPKs) play an important role in ischemia/reperfusion (I/R) injury. Previous evidence suggests that p38 MAPK inhibition before ischemia is cardioprotective. However, whether p38 MAPK inhibition during ischemia or reperfusion provides cardioprotection is not well known. We tested the hypothesis that p38 MAPK inhibition at different times during I/R protects the heart from arrhythmias, reduces the infarct size, and attenuates ventricular dysfunction. Adult Wistar rats were subject to a 30-minute left anterior descending coronary artery occlusion, followed by a 120-minute reperfusion. A p38 MAPK inhibitor, SB203580, was given intravenously before left anterior descending coronary artery occlusion, during ischemia, or at the onset of reperfusion. The results showed that SB203580 given either before or during ischemia, but not at the onset of reperfusion, decreased the ventricular tachycardia/ventricular fibrillation (VT/VF) incidence and heat shock protein 27 phosphorylation, and increased connexin 43 phosphorylation. The infarct size and cytochrome c level was decreased in all SB203580-treated rats, without the alteration of the total Bax/Bcl-2 expression. The ventricular function was improved only in SB203580-pretreated rats. These findings suggest that timing of p38 MAPK inhibition with respect to onset of ischemia is an important determinant of therapeutic efficacy.

Key Words: p38 MAPK, ischemia/reperfusion injury, arrhythmias

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INTRODUCTION

Acute myocardial infarction (AMI) is the main cause of death in most countries around the world. The World Health Organization predicts that the major cause of mortality in 2030 will still be ischemic heart disease.[1] Currently, the most effective method of reducing mortality in these patients is rapid reperfusion to reduce ischemic injury.[2] However, myocardial injury from reperfusion has been shown to lead to ventricular dysfunction, ventricular arrhythmias, and finally myocardial cell death.[3,4] Therefore, early reperfusion with the least amount of reperfusion injury and mortality after ischemia/reperfusion (I/R) remains an important clinical target.

Multiple mitogen-activated protein kinases (MAPKs) are known to play a crucial role in the myocardial response to cardiac ischemia. Growing evidence suggests p38 MAPK, one among 5 MAPK cascades, is activated by myocardial I/R.[5,6] Moreover, the inhibition of p38 MAPK activation, using direct (selective) agents[7] or by other pharmacological treatments,[8,9] has been shown to reduce myocardial infarct size and improve myocardial function after I/R injury. Although a number of studies demonstrated that the inhibition of p38 MAPK before ischemia was cardioprotective in ischemic heart models of isolated perfused rat and rabbit hearts,[5,10] as well as in the in vivo models of mouse and pig,[7,11] its effect on cardiac arrhythmias, cardiac function, and the infarct size administered after ischemia or during reperfusion is still unclear.[12] Because patients with AMI often arrive at the hospital after arterial occlusion, adoption of clinical treatments such as p38 MAPK inhibition will occur more rapidly if they can be administered following onset of symptoms.

In the present study, we determined the effects of p38 MAPK inhibitor, SB203580, on arrhythmias, cardiac function, and infarct size when administered at various times during I/R periods in the in vivo rat model. We tested the hypothesis that the inhibition of p38 MAPK after coronary artery occlusion and during reperfusion can protect the heart from fatal arrhythmias, reduce the infarct size, and improve ventricular functions, similar to inhibition of p38 MAPK before the coronary artery occlusion.

METHODS

Ethical Approval

This study was approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, Chiang Mai University, and the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care (NIH publication 85-23, revised in 1985). Adult male Wistar rats weighing 300–350 g were obtained from the National Animal Center, Salaya Campus,

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From the *Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand; †Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand; and ‡Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand.

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S. Surinkaew and S. Kumphune contributed equally in this work.

Reprints: Nipon Chattipakorn, MD, PhD, Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand (e-mail: nchattip@gmail.com).

Mahidol University, Bangkok, Thailand. All animals were fed with normal rat chow and water ad libitum before the study.

Surgical Preparation of Myocardial I/R Model in Rats

Rats were anesthetized using an intramuscular injection of zolitil (50 mg/kg) and xylazine (0.15 mL/kg). To assess the adequacy of anesthesia, the parameters such as responsiveness, blood pressure, and heart rate were monitored throughout the surgical procedures. Tracheostomy was performed, and the rat was ventilated with room air from a positive ventilator (model 683, Harvard Apparatus, MA), which was started immediately with a tidal volume of 8%–10% of body weight and ventilator rate of 70–110 breaths per minute to maintain PCO_2, PO_2, and pH parameters under physiological condition.[13] The electrocardiogram (ECG) lead II was recorded throughout the experiment. The right carotid artery was cannulated for measuring the left ventricular (LV) pressure and volume using the pressure–volume (P-V) conductance catheter (SciSense; Ontario, Canada). The left femoral vein was cannulated for the administration of drug or vehicle. A left thoracotomy was performed via the fourth intercostal space to expose the heart, and the pericardium was opened. The left anterior descending coronary artery (LAD) was identified and ligated at approximately 2 mm from its origin by a 5–0 silk suture with a traumatic needle.[14] Both ends of the thread were passed through a small vinyl tube to form a snare.[14] Ischemia was confirmed by an ST elevation on the ECG and the change in color of the myocardial tissue of the ischemic area. After 30 minutes of ischemia, the ligature was loosened, and the ischemic myocardium was reperfused for 120 minutes. At the end of the protocol, the hearts were quickly excised when the animals were deeply anesthetized. Sham-operated animals received all the above described surgical procedures, except that no LAD occlusion was performed.

Experimental Groups

Seventy-six rats were used in this study. Rats were randomly allocated to 1 of the 7 groups (Fig. 1A). In a sham group (n = 4), surgery was done without LAD occlusion. In the I/R group, rats were subject to a 30-minute LAD occlusion followed by a 120-minute reperfusion and were divided into 6 subgroups (n = 12/group) as illustrated in Figure 1A. In these six I/R subgroups, rats were assigned to receive either vehicle (normal saline solution) or 2-mg/kg SB203580HCl (Tocris, Ellisville, MO),[15] a p38 MAPK inhibitor, intravenously at 3 different time points of I/R: (1) 15 minutes before ischemia (pretreatment), (2) 15 minutes after the LAD occlusion (during ischemia), or (3) at the onset of reperfusion. Vehicle or drug of the same volume was administered intravenously via the femoral vein at 0.33 mL/min for 3 minutes.[14]

Arrhythmia Determination

After surgical preparation, the ECG lead II was recorded using PowerLab 4/25T (AD Instruments, Inc). Arrhythmias were characterized in accordance with the Lambeth Conventions,[16] and the scores were tabulated for the entire experimental period using a score described previously by Curtis and Walker.[17] The score was based on the frequency and duration of arrhythmias

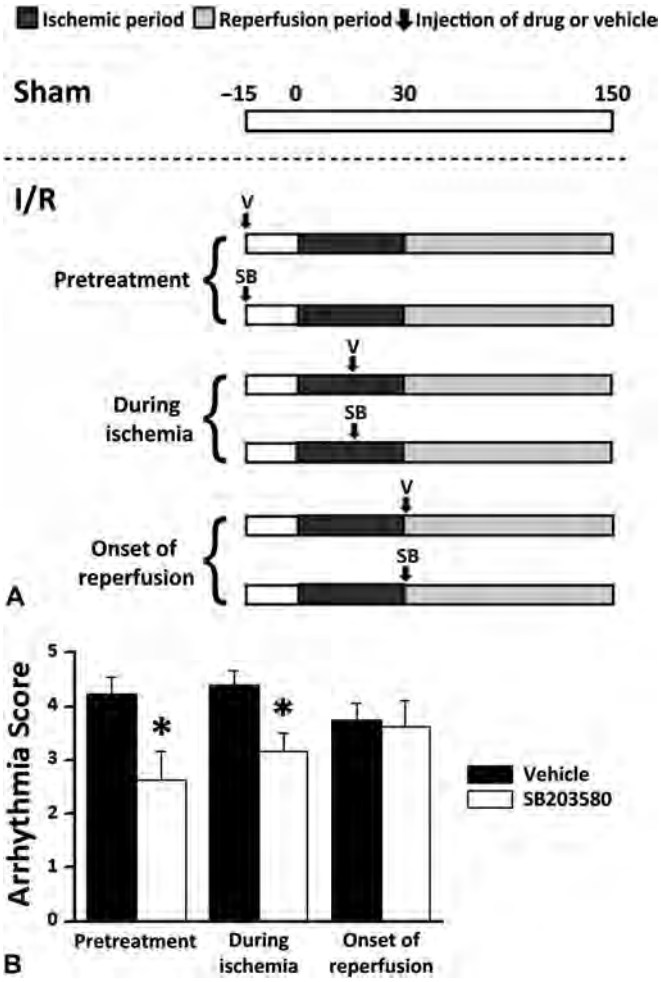

FIGURE 1. Study protocol and arrhythmia scores. Study protocol for experimental groups and timing of SB203580 (SB) or normal saline solution (vehicle, V) administration (A) and effect of SB203580 on the arrhythmia scores in ischemia/reperfusion (I/R) rats (B). *$P < 0.05$ versus vehicle group.

detected (1 was the lowest and 5 was the highest arrhythmia incidence).[18] The criteria of arrhythmia score was as follows: 0: <50 ventricular premature beats; 1: 50–499 ventricular premature beats; 2: >500 ventricular premature beats and/or one episode of spontaneously reverting ventricular tachycardia or ventricular fibrillation (VT/VF); 3: more than one episode of spontaneously reverting VT/VF (<1 minute total combined duration); 4: 1–2 minutes of total combined VT/VF; 5: >2 minutes of VT/VF.[17]

Cardiac Function Determination

The heart rate, end-systolic pressure (ESP) and end-diastolic pressure (EDP), maximum and minimum pressure (P_{max}, P_{min}), maximal (dP/dt_{max}) and minimum (dP/dt_{min}) slope of LV pressure waveform, stroke volume, cardiac output, and stroke work (SW) were measured and recorded using the PV loop system (SciSense) and were assessed 15 minutes before the LAD occlusion (baseline), after 30 minutes of ischemia, and at 90 minutes of the reperfusion period.

Infarct Size Determination

At the end of each experiment, the heart was excised and mounted on the modified Langendorff apparatus via the aorta.[19–21] Cold saline solution was used to flush out the blood, after which the LAD was reoccluded and 1-mL Evans blue dye (0.5%) was injected to define the area at risk.[22] Then, the hearts were frozen and sliced from apex to base into 7–8 transverse sections of approximately 1-mm thickness.[22] Heart slices were incubated in 1% buffered 2,3,5-triphenyltetrazolium chloride (TTC) (pH 7.4) at 37°C for at least 15 minutes to define the necrotic myocardium,[22] followed by placement in 10% formaldehyde (15–20 hours) to enhance the contrast between the stained and unstained TTC tissue.[23] The infarct area (TTC negative) and the nonischemic area (Evans blue–stained area) were determined by the ImageTool software version 3.0. The infarct size was calculated depending on the weight of each slice according to the formula of Reiss et al.[24–26]

Western Blot Analysis

At the end of each experiment, the heart was rapidly excised, and then the whole ventricular tissue was collected, quickly frozen in liquid nitrogen, and stored at −80°C until analysis. Heart proteins were lysed with extraction buffer (20 mmol/L Tris–HCl, 1 mmol/L Na_3VO_4, 5 mmol/L NaF) and separated by electrophoresis on 10% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then were transferred onto a polyvinylidene difluoride (PVDF) membranes. After immunoblots were blocked for 1 hour with 5% nonfat dry milk in Tris-buffer saline (pH 7.4) containing 0.1% Tween 20, they were probed overnight at 4°C with the primary antibodies that recognize phospho-HSP27 (Ser82); a downstream substrate of p38 MAPK, connexin 43 (Cx43) and phospho-Cx43 (Ser368); a marker of intercellular electrical communication, Bcl-2, cytochrome c (Cell Signaling Technology, Danvers, MA) and Bax (Santa Cruz Biotechnology, Inc, CA); a marker of apoptosis, and actin (Sigma-Aldrich, Tokyo, Japan); a loading control, followed by 1 hour of incubation at room temperature with the horseradish peroxidase–conjugated secondary antibody. The fold phosphorylation of Cx43 at Ser368 per total Cx43 was measured. The Cx43 antibody detected the total Cx43 protein at the molecular weight that ranged from 39 to 44 kDa and phosphorylated Cx43 (S368) at molecular weight ranging from 42 to 46 kDa. The blots were visualized by ECL reagent. The film images of the western blots were scanned and were analyzed using ImageJ (NIH image) analysis software.[27] For quantitation of the proteins of interest, phosphorylated proteins were normalized to total protein expression.

Statistical Analysis

All data are expressed as mean ± SEM, and statistics were calculated using SPSS (Statistical Package for Social Sciences, Chicago, IL). Drug-induced changes in arrhythmia scores, time to VT/VF onset, infarct size, as well as hemodynamic parameters at baseline, during ischemia, and reperfusion periods were analyzed using one-way ANOVA and followed by a Fisher least significant difference (LSD) test. Alterations of protein expression were analyzed using Mann–Whitney *U* test. Comparisons between groups for the VT/VF incidence and mortality rate were performed using a χ^2 test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Occurrence of Arrhythmias During I/R

There was no arrhythmia incidence in sham rats. In vehicle-treated I/R rats, before ischemia, during ischemia, or at the onset of reperfusion, arrhythmias were detected and defined as baseline arrhythmia score (4.2 ± 0.3, 4.4 ± 0.3 and 3.8 ± 0.3, respectively). After treatment with SB203580 either before LAD occlusion or during ischemia, the arrhythmia scores were significantly ($P < 0.05$) decreased compared with the vehicle-treated rats of each group (Fig. 1B). However, SB203580 administration at the onset of reperfusion did not reduce the arrhythmia scores (Fig. 1B). There were no significant differences in arrhythmia scores among all vehicle control groups. The occurrence of VT/VF was observed in all the vehicle-treated groups (Fig. 2A). Treatment of SB203580 either before LAD occlusion or during ischemia significantly reduced the VT/VF incidence during the whole period of I/R (38% and 33% reduction, respectively) (Fig. 2A). However, SB203580 treatment did not alter the occurrence of VT/VF, when given at the onset of reperfusion (Fig. 2A). The time to VT/VF onset after LAD occlusion was not different when vehicle or SB203580 was administered before ischemia, during ischemia, or at the onset of reperfusion (vehicle groups; 380 ± 17 seconds, 367 ± 19 seconds, 391 ± 18 seconds, SB203580 groups; 400 ± 15 seconds, 368 ± 31 seconds, 358 ± 22 seconds, respectively) (Fig. 2B).

SB203580 Inhibited p38 MAPK Activity

Assessment of the effectiveness of SB203580 to inhibit p38-MAPK activity was performed by measuring the phosphorylation of downstream substrate HSP27. In I/R rats, the phosphorylation of HSP27 was significantly ($P < 0.05$) increased in all groups compared with the sham group (Figure 3A). Administration of SB203580 significantly inhibited p38 MAPK activity, which in turn attenuated the phosphorylation of its downstream substrate HSP27 when given before and during LAD occlusion compared with vehicle-treated groups (Fig. 3A). However, SB203580 did not decrease the level of HSP27 phosphorylation when given at the onset of reperfusion (Fig. 3A).

SB203580 Reduced Incidence of Arrhythmia by Increasing Cx43 Phosphorylation

In myocardial ischemia, p38 MAPK activation could cause the dephosphorylation of Cx43 and induce the loss of cellular communication via gap function, resulting in cardiac arrhythmia. In the present study, we measured the effect of SB203580 on Cx43 phosphorylation. The results showed that the phosphorylation of Cx43 was significantly ($P < 0.05$) decreased in vehicle I/R groups compared with the sham group (Fig. 3B). However, SB203580 given before or during ischemia increased the phosphorylated Cx43 compared with the vehicle-treated group (Fig. 3B). SB203580 given at the

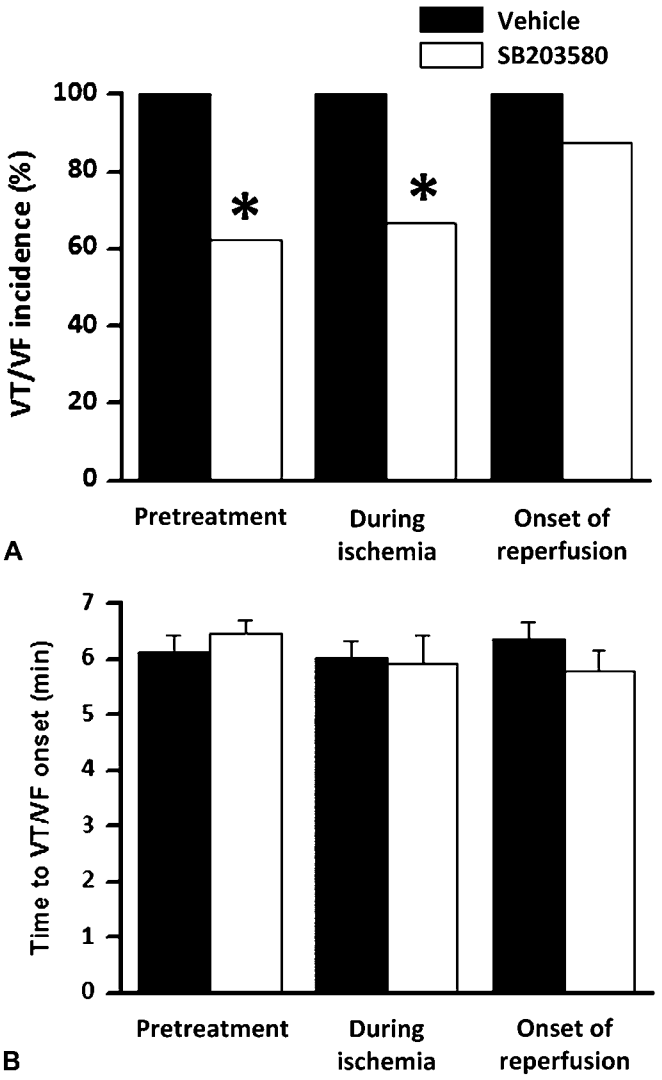

FIGURE 2. Arrhythmia determination. The incidence of VT/VF (A) and time to VT/VF onset (B) in ischemia/reperfusion rats. *$P < 0.05$ versus vehicle group.

onset of reperfusion did not alter the phosphorylated Cx43 (Fig. 3B).

Infarct Size

Administration of SB203580 at any times of I/R (ie, pretreatment, during ischemia, or at the onset of reperfusion) significantly decreased the infarct size (30%, 31%, and 27% reduction, respectively) compared with that in the vehicle-treated group (42 ± 4%, 46 ± 2% and 45 ± 3%, respectively) (Fig. 4).

Bax, Bcl-2, and Cytochrome c Level

The expression of Bax to Bcl-2 and the level of cytochrome c were increased in the vehicle-treated and SB203580-treated I/R rats compared with the sham group (Figs. 5A, B). Although the infarct size was decreased in the SB203580-treated groups, administration of SB203580 at any time of I/R injury did not change the ratio of Bax and Bcl-2 expression compared with the vehicle-treated rats of the same group (Fig. 5A). However, SB203580 administration at any times of I/R injury, pretreatment, during ischemia, or onset of reperfusion significantly ($P < 0.05$) decreased the mitochondrial cytochrome c release (6%, 2%, and 8% reduction, respectively) compared with that in the vehicle-treated group (Fig. 5B). Furthermore, there was no significant difference in mortality rate between vehicle- and SB203580-treated group either before ischemia, during ischemia, or at the onset of reperfusion (vehicle groups: 8%, 25%, and 33%; SB203580 groups, 17%, 33%, and 33%, respectively).

Effect of Intervention on Hemodynamic Parameters

Changes in hemodynamics in the I/R group are summarized in Tables 1–3. At the baseline, no differences in hemodynamic parameters were found between groups (Table 1). Our results showed that the ESP, and dP/dt_{max} were attenuated by the ischemia and reperfusion. Moreover, the stroke volume and cardiac output were significantly decreased during ischemia. Pretreatment of SB203580 partially improved ESP, P_{max}, P_{min},

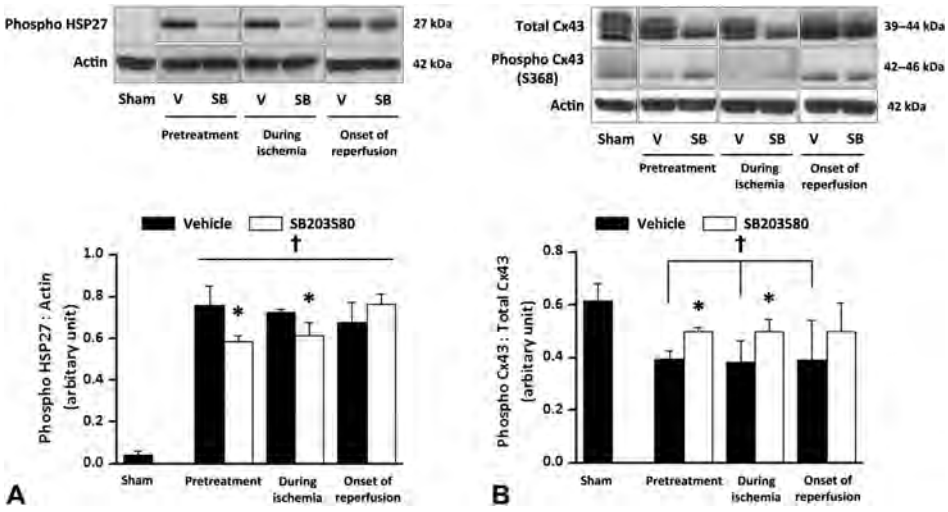

FIGURE 3. HSP27 and Cx43 phosphorylation. Effects of SB203580 (SB) on myocardial HSP27 phosphorylation (A) and Cx43 protein level (B) in heart tissue. Upper panels indicate representative immunoblots of myocardial rat ventricle tissue from each of the treatment regimes. Lower panels indicate quantitative data of phosphorylation of HSP27 and Cx43 normalized to total protein. Western blot analysis results were taken from the groups that were treated with vehicle (V) or SB before ischemia, during ischemia or at the onset of reperfusion in ischemia/reperfusion rats. †$P < 0.05$ versus sham group, *$P < 0.05$ versus vehicle group.

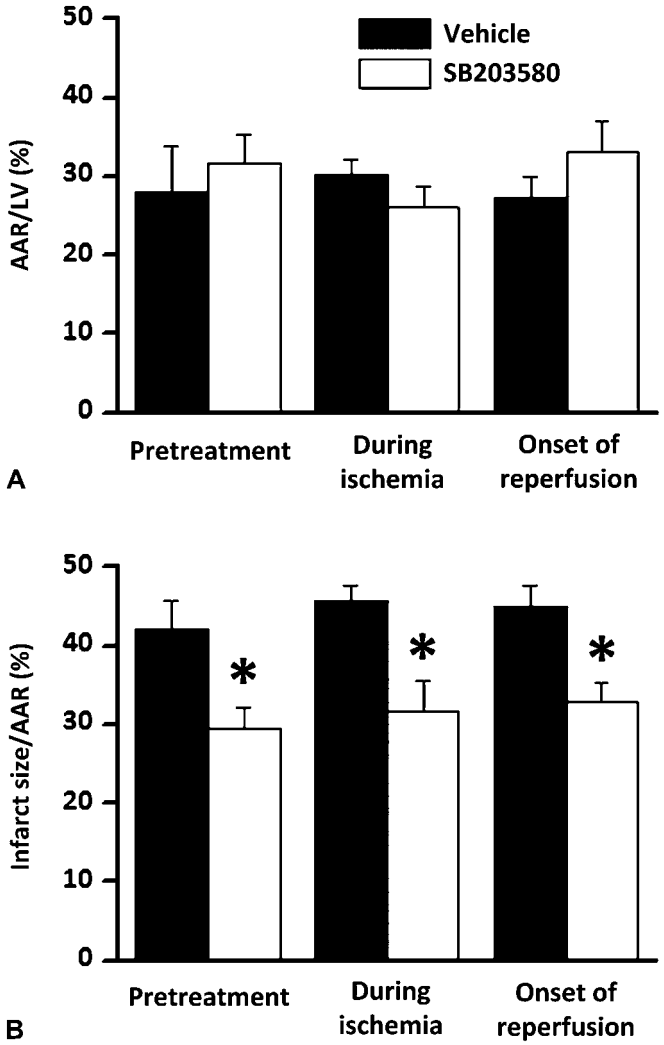

FIGURE 4. Infarct size determination. Effects of SB203580 on the infarct size in ischemia/reperfusion rats). A, The percentage of AAR/LV volume. B, The percentage of infarction/AAR. *$P < 0.05$ versus vehicle group. AAR, area at risk; LV, left ventricular.

dp/dt_{max} during ischemic and reperfusion periods. However, SB203580 administration during ischemia or at the onset of reperfusion period did not improve the hemodynamic parameters. Although SB203580 treatment could reduce the infarct size at any given study periods, the cardiac function could be improved only when SB203580 given before ischemia (Tables 2, 3).

DISCUSSION

The major findings of this study are that in the setting of I/R injury, administration of SB203580 before or during ischemia decreased the arrhythmia scores, the incidence of VT/VF, the phosphorylation of HSP27, and increase the Cx43 phosphorylation, whereas SB203580 given at the onset of reperfusion did not provide those beneficial effects. However, SB203580 decreased the infarct size in all I/R groups. Furthermore, SB203580 reduced cytochrome c release in all I/R groups without altering total Bax/Bcl-2 expression. LV function was improved only when SB203580 was administered before ischemia.

p38 MAPK has been shown to be involved in myocardial injury and cell death, and the inhibition of p38 MAPK activation in the ischemic heart has been shown to reduce cell death, infarct size, and attenuate the degradation of LV function.[3,5–8,11] Despite these potential cardioprotective benefits, the effects of p38 MAPK inhibitor given during myocardial I/R on postischemic fatal arrhythmias has never been elucidated. Ischemic-induced fatal arrhythmias are known to be responsible for high mortality in AMI patients.[28–30] Several studies demonstrated the beneficial effects of pharmacological interventions on cardiac electrophysiological alterations, such as decreased VF incidence and prolonged time to the first occurrence of VF during I/R.[25,26] However, this is the first study to demonstrate the effect of p38 MAPK inhibitor, SB203580, on cardiac arrhythmias during myocardial I/R in an in vivo rat model. In I/R rats, SB203580 given before or during ischemia decreased the incidence of VT/VF and the arrhythmia scores, whereas SB203580 given at the onset of reperfusion did not protect the heart from arrhythmia during I/R. The possible underlying mechanism of p38 MAPK inhibition in a reduction of arrhythmia could be due to the improvement in cellular communication via Cx43, which is the major gap junction protein found in adult mammalian hearts.[31]

During I/R, dephosphorylation of Cx43 occurred, leading to the loss of intercellular electrical communication via gap junctions in the ischemic heart, results in conduction abnormalities, and reentrant arrhythmias.[32,33] A previous study also demonstrated that in heterozygous Cx43 knockout mice subjected to acute ischemia, a higher incidence of arrhythmogenesis including an increase in the frequency of premature ventricular beats, spontaneous VT, and rapid onset of the first run of VT was observed.[4] Cx43 has been shown to be regulated by p38 MAPK under ischemia,[34] ischemic preconditioning,[34] and α-adrenergic stimulation.[35,36] Therefore, under ischemic condition, p38 MAPK activation could lead to dephosphorylation of Cx43,[34] resulting in decreased gap junction communication and increased occurrence of arrhythmias. However, the actual link between p38 MAPK, Cx43, and arrhythmogenesis in response to I/R has never been investigated.

The present study demonstrates that SB203580 given only before or during ischemia in I/R rats increased the phosphorylation of Cx43 at Ser368, which was associated with decreased arrhythmia scores and VT/VF incidence in the SB203580-treated rats. Because increased Cx43 phosphorylation at the Ser368 site has been shown to enhance myocardial resistance to ischemic injury,[37] the increased phosphorylated Cx43 by p38 MAPK inhibition could be responsible for arrhythmia reduction found in this study. Furthermore, phosphorylation of Cx43 at this site has been shown to limit the infarct size via the gap junction–dependent mechanisms.[38] Therefore, this together with decreased cytochrome c release could be responsible for infarct size reduction found in this study.

A previous study demonstrated that the activation of p38 MAPK reduced cardiac contractility in cultured rat cardiomyocytes, and that inhibition of p38 MAPK reversibly

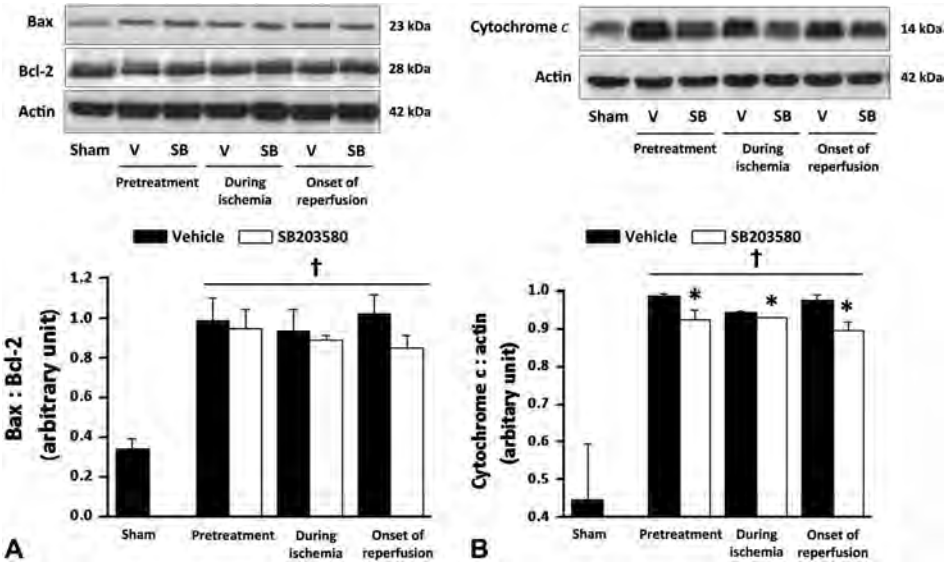

FIGURE 5. Bax, Bcl and cytochrome c expression. Immunoblots (upper panels) and quantitation (lower panels) of Bax and Bcl-2 expression (A) and the expression of mitochondrial cytochrome c release (B) in ventricle hearts treated with SB203580 (SB) before ischemia, during ischemia, or at the onset of reperfusion in ischemia/reperfusion rats, compared with the vehicle (V) control of each group †$P < 0.05$ versus sham group, *$P < 0.05$ versus vehicle group.

enhanced this effect by increasing the myofilament response to calcium.[39] We observed that timing of p38 MAPK inhibition with respect to onset of ischemia importantly modulated the effectiveness of the drug; attenuation of LV dysfunction was only observed when SB203580 was administered before the onset of ischemia, despite the fact that infarct size was decreased in all given periods of SB203580. This effect could be because of the protective effect of SB203580 on preventing the p38 MAPK activation, which was initiated during ischemia. Therefore, inhibition of p38 MAPK after ischemia, in which p38 MAPK was already activated, could be too late and thus did not attenuate cardiac dysfunction.

SB203580 action on p38 MAPK activation was confirmed by our findings that SB203580 significantly inhibited HSP27 phosphorylation, that is,. a downstream substrate of p38 MAPK activity, when given before or during myocardial I/R. However, its administration at the onset of reperfusion did not inhibit HSP27 phosphorylation. In myocardial I/R, it has been shown previously that p38 MAPK was activated during ischemia and was reactivated during reperfusion.[5] Our results that SB203580 effectively decreased HSP27 phosphorylation when given before ischemia as well as during ischemia but not at reperfusion, indicated that activation of HSP27 during ischemia occurred late after an LAD occlusion, whereas HSP27 reactivation occurred immediately after reperfusion. Although SB203580 did not reduce HSP27 phosphorylation when administered at the onset of reperfusion, it decreased the infarct size and the level of the cytochrome c release. This finding implied that the effect of SB203580 on the infarct size reduction may occur by means of not only the decreased HSP27 phosphorylation but also the other downstream transcription factors such as p53,[40] STAT1 (signal transducer and activator transcription 1),[41] and CHOP (C/EBP homologous protein),[42] which have been shown to involve in cellular apoptosis. However, the actual mechanism regarding the inhibition of p38 MAPK during reperfusion will need further investigation in the future.

TABLE 1. Cardiac Function Parameters at Baseline in I/R Rats Treated With Vehicle or SB203580

	Pretreatment		During Ischemia		Onset of Reperfusion	
Hemodynamic Parameters	**V**	**SB**	**V**	**SB**	**V**	**SB**
HR (beats/min)	264 ± 14	271 ± 18	265 ± 20	274 ± 12	262 ± 10	266 ± 8
ESP (mm Hg)	148 ± 7	164 ± 14	144 ± 6	149 ± 12	141 ± 9	157 ± 8
EDP (mm Hg)	15 ± 4	13 ± 6	13 ± 7	13 ± 6	14 ± 6	14 ± 3
P_{max} (mm Hg)	148 ± 6	167 ± 13	150 ± 6	150 ± 12	146 ± 8	161 ± 7
P_{min} (mm Hg)	49 ± 4	51 ± 3	47 ± 4	44 ± 6	46 ± 5	44 ± 4
dP/dt_{max} (mm Hg/s)	9577 ± 752	10,316 ± 631	8947 ± 1134	8766 ± 1169	7948 ± 1576	8882 ± 777
dP/dt_{min} (mm Hg/s)	−4304 ± 399	−4572 ± 467	−4253 ± 359	−4267 ± 413	−4442 ± 290	−4978 ± 391
Stroke volume (mL)	0.17 ± 0.01	0.20 ± 0.02	0.21 ± 0.02	0.19 ± 0.03	0.18 ± 0.02	0.18 ± 0.01
Cardiac output (mL/min)	50 ± 4	55 ± 7	48 ± 7	50 ± 9	44 ± 4	49 ± 5
Stroke work (mm Hg/mL)	16 ± 2	21 ± 3	18 ± 2	15 ± 5	14 ± 2	18 ± 2

*V, vehicle; SB, SB203580-p38 MAPK inhibitor; HR, heart rate; P_{max}, maximum pressure; P_{min}, minimum pressure; dP/dt_{max}, maximal slope of left ventricle pressure waveform; dP/dt_{min}, maximum slope of left ventricle pressure waveform.

TABLE 2. Cardiac Function Parameters at Ischemic Period in I/R Rats Treated With Vehicle or SB203580

	Pretreatment		During Ischemia		Onset of Reperfusion	
Hemodynamic Parameters	**V**	**SB**	**V**	**SB**	**V**	**SB**
HR (beats/min)	251 ± 15	274 ± 23	249 ± 14	280 ± 8	269 ± 15	290 ± 20
ESP (mm Hg)	124 ± 9	155 ± 6*	141 ± 10	126 ± 12	131 ± 10	133 ± 7
EDP (mm Hg)	15 ± 4	18 ± 4	15 ± 7	15 ± 9	15 ± 5	14 ± 4
P_{max} (mm Hg)	133 ± 7	159 ± 6*	145 ± 9	131 ± 11	134 ± 10	138 ± 8
P_{min} (mm Hg)	51 ± 4	61 ± 2*	51 ± 6	46 ± 7	50 ± 5	49 ± 4
dP/dt_{max} (mm Hg/s)	9580 ± 768	12,246 ± 414*	10,130 ± 1144	9281 ± 1366	10,063 ± 950	9822 ± 851
dP/dt_{min} (mm Hg/s)	−3611 ± 561	−4093 ± 320	−3875 ± 495	−3888 ± 503	−3430 ± 238	−3759 ± 444
Stroke volume (mL)	0.09 ± 0.01	0.11 ± 0.03	0.17 ± 0.02	0.12 ± 0.03	0.09 ± 0.02	0.10 ± 0.01
Cardiac output (mL/min)	25 ± 5	26 ± 4	39 ± 5	35 ± 8	24 ± 6	24 ± 3
Stroke work (mm Hg/mL)	7 ± 1	10 ± 2	11 ± 2	9 ± 3	8 ± 4	8 ± 2

*$P < 0.05$ versus vehicle.
V, vehicle; SB, SB203580-p38 MAPK inhibitor; HR, heart rate; P_{max}, maximum pressure; P_{min}, minimum pressure; dP/dt_{max}, maximal slope of left ventricle pressure waveform; dP/dt_{min}, maximum slope of left ventricle pressure waveform.

Previous studies demonstrated that SB203580 reduced the infarct size when it was given before ischemia in both the in vivo[7,11] and ex vivo rabbit I/R model.[10] In the present study, our results demonstrated further the beneficial effect of infarct size reduction by SB203580 given even during ischemia or at the onset of reperfusion. This beneficial effect was similar to when SB203580 was given before myocardial ischemia. These findings suggest that myocardial insult leading to myocardial cell death occurred later during reperfusion[43] as confirmed by equal infarct size reduction by p38 MAPK inhibitor given before or during ischemia and at the onset of reperfusion. This is also consistent with a previous report that myocardial ischemia alone only provoked myocardial damage, and that reperfusion activated apoptosis and increased the myocardial injury.[44] In apoptosis, Bax and Bcl-2 are proteins that play an important role in mitochondrial outer membrane permeabilization pore regulation. Bax promote pore formation on mitochondrial outer membrane, whereas Bcl-2 prevents this phenomenon. The releasing of cytochrome c from mitochondria also occurs as a process in apoptosis and regulates cellular morphological alteration in apoptosis. Therefore, Bax, Bcl-2, and cytochrome c have been used as marker proteins for apoptotic. Our results found that cytochrome c release was also decreased in all SB203580-treated groups, directly associated with infarct size reduction. However, the decreased infarct size was not related to total Bax/Bcl-2 expression. It is possible that this unchanged pattern of Bax and Bcl-2 was the result of the limitation of the method used in this study because we used the whole heart homogenate, rather than proteins extracted from the remote area, area at risk, and the infarct area. Furthermore, direct apoptosis assessment such as TUNEL assay was not performed in this study. The reduction in cytochrome c release was small and may not have significant physiological effect in this study. Nevertheless, our findings that SB203580 reduced cytochrome c level, without changing the Bax/Bcl-2 level, suggested that the decreasing of mitochondrial cytochrome c release may be involved in the suppression of mitochondrial permeability transition pore opening that prevented myocardial apoptosis.[45] Future studies

TABLE 3. Cardiac Function Parameters at Reperfusion Period in I/R Rats Treated With Vehicle or SB203580

	Pretreatment		During Ischemia		Onset of Reperfusion	
Hemodynamic Parameters	**V**	**SB**	**V**	**SB**	**V**	**SB**
HR (beats/min)	261 ± 16	263 ± 15	290 ± 8	253 ± 24	252 ± 13	281 ± 14
ESP (mm Hg)	141 ± 14	162 ± 11	167 ± 11	150 ± 13	129 ± 9	135 ± 16
EDP (mm Hg)	12 ± 3	17 ± 2*	14 ± 4	12 ± 10	13 ± 5	13 ± 8
P_{max} (mm Hg)	146 ± 10	163 ± 11	170 ± 10	154 ± 13	132 ± 9	140 ± 15
P_{min} (mm Hg)	42 ± 3	58 ± 2*	52 ± 6	46 ± 6	44 ± 5	44 ± 7
dP/dt_{max} (mm Hg/s)	7951 ± 782	11,553 ± 473*	10,446 ± 1229	9295 ± 1230	8788 ± 944	8805 ± 1372
dP/dt_{min} (mm Hg/s)	−4666 ± 749	−4558 ± 980	−5239 ± 574	−4477 ± 897	−4010 ± 730	−4530 ± 903
Stroke volume (mL)	0.09 ± 0.02	0.08 ± 0.02	0.14 ± 0.02	0.08 ± 0.03	0.08 ± 0.02	0.11 ± 0.01
Cardiac output (mL/min)	25 ± 5	20 ± 6	37 ± 3	24 ± 10	19 ± 4	29 ± 2
Stroke work (mm Hg/mL)	9 ± 2	7 ± 2	14 ± 1	8 ± 4	5 ± 2	8 ± 2

*$P < 0.05$ versus vehicle.
V, vehicle; SB, SB203580-p38 MAPK inhibitor; HR, heart rate; P_{max}, maximum pressure; P_{min}, minimum pressure; dP/dt_{max}, maximal slope of left ventricle pressure waveform; dP/dt_{min}, maximum slope of left ventricle pressure waveform.

are needed to verify the exact mechanisms of p38 MAPK-mediated apoptosis under myocardial I/R injury.

CONCLUSIONS

Our study demonstrated that the inhibition of p38 MAPK activation by SB203580 in an in vivo rat model before or during the coronary artery occlusion attenuated ischemia-induced fatal arrhythmias. However, SB203580 given at the onset of reperfusion did not reduce fatal arrhythmia in these I/R rats. These findings suggest that timing of p38 MAPK inhibition with respect to onset of ischemia is an important determinant of therapeutic efficacy.

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Roles of p38-MAPK in Insulin Resistant Heart: Evidence from Bench to Future Bedside Application

Sarawut Kumphune[1,2], Siriporn Chattipakorn[1,3] and Nipon Chattipakorn[1,4,*]

[1]*Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, THAILAND;* [2]*Biomedical Research Unit in Cardiovascular Sciences and Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000 THAILAND;* [3]*Department of Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai, 50200, THAILAND;* [4]*Cardiac Electrophysiology Unit, Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, THAILAND*

Abstract: Insulin resistance is associated with the impairment of the response of insulin receptor to insulin, resulting in the reduction of glucose uptake, leading to the alteration of myocardial glucose metabolism, impairment of cardiac electrophysiology, and increased susceptibility to ischemia-induced myocardial injury. Insulin resistance is associated with the impairment of the intracellular insulin signal transduction pathway. Among the MAPK family, p38-MAPK is a serine/threonine protein kinase, which has been shown to play an important role in cellular responses to various kinds of stress, including insulin resistance. Since growing evidence indicates the involvement of p38-MAPK in cardiovascular dysfunction, it is possible that the activation of p38-MAPK is responsible in part as a causative mechanism for cardiovascular complications in the insulin resistant heart. In addition, several anti-diabetic drugs have been shown to affect the myocardial p38-MAPK pathway. The effect of these drugs on p38-MAPK could be associated with their cardiovascular results in patients with insulin resistance. In this article, the signal transduction pathways of myocardial p38-MAPK activation in the insulin resistant heart, as well as the effects of anti-diabetic drugs on the myocardial p38-MAPK pathway, are comprehensively reviewed. Furthermore, the possible therapeutic approach regarding the utilization of a p38-MAPK inhibitor in diabetes patients to prevent cardiovascular complications is also addressed.

Keywords: Diabetes, insulin resistance, p38-MAPK, anti-diabetic drugs, cardiovascular complications.

INTRODUCTION

Diabetes mellitus impacts around 285 million people around the world and is expected to reach 438 million in less than 15 years [1]. It has been shown that diabetes patients have a two to four fold higher risk of cardiovascular disease than the non-diabetic subjects [2]. Insulin resistance is a prediabetic condition, which refers to the impairment of insulin receptor function in response to insulin binding in target tissues [3]. Previous reports from both pre-clinical and clinical studies demonstrated that insulin resistance is associated with cardiac dysfunction [4-8]. The impairment of intracellular insulin signaling, particularly in the insulin resistant heart, has been shown to be involved in the alteration of myocardial glucose metabolism,[9] impairment of cardiac electrophysiology, abolishment of the cardioprotective effect of insulin that worsens ischemia-induced myocardial injury,[10-12] alteration of systolic and diastolic function,[13] left ventricular (LV) hypertrophy, [14] dilated cardiomyopathy, and cardiac fibrosis [15].

p38-Mitogen Activate Protein Kinase (MAPK) is a serine/threonine kinase that is activated in response to variety of cellular stresses [16]. Growing evidence indicates that the activation of p38-MAPK during myocardial ischemia/reperfusion could aggravate lethal injury, and that inhibition of p38-MAPK activity, by using pharmacological inhibitors, could attenuate the myocardial injury and infarction [17-22]. Recently, evidence has strongly demonstrated the linkage between myocardial increased p38-MAPK activation and insulin resistance [23]. Moreover, since several anti-diabetic drugs have been shown to activate the myocardial p38-MAPK pathway, and possibly cause subsequent adverse effects on the heart [24-30], extensive understanding regarding the role of p38-MAPK and insulin resistance in the heart should provide promising therapeutic as well as preventive strategies in diabetes patients in the future. In this article, the comprehensive review of the association between the signaling pathways of insulin resistance and their involvement in myocardial p38-MAPK activation, as well as the effect of anti-diabetic drugs on the myocardial p38-MAPK pathway is presented, and the consistent as well as controversial reports regarding these issues are discussed.

*Address correspondence to this author at the Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, THAILAND; Tel: +66-53-945329;
Fax: +66-53-945368; E-mail: nchattip@med.cmu.ac.th

INSULIN RESISTANCE AND MYOCARDIAL P38-MAPK

Insulin resistance is a pre-diabetic condition in which hyperinsulinemia with euglycemia can be observed [31]. It is well known that obesity, aging, and inappropriate lifestyle are major risk factors for developing insulin resistance [32]. Chronic inflammation together with increased plasma insulin and free fatty acid levels have been reported under an insulin resistant condition [3]. Interestingly, the activation of p38-MAPK has been demonstrated since the development of insulin resistance [23]. A progression from insulin resistance that leads to type 2 diabetes is characterized by the presence of a hyperglycemic condition. The biochemical changes occurring in both insulin resistance and type 2 diabetes are capable of activating p38-MAPK as illustrated in (Fig. **1**).

Hyperinsulinemia and High Plasma Free Fatty Acid Activate p38-MAPK

The major insulin signaling pathway impairment downstream of insulin receptor is the signaling through the phosphatidylinositide-3 kinase (PI3K)/Protein Kinase B (PKB, or Akt) pathway [33]. In the heart, a balance between the PI3K-Akt signaling cascade and the classical mitogenic cascade (Ras-ERK) co-operatively maintains normal cardiac growth, metabolism, and functions [34]. It has been shown that insulin resistance predominantly affects the PI3K-Akt cascade, rather than the Ras-ERK cascade, by de-sensitizing the insulin receptors [34].

Plasma free fatty acid (FFA) has been shown to be related to insulin resistance. The increase in plasma FFA has been suggested

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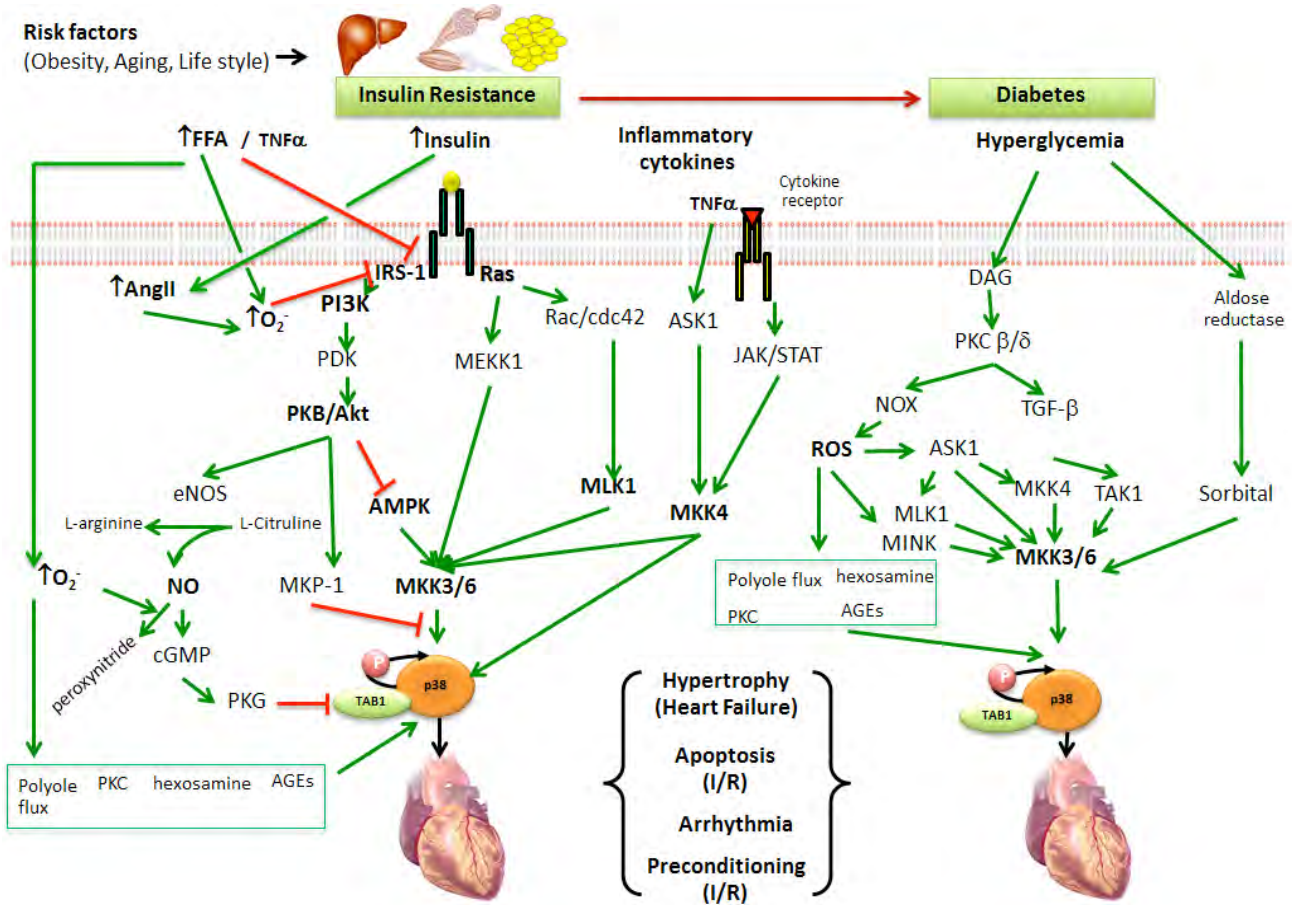

Fig. (1). Signal transduction of p38-MAPK activation in insulin resistance.
The biochemical changes in insulin resistance such as increase in free fatty acid and plasma insulin, as well as chronic inflammation and, lead to p38-MAPK activation. The major signaling pathway affected by the insensitivity of insulin receptor is PI3K/Akt cascade. p38-MAPK can be indirectly activated via the augmentation of the signal inactivation mechanism. Inflammatory cytokines can activate the MAPK kinase upstream of p38-MAPK. In addition, hyperglycemia that occurs as a result of the progression to diabetes can also activate p38-MAPK, predominantly by reactive oxygen species and diacylglycerol, via an activation of MAPK kinase upstream of p38-MAPK.

to be associated with the impairment of insulin signaling and responses, which consequently lead to p38-MAPK activation in the heart. In this regards, FFA has been shown to inhibit the association and activation of insulin receptor substrate-1 (IRS-1) to the intracellular compartment of insulin receptor, which subsequently reduced the PI3K-Akt activation [35; 36]. Normally, activated PKB/Akt can phosphorylate AMP-activated protein kinase (AMPK),[37] leading to the inactivation of AMPK, which is an upstream activator of MKK3/6-p38-MAPK [38; 39]. Therefore, reduction of PI3K-Akt activation in insulin resistance could indirectly activate p38-MAPK (Fig. **1**). In addition, impairment of PI3K-Akt signaling could reduce the phosphatase activity of MAPK phosphatase-1 (MKP-1) and hence activate p38-MAPK [40; 41]. Interestingly, the impairment of PI3K-Akt activation has been shown to reduce the nitric oxide (NO) production via endothelial nitric oxide synthase (eNOS) phosphorylation and hyperglycemia-induced eNOS glycosylation by O-GlcNAc modification [34]. Since NO has been shown to activate cGMP and protein kinase G-I (PKG I), which subsequently interfere with TAB1 association and induction of p38-MAPK autophosphorylation [42], the reduction of NO as a result of impaired PI3K-Akt activation could further increase p38-MAPK activation (Fig. **1**).

In addition, high plasma FFA concentration has been shown to increase the myocardial fatty acid uptake rate and oxidation. The β-oxidation of fatty acid in cardiomyocytes primarily occurs in the mitochondria and for a less extent in myocardial peroxisomes [43; 44], where uncoupling of oxidative phosphorylation as well as ROS production occurred [45; 46]. The increased ROS production and oxidative stress have been shown to cause stress-sensitive serine/threonine kinase signaling activation, which was capable of phosphorylating IR and IRS protein in serine and threonine residues, thus reducing the sensitivity of tyrosine phosphorylation by insulin stimulation [47; 48]. Furthermore, it has been shown that FFA not only induces oxidative stress, but also reduces the intracellular antioxidant molecules such as glutathione [49; 50]. This phenomenon aggravates the insulin signaling impairment, thus worsening the insulin resistant condition.

The overproduction of mitochondrial superoxide has been shown to increase the formation of advanced glycation end products (AGEs), polyole pathway flux, activation of protein kinase C (PKC) isoforms, and increase the activity of hexosamine pathway [51]. These metabolic products have been shown to activate p38-MAPK [52-56] (Fig. **1**). Under an insulin resistant condition, the pancreatic cells produce a high amount of insulin. The hyperinsulinemia in insulin resistance is known as a strong stimulant of a classical Ras-MEKK1signaling pathway, which possibly indirectly activates the MKK3/6-p38-MAPK signaling [57; 58]. In addition, Ras phosphorylation could also activate Rac/cdc42 and subsequently activate

MLK1, which is an upstream kinase activated MKK3/6 –p38-MAPK cascade,[59; 60] thus leading to increased p38-MAPK activation (Fig. **1**). Furthermore, hyperinsulinemia has been shown to increase the plasma level of angiotensin II (Ang II), an octapeptide that is a potent vasopressor, via the hyperinsulin- induced Renin-Angiotensin system [61]. Increased Ang II not only promotes vasoconstriction, but also produces superoxide, which can mediate the conversion of NO to peroxynitrite and resulting in the reduction of NO bioavailability,[62] which could restrain the NO-cGMP-PKG I pathway and enhance TAB1-induced p38-MAPK autophosphorylation (Fig. **1**) [42].

Inflammatory Cytokines Induce p38-MAPK Activation

In an insulin resistant condition, inflammatory cytokines, such as TNF-α, IL-6, IL-1, IL-18, monocyte chemotactic protein (MCP)-1, and C-reactive protein (CRP), have been released [63-68]. These cytokines have been shown to activate p38-MAPKvia either the apoptosis signal-regulating kinase 1 (ASK1) pathway or JAK/STAT-MKK4 pathway [69]. Moreover, it has been shown that TNF-α could induce serine phosphorylation and reduce tyrosine phosphorylation of IRS-1, resulting in the impairment of the IRS-1/PI3K/Akt pathway [70]. Less Akt phosphorylation under this circumstance could attenuate NO generation, which could indirectly stimulate TAB1-induce-p38-MAPK autophosphorylation as previously discussed,[42] leading to increased p38-MAPK activation.

Progression of Insulin Resistance to Type-2 Diabetes and p38-MAPK Activation

One of the most serious progressions of insulin resistance is its transition to type 2 diabetes, a condition in which the plasma glucose level is increased above the normal reference range. The hyperglycemic condition has been shown to activate the aldose reductase activity and cause the generation of sorbital,[71-73] which is known as a potent activator of p38-MAPK (Fig. **1**) [74]. Furthermore, the increasing in the plasma glucose level also enhances *de novo* synthesis of diacylglycerol (DAG) and consequently activates protein kinase C (PKC) [75]. The PKC activation is an important signal transduction, which triggers the transforming growth factor beta 1 (TGF-β) activation [52], which subsequently activates the MKK3/6-p38-MAPK cascade (Fig. **1**) [55].

Hyperglycemia is also known to cause increased reactive oxygen species (ROS) production via mitochondrial ROS generation pathway, as described in the previous session, which could lead to NAD(P)H oxidase (NOX) activation [76]. NOX-derived ROS has been shown to activate various redox-sensitive kinases such as Misshapen/NIKs-related kinase (MINK),[77] which is capable of activating the MKK3/6-p38-MAPKpathway [78]. NOX also generates further amounts of ROS, particularly superoxide anion (O_2^-) and increases the overall oxidative stress. The elevation of the O_2^-level could also result in the formation of advanced glycation end products (AGEs), polyole pathway flux, activation of protein kinase C (PKC) isoforms, and increased activity of hexosamine pathway [51], which could activate p38-MAPK [52-56], as previously discussed (Fig. **1**). Moreover, high superoxide ion could lead to the reduction of NO bioavailability,[79] resulting in TAB1-induced p38-MAPK autophosphorylation,[42] and finally activating p38-MAPK (Fig. **1**). In addition, high plasma glucose also activates apoptosis signal-regulating kinase 1 (*ASK1*) [80], which is the MAPKK kinase capable of dual activating JNK and p38-MAPK via MKK4 and MKK3/6, or mixed linage kinase 1(MLK)1 (Fig. **1**) [81].

It has been reported that the activation of myocardial p38-MAPK plays an important role in the pathophysiological mechanism of cardiomyopathies such as ischemia-induced cardiomyocyte injury and apoptosis,[19] cardiac arrhythmia,[82] and cardiac hypertrophy [83]. Since several biochemical changes occurring under an insulin resistant condition could lead to an activation of p38-MAPK in the heart, this association may be a mechanistic explanation of cardiac complications found in insulin resistant subjects [7; 84-90]. Therefore, targeting p38-MAPK activation by using a p38-MAPK inhibitor in insulin resistance may be a promising therapeutic strategy to prevent or reduce early cardiac complications in this group of patients.

EFFECT OF ANTI-DIABETIC DRUGS ON MYOCARDIAL P38-MAPK SIGNALING

Anti-diabetic drugs have been used for many decades for glycemic control as well as to enhance insulin sensitivity [91]. Although these drugs are shown to provide therapeutic benefits in patients with insulin resistance and diabetes, some of these anti-diabetic drugs have been shown to cause adverse effects in the heart [3]. Since p38-MAPK plays an important role in facilitating undesirable cardiac effects in insulin resistance and diabetes, it is necessary to understand the association between these anti-diabetic drugs and their effects on p38-MAPK activation. In this section, the molecular mechanism of anti-diabetic drugs that have been reported to affect myocardial p38-MAPK will be comprehensively discussed.

Thiazolidinediones (Pioglitazone, Rosiglitazone, Ciglitazone, Troglitazone)

Thiazolidinediones (TZD) was introduced to the pharmaceutical market in 1997 [92]. The main mechanistic action of these drugs is their ability to stimulate nuclear peroxisome proliferators activated receptor (PPAR)-γ, which is known to control the transcription process of genes enhancing insulin sensitivity [93]. TZD acts through its association to the PPAR-γ receptor and subsequently mediates retinoic-X receptor (RXR-receptor) binding. This receptor activation initiates the transcription process of genes that are involved in carbohydrate and lipid metabolism and suppress the expression of TNF-α [94]. Although the TZD has been shown to be beneficial in many studies, the results from meta-analysis of clinical studies on the cardiovascular effects of TZD revealed that TZD is associated with significantly higher odds ratios of congestive heart failure and myocardial infarction [95]. Currently, the drugs in this group are no longer available for use in diabetes patients in many countries.

In pre-clinical studies, TZD has been shown to affect the myocardial p38-MAPK signaling and consequently resulting in various undesirable biological responses in the heart [96-99]. During ischemia/reperfusion (I/R), p38-MAPK activation has been shown to mediate cellular apoptosis [100-102]. Treatment of rosiglitazone was shown to reduce cardiomyocyte death by inhibiting p38-MAPK activation (Fig. **2**) [103]. Similarly, treatment of pioglitazone in the ischemia/reperfusion model also reduced the infarct size, preserved mitochondrial ultrastructure, down regulation of pro-apoptotic protein Bax, and upregulation of anti-apoptotic protein Bcl-2 (Fig. **2**) [28]. This anti-apoptotic activity was mediated by down regulation of p38-MAPK [28]. However, Ye *et al* reported inconsistent findings in which pioglitazone could reduce the infarct size in an ischemia/reperfusion model, but its mechanism was not associated with p38-MAPK (Table **1**) [104]. Although using a similar animal model and study protocol of ischemia/reperfusion, the dosages and routes of drug administration were different between those studies. A higher concentration of the drug, given intravenously, could down-regulate p38-MAPK, whereas oral administration of the less concentrated drug did not affect p38-MAPK activation (Table **1**). This could possibly explain the inconsistency between these two studies. In addition, pioglitazone was shown to reduce the duration of atrial fibrillation (AF) without any changes in p38-MAPK signaling,[29] whereas rosiglitazone was shown to increase the ventricular fibrillation (VF) incidence [105]. However, the latter study still lacks the information of the linkage between the activation of p38-MAPK and those arrhythmia incidences.

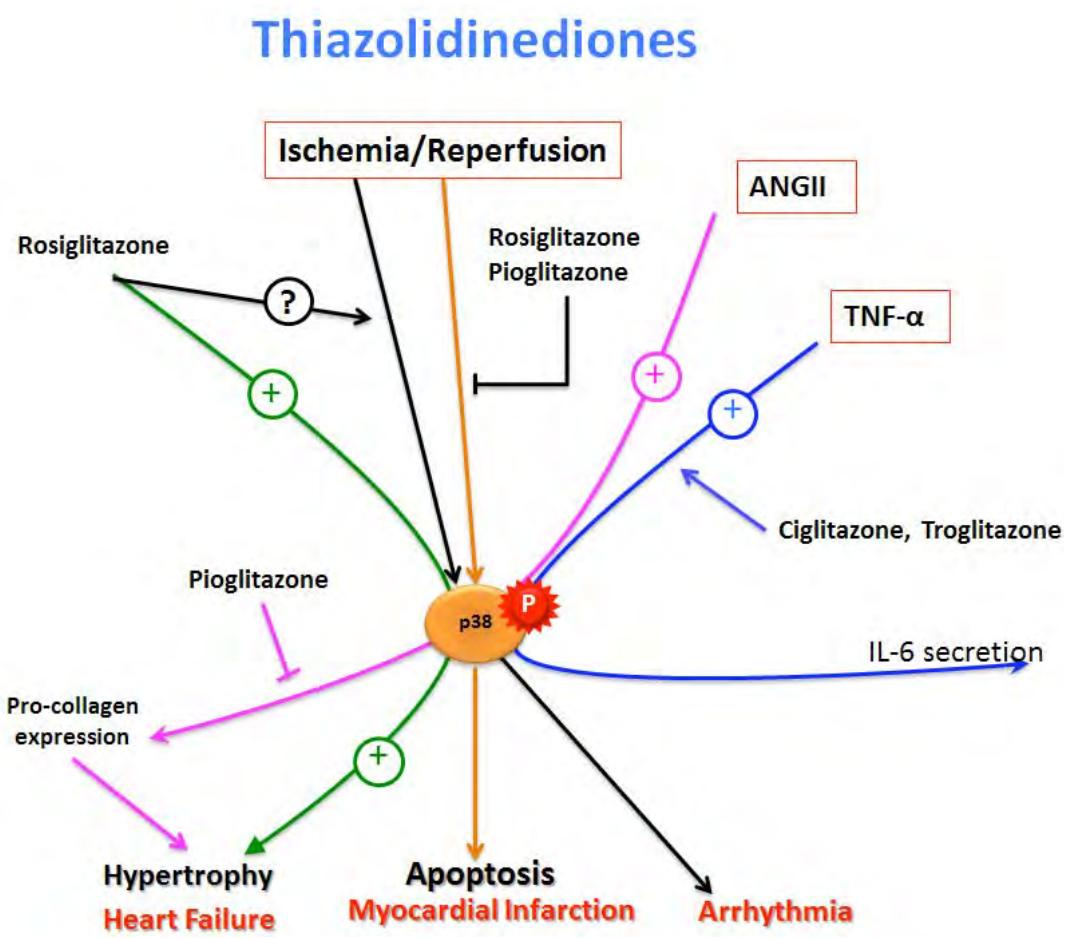

Fig. (2). Effect of Thiazolidinediones on p38-MAPK activation in the insulin resistant heart. Thiazolidinediones cause p38-MAPK activation and consequently result in various biological responses in the heart. Ischemia/Reperfusion cause p38-MAPK to mediate cellular apoptosis. Rosiglitazone and pioglitazone reduced cardiomyocytes death via inhibiting p38-MAPK activation. However, rosiglitazone could inhibit ischemia but induced fatal arrhythmia. Treatment cardiomyocytes with rosiglitazone induced p38-MAPK activation and hypertrophy. However, pioglitazone could inhibit p38-MAPK mediate angiotensin II induced pro-collagen expression, cell length and hypertrophy.

TZD has also been shown to induce cardiac hypertrophy [106]. Rosiglitazone has been shown to induced p38-MAPK activation and hypertrophy (Fig. **2**) [27]. In contrast, pioglitazone has been shown to have anti-hypertrophic effect without changes in the p38-MAPK activation (Table **1**) [25; 107]. Although treatment of pioglitazone alone could not affect the p38-MAPK, the combined treatment of pioglitazone with pravastatin has been shown to inhibit p38-MAPK mediated angiotensin II induced pro-collagen expression and hypertrophy [25]. These findings suggested the detrimental effect of p38-MAPK activation, which seem to be prevented by TZD administration. However, TZD (ciglitazone and troglitazone) have also been shown to enhance TNF-α induced IL-6 expression in cardiac fibroblast, with this activity requiring p38-MAPK activation [30]. Since the pro-inflammatory cytokines has been shown to act as a strong modulator for cardiac fibrosis,[108] receiving TZD could possibly cause cardiac fibrosis. All of these studies suggested that the effects of drugs in the TZD group on p38-MAPK depended in part on the concentration and route of administration. These findings regarding the effect of TZD on p38-MAPK are summarized in (Table **1**).

Biguanides (Metformin)

Metformin has been available since 1950s when it was reported to reduce blood glucose levels in patients [109]. The molecular mechanisms of the action of metformin is thought to improve insulin sensitivity via its modification of the insulin signaling pathway at a post-receptor level, possibly AMPK [110; 111]. Metformin has been shown to reduce gluconeogenesis and glycogenolysis via the increased hepatic insulin sensitivity, and increased glucose uptake by the upregulation of glucose transporter (GLUT)-4 and GLUT-1 transporters [112].

Evidence demonstrated that metformin could affect myocardial p38-MAPK signaling in the heart. The purposed mechanism of metformin influence p38-MAPK activation is illustrated in (Fig. **3**). Treatment of cardiac cells with metformin increased AMPK-induced p38-MAPK activation, a similar process found in p38-MAPK activation in myocardial ischemia or in cell treated with AMP analog, so-called AICAR [113]. An *in vitro* treatment of metformin potentially activates p38-MAPK-mediate Bax translocation in response to ischemia/reperfusion, resulting in increased cellular apoptosis (Fig. **3**) [24]. It is noteworthy that this is the only study that suggested that metformin could possibly aggravate cardiac cell death during ischemia/reperfusion injury and led to a doubt in the safety issue of using this drug. In contrast, several *in vivo* studies demonstrated the cardioprotective ability of metformin in the ischemia/reperfusion model [114; 115]. However, the evidence regarding the direct effect of the drug on myocardial p38-MAPK activation in ischemia/reperfusion studies has not been mentioned. Besides the ischemia/reperfusion study, metformin administration demonstrated a beneficial effect to the heart by enhancing intracel-

Table 1. Effect of Thiazolidinediones on Myocardial p38-MAPK Activation.

Mode of Drug	Drugs	Dosage	Study model	Outcome	Effect on p38-MAPK	References
Insulin Sensitizers	Ciglitazone Troglitazone Rosiglitazone	10 μM for 1 hour pretreatment	*In vitro*: Isolated human cardiac fibroblast stimulated with 0.1-10 ng/ml TNF-α	Increased TNF-α induced IL-6 secretion by ciglitazone and troglitazone, but not rosiglitazone	↑p38-MAPK	[30]
	Pioglitazone	400 mg /kg for 2 weeks	*In vitro*: - Adult rat ventricular cardiomyocytes - Transgenic mice KO-PPARγ - Transgenic rats overexpressing renin	Pioglitazone reduced cell length in wild-type, not PPARγ KO mice, or PPARγ KO mice + pioglitazone	No change on p38-MAPK	[107]
		1-10 μM for three hours pretreatment Pioglitazone treatment in combination with 0.1μM pravastatin	*In vitro*: Isolated mouse cardiac fibroblast stimulated with 1μM ANGII for 24h	5 μM Pioglitazone decreased ANGII- induced NADPH oxidase expression, superoxide anion production, procollagen-1 expression	Pioglitazone alone had no change on p38-MAPK Pioglitazone + pravastatin ↓ p38-MAPK	[25]
Insulin Sensitizers	Pioglitazone	5 mg/kg orally daily for 14 days before the onset of tachypacing and continued for 28 days	*In vivo*: Rabbit + Ventricular tachypacing at 380-400 bpm for 4 weeks ±pioglitazone	Pioglitazone reduced the duration of AF, attenuate atrial structural remodeling, reduction in interatrial activation time, and atrial fibrosis Reduced TGF-β1, TNF-α	No change on p38-MAPK but ↑ERK1/2	[29]
		3 mg/kg, i.v. 24 hours before I/R	*In vivo*: Adult male Sprague-Dawley rats subjected to 30 minutes Ischemia/4 hours reperfusion	Pioglitazone reduced infarct size, mitochondrial ultrastructure damage, downregulation of Bax, upregulation of Bcl-2	Down-regulation of p38-MAPK	[28]
		2.5 mg/kg/d by oral gavage five days before I/R	*In vivo*: Adult male Sprague-Dawley rats subjected to 30 minutes Ischemia/4 hours reperfusion	Pioglitazone reduced infarct size. The effect was additive in combination of TAK-491 and Pioglitazone. The left ventricular functions were improved. Only Pioglitazone decreased COX2 expression	No change on p38-MAPK	[104]
	Rosiglitazone	10 mg/kg BW/day in chow for 4 weeks	*In vivo*: Cardiac specific PPARγ knockout mouse	Rosiglitazone induced hypertrophy in both control and knockout mice.	↓p38-MAPK	[27]
		3 mg/kg/day oral gavage 5 weeks post-ischemia	*In vivo*: Adult male New Zealand white rabbits fed with high cholesterol diet for eight weeks, subjected to 60 min Ischemia/four hours reperfusion	Rosiglitazone reduced high cholesterol increased post-ischemia myocardial apoptosis	↓ p38-MAPK	[103]

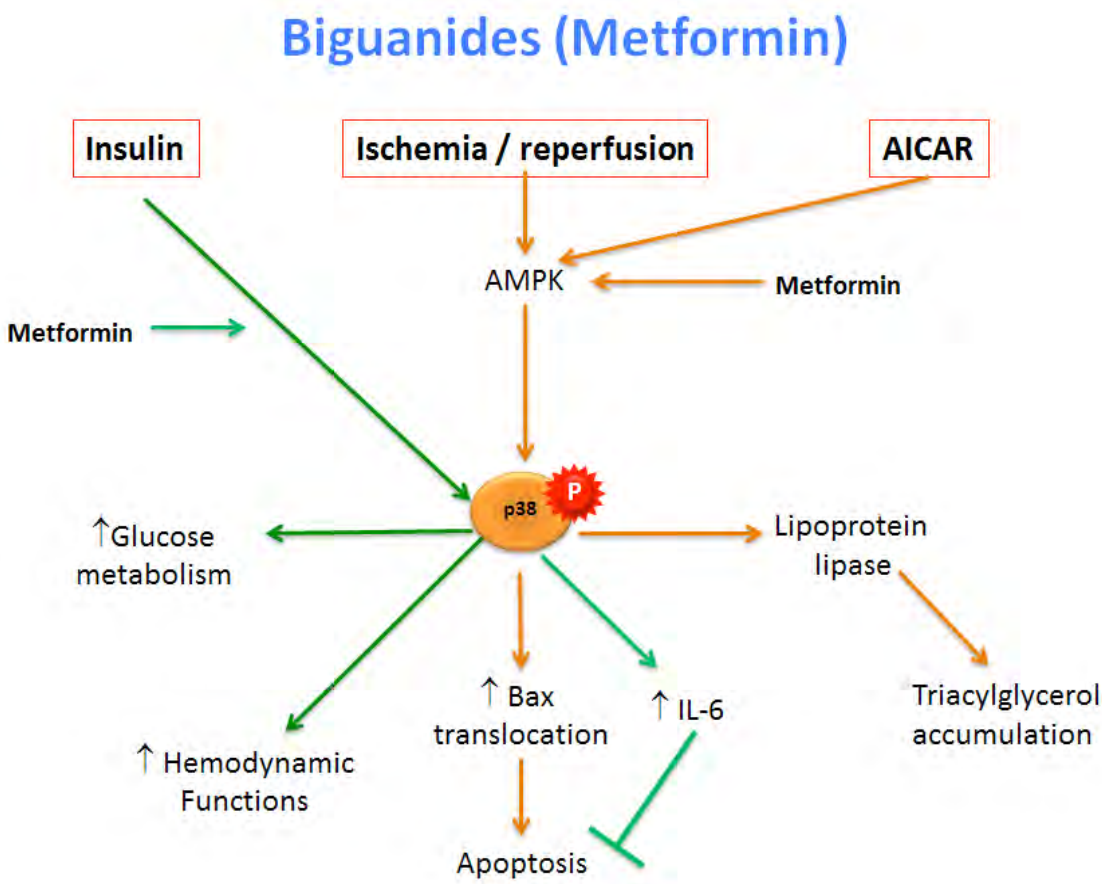

Fig. (3). Effect of biguanides drugs on p38-MAPK activation in the insulin resistant heart. Metformin increased AMPK activity, activated p38-MAPK-mediated Bax translocation in response to ischemia/reperfusion and resulted in increased cellular apoptosis. However, activation of p38-MAPK by metformin could increase IL-6 production, which is believed to be a late cardioprotection mechanism. Moreover, metformin could enhance intracellular metabolism and cardiac function via activation of p38-MAPK.

lular metabolism and cardiac function via p38-MAPK activation [116]. The previous study by Du *et al* demonstrated that activation of p38-MAPK by metformin could increase IL-6 production,[26] which is claimed as a late cardioprotective mechanism [117; 118]. Moreover, activation of p38-MAPK, together with other survival kinases, in ischemia preconditioning has been shown to protect the heart from ischemia/reperfusion injury and death [119; 120] . At this point, it could be speculated that the cardioprotective effect of metformin, seen in *in vivo* ischemia/reperfusion studies,[26; 116] may be due to metformin-induced p38-MAPK activation. Although p38-MAPK activation can cause an undesirable effect in the heart, metformin may exert its cardioprotection by pharmacological pre-conditioning. Since the association between metformin and p38-MAPK in the heart is still limited, intensive investigation on molecular signaling of metformin preconditioning associated with p38-MAPK will provide useful information and better understanding regarding the cellular effects of this drug. The studies concerning the effect of metformin on myocardial p38-MAPK are summarized in (Table **2**).

Sulfonylureas (Glibenclamide, Gliclazide, Glipizide, Glimepiride)

Anti-glycemic activity of sulfonylureas was first discovered in 1942 and was not used as anti-diabetic drug for type 2 DM until 1955 [3]. The mechanism of action is known to directly enhance glucose induced insulin secretion from β-cells [121]. The Sulfonylureas can bind to the transmembrane sulfonylurea receptor (SUR-1) and induce the closing of the potassium sensitive ATP channel, thus reducing cellular efflux of potassium, reducing membrane polarization, increasing calcium influx, and resulting in the release of pre-formed insulin granules [122]. Although sulfonylureas have therapeutic potential against diabetes, it has been shown to worsen the angina symptoms in diabetes patients with existing coronary artery disease, and increase the risk of in-hospital mortality among diabetic patients undergoing coronary angioplasty for acute myocardial infarction [123; 124].

It has been shown that the cardioprotective effect of ischemic preconditioning (IPC) is mediated via p38-MAPK activation [119; 120]. In addition, Bugge and Ytrehus reported that bradykinin could protect the heart from ischemia and exerts the protective effect, similar to IPC, via p38-MAPK activation (Fig. **4**) [125; 126]. The *in vitro* treatment of sulfonylureas including glibenclamide and gliclazide has been shown to abolish the cardioprotective effect of IPC, as well as the beneficial effect of bradykinin (Fig. **4**) [127; 128]. However, there was no evidence indicating the direct effect of glibenclamide and gliclazide treatment on myocardial p38-MAPK. In addition, the information regarding the effects of sulfonylureas such as IPC may not be useful in a real clinical setting. Currently, limited data are available regarding the effects of sulfonylureas on p38-MAPK activation in an insulin resistant heart subjected to ischemia/reperfusion, as well as in other cardiomyopathies such as cardiac fibrosis and cardiac hypertrophy. Future investigation on this issue will provide useful information regarding the adverse effect of the drugs reported in clinical studies [110,111].

Table 2. Effect of Biguanides drugs on myocardial p38-MAPK activation.

Mode of Drug	Drugs	Dosage	Study model	Outcome	Effect on p38-MAPK	References
Insulin Sensitizers	Biguanides (Metformin)	3 mM Metformin 30 minutes prior to ischemia	*In vitro:* Neonatal rat ventricular myocytes subjected to 3hrs simulate ischemia	Metformin stimulate Bax translocation in response to p38-MAPK MAPK	↑p38-MAPK	[24]
	Metformin	1 mM Metformin 12 hours	*In vitro:* Adult mice cardiac fibroblast treated with AICAR or Metformin	Metformin dose-dependently increased IL-6 production	↑p38-MAPK	[26]
	Metformin	2 mM Metformin	*In vitro:* Rat cardiac myoblast cell line (H9c2) treatment with metformin in buffer containing insulin for 8 hours *Ex vivo*: Isolated male Sprague-Dawley heart perfused with metformin	*In vitro:* Metformin accelerate glycolysis *Ex vivo*: Metformin enhanced cardiac output, heart rate, and hydraulic work	*In vitro:* ↑p38-MAPK *Ex vivo*: ↑p38-MAPK	[116]
	Metformin	300, 600 mg/kg/d in drinking water for 2 weeks	*In vivo:* Adult male Sprague-Dawley rats treated with Metformin for 2 weeks *Ex vivo*: The hearts were isolated and perfused with AMPK activator (0.5 mM AICAR)	Both AMPK agonist AICAR and Metformin increased AMPK activity, and increased cardiac endothelial lipoprotein lipase translocation, increased triacylglycerol (TAG) accumulation in cardiomyocytes	↑p38-MAPK	[113]

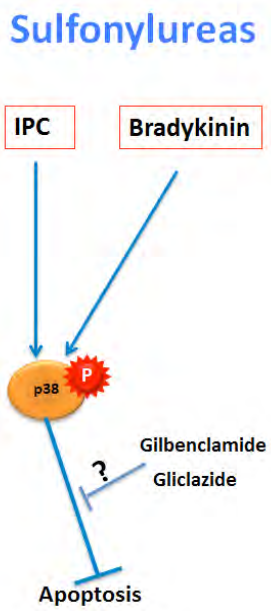

Fig. (4). Effect of sulfonylureas on p38-MAPK activation in the insulin resistant heart. Glibenclamide and gliclazide abolished the cardioprotective effect p38-MAPK mediated ischemia preconditioning, and bradykinin.

Incretins (Exendin-4, Liraglutide, Vildagliptin, Sitagliptin)

The glucagon-like peptide-1 (GLP-1) as well as glucose-dependent insulinotropic polypeptide (GIP), are secreted from the small intestine when being stimulated by food intake, and can activate insulin secretion [129]. The GLP-1 molecule is normally rapidly degraded by the activity of the dipeptidyl peptidase enzyme (DPP-IV). There are several anti-diabetic drugs in this group including GLP-1 analogues such as exedin-4 and liraglutide, and DDP-IV inhibitors such as vildagliptin and sitagliptin. GPL-1 has been shown to reduce cardiac cell death by the down-regulation of Bax and up-regulation of Bcl-2,[130] and increased cell survival via the cAMP mediated inhibition of p38-MAPK (Fig. **5**) [131]. Currently, there is only one study investigating the association between GLP-1 and p38-MAPK in the low-flow ischemia model of Wistar rats [132]. This study demonstrated that GLP-1 increased the *in vitro* kinase activity of p38-MAPK. These inconsistent findings regarding GLP-1 and p38-MAPK will need further investigation, especially in terms of the direct effect of GLP-1 on myocardial p38-MAPK activation, in the presence and absence of a p38-MAPK inhibitor, as well as the sensitivity to infarction. Moreover, GLP-1 has been reported to increase NO production via p38-MAPK induced nitric oxide synthase 2 (NOS2) production [133]. In addition, it has been shown that GLP-1enhanced NO production not only reduced cell death,[130] but also increased the glucose uptake [133] and improved the functional recovery of the post-ischemic myocardium [132]. Recently, Chinda *et al* demonstrated that vildagliptin could protect the heart from ischemia/reperfusion injury [134]. However, the direct effect of vildagliptin on myocardial p38-MAPK activation is still unclear. Further experiments concerning the effect of the drugs on myocardial p38-MAPK in the ischemia/reperfusion model, as well as in other cardiomyopathies, will provide useful information not only in clinical application, but also in terms of mechanistic insight on p38-MAPK activation. The studies concerning the effect of incretin on p38-MAPK are summarized in (Table **4**).

Effect of p38-MAPK Inhibitor on Insulin Resistant Heart

The activation of p38-MAPK is known to be associated with pathophysiology in cardiovascular dysfunction [19; 135-138]. The

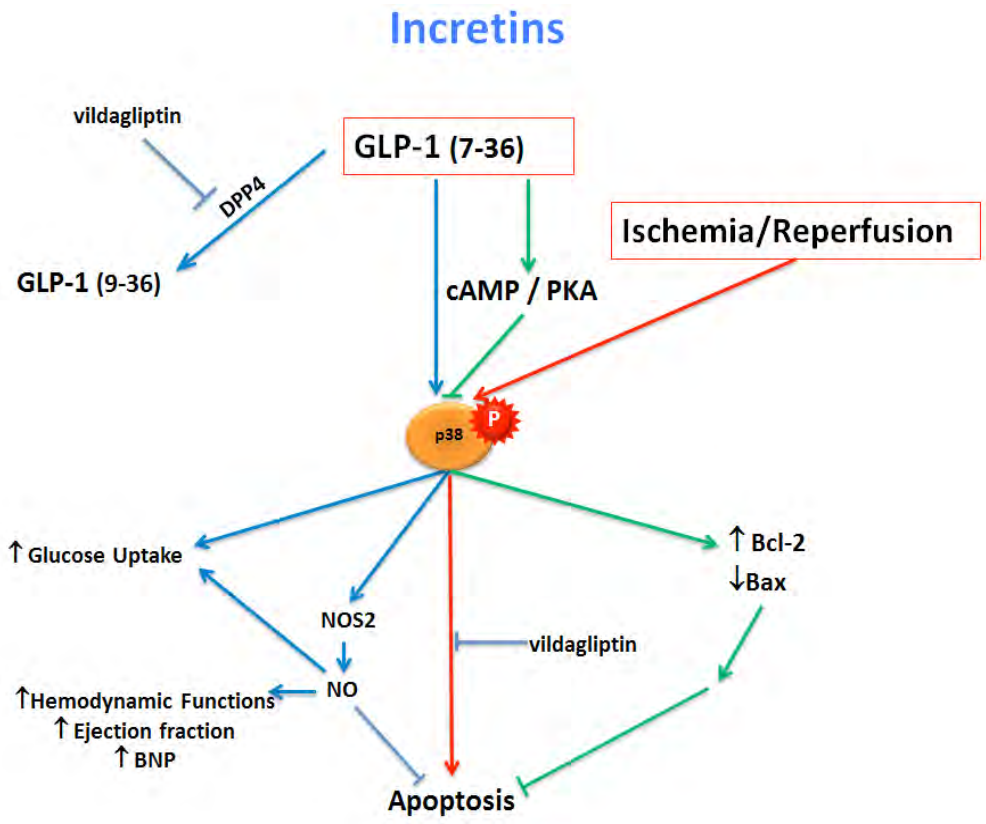

Fig. (5). Effect of incretin on p38-MAPK activation in the insulin resistant heart.GPL-1 promotes a cardioprotective effect by enhancing p38-MAPK mediating Bcl-2 expression and nitric oxide production. Moreover, GLP-1 enhanced glucose uptake and improved functional recovery of the post ischemic myocardium. Vildagliptin, the DPP-4 inhibitor, protected the heart from ischemia/reperfusion injury.

Table 3. Effect of Sulfonylureas drugs on myocardial p38-MAPK activation.

Mode of Drug	Drugs	Dosage	Study model	Outcome	Effect on p38-MAPK	References
Insulin secretagogues	Gliclazide	1, 10, 30, and 100 µM	*In vitro:* Human trabecular muscle section from the right atrial appendage.	Cardioprotection of ischemia preconditioning was abolished in all concentration of glibenclamide, and by supratherapeutic concentration of gliclazide	Both Gliclazide and Glibenclamide did not block p38-MAPK activation	[127]
	Glibenclamide	0.1, 1, 3, and 10 µM	Sections were incubate in simulated ischemia (sI) buffer for 90 min and incubated in reoxygenation buffer for 120 min. Preconditioning was performed by 5 minutes sI / 5 minutes reoxygenation			
	Glibenclamide	1 µM before and during preconditioning	*In vitro:* Rat neonatal cardiac fibroblast subjected to simulated ischemia and preconditioning by 1 µM 5,hydroxydecanoate (5HD). Bradykinin was administered 30 minutes prior to lethal ischemia	Bradykinin and preconditioning with 5HD reduced cardiac fibroblast cell death via activation of p38-MAPK MAPK. Glibenclamide reduced cell survival as well as abolish preconditioning	Not mentioned	[128]

Table 4. Effect of incretin on myocardial p38-MAPK activation.

Mode of Drug	Drugs	Dosage	Study model	Outcome	Effect on p38-MAPK	References
Incretin	GLP-1	500 pM	*Ex vivo*: Langendorff-perfused rat heart perfused with GLP-1 for 30 minutes followed by 30 minutes insulin perfusion. Hearts subjected to low-flow ischemia in the presence and absence of GLP-1 pretreatment	GLP-1stimulated myocardial glucose uptake through increased myocardial nitric oxide production p38-MAPK activation, and GLUT-4 translocation GLP-1 enhanced functional recovery of postischemic myocardium	↑p38-MAPK	[132]
	GLP-1(7-36) amide	2.5 pM/kg/minutes for 48 hours	*In vivo*: Mongrel dogs were induced to be dilated cardiomyopathy by rapid right ventricular pacing (240/min) for 28 days. The hemodynamic parameters were collected. GLP-1 was infused for 48 hours.	GLP-1 stimulate myocardial glucose uptake via p38-MAPK dependent pathway, mediating chronic hibernation and late phase of ischemic preconditioning	↑p38-MAPK	[133]

inhibition of p38-MAPK activation has been proposed to provide therapeutic potential [19; 139-141]. The majority of findings in insulin resistance, as well as diabetic models, suggested that the activation of p38-MAPK could aggravate cardiomyopathy [23]. Therefore, the inhibition of p38-MAPK by pharmacological inhibitors may possibly be beneficial in the insulin resistant heart. Evidence demonstrated that the administration of p38-MAPK inhibitors in insulin resistant, or diabetic models, improved left ventricular function and endothelial function, and reduced cardiac inflammation [142]. In addition, treatment of p38-MAPK inhibitor, SB203580, has been shown to improve cardiac contractile functions in insulin resistance and hyperleptinemia,[143] and also inhibited advanced glycation end products (AGEs), induced overexpression of collagen type I in cardiac fibroblasts, which were implicated in cardiac fibrosis [144]. These findings point out the usefulness of p38-MAPK inhibition in diabetes. Recently, the cardioprotective effects of the p38-MAPK inhibitor, SB203580, was demonstrated in an ischemic/reperfusion injury in rats [82; 145] However, information regarding p38-MAPK inhibition in ischemia/reperfusion injury of the diabetic heart has never been demonstrated elsewhere.

Insulin resistance and type 2 DM are known to enhance susceptibility to myocardial ischemia/reperfusion,[146] which is possibly due to the high basal level of activated p38-MAPK in the insulin resistant heart [147]. As previously discussed, treatment with some anti-diabetic agents could also enhance p38-MAPK phosphorylation [24; 26; 113; 116; 132; 133]. Any attempt to maintain glycemic control, by using anti-diabetic agents, especially in patients with existing coronary artery disease, may require caution since they could worsen the clinical outcomes, particularly the drugs that could cause p38-MAPK activation. Despite these facts, only limited knowledge is currently available from both basic and clinical studies regarding the roles of anti-diabetic drugs on p38-MAPK in the heart. Future studies are needed to extensively investigate these issues to warrant their clinical application in patients with insulin resistance and diabetes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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S.N. Pramojanee[1], M. Phimphilai[3], S. Kumphune[2,5], N. Chattipakorn[2,4], and S.C. Chattipakorn[1,2]*

[1]Department of Oral Biology and Diagnostic Science, Faculty of Dentistry; [2]Neurophysiology Unit, Cardiac Electrophysiology Research and Training Center, Faculty of Medicine; [3]Department of Medicine, Faculty of Medicine; [4]Biomedical Engineering Center, Chiang Mai University, Chiang Mai 50200, Thailand; and [5]Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand; *corresponding author, s.chat@chiangmai.ac.th

Decreased Jaw Bone Density and Osteoblastic Insulin Signaling in a Model of Obesity

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APPENDIX

MATERIALS & METHODS

Plasma Analysis

Plasma glucose and cholesterol concentrations were determined by colorimetric assay with a commercially available kit (Biotech, Bangkok, Thailand). Plasma insulin was measured by Sandwich ELISA (LINCO Research, St. Charles, MO, USA). Plasma osteocalcin was measured by ELISA (Takara Bio Inc., Tokyo, Japan). Insulin resistance was assessed by the Homeostasis Model Assessment (HOMA) (Haffner *et al.*, 1997). A higher HOMA index indicates a higher degree of insulin resistance.

Preparation of Primary Osteoblastic Culture

Rat osteoblasts from calvariae were isolated by the enzymatic isolation method, as described in a previous study (Fulzele *et al.*, 2010). Bone-derived cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 mg/mL ascorbic acid. Isolated cells were tested for osteoblastic phenotypes by the alkaline phosphatase (ALP) activity and Alizarin red staining methods, as described in a previous study (Perrini *et al.*, 2008). For cell culture experiments, at least 4 osteoblastic cultures derived from different rats in each dietary group (n = 6 rats *per* group) were used.

Cell Proliferation and Apoptosis Assay

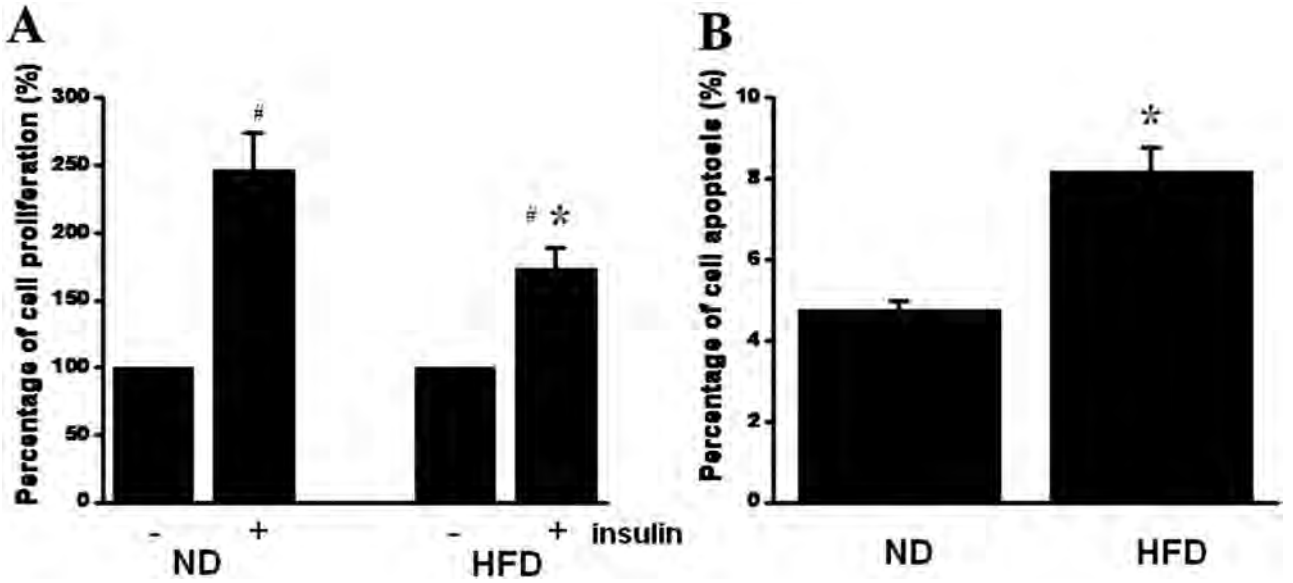
The osteoblasts isolated from ND and HFD groups were plated in 96-well plates at a density of 1 x 10^3 cells/well and cultured in DMEM supplemented with 10% FBS for 24 hrs. Cells were starved by DMEM supplemented with 1% FBS for 24 hrs before stimulation with 10 nM insulin for 24 hrs. Cell proliferation was measured by the Alamarblue™ fluorometric cell viability assay (Biosource, Carlsbad, CA, USA). In addition to the Alamarblue™ assay, a TUNEL assay was used to determine cellular apoptosis. The osteoblasts isolated from ND and HFD groups were plated on SPL cell culture slides (SPL Life Sciences Inc., Seoul, Korea) at a density of 5 x 10^3 cells/well and cultured in DMEM supplemented with 10% FBS for 24 hrs. Cells were starved by DMEM supplemented with 1% FBS for 24 hrs before stimulation with 10 nM insulin for 24 hrs. Cells were then fixed with 4% paraformaldehyde in PBS, permeabilized with 0.25% Triton® X-100 in PBS, and assessed for apoptosis with the Click-iT® TUNEL Alexa Fluor® 594 (Invitrogen, Carlsbad, CA, USA) imaging assay according to the manufacturer's instructions. The percentages of TUNEL-positive cells were randomly counted and calculated as the number of TUNEL-positive cells *per* total cells. Cell proliferation/apoptosis was given as percentage of control (non-insulin-stimulated cells).

Micro-computed Tomography (micro-CT) of the Mandible

Mandibles (n = 5 in each group) were embedded in polymethyl methacrylate (PMMA) and imaged at the molar region, as described previously (Abbassy *et al.*, 2010), with micro-CT (Skyscan 1072 microCT, Skyscan, Aartselaar, Belgium). Three-dimensional images of each hemi-mandible were acquired with a resolution voxel size of 15 μm/pixel. Raw data were obtained by rotating the sample stage 360 degrees. Slice images were then prepared, and reconstruction of the specimens for analysis was obtained with ANT software (Skyscan, Aartselaar, Belgium). The following parameters were measured: tissue volume (TV), bone volume (BV), and percentage of bone volume (BV/TV). Four properties of the trabeculae were calculated: trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and percentage of porosity (%porosity).

Assessment of Bone Formation Rate with Tetracycline

Rats were injected intra-peritoneally with tetracycline (7.5 mg/kg BW) on the 1st, 7th, and 14th days after the end of 12 wks of either diet (n = 5/group). At the end of the treatment, all animals were sacrificed. The mandible of each rat was used to determine the bone apposition rate.



Appendix Figure. Decreased osteoblastic proliferation and increased osteoblastic apoptosis in HFD-fed rats. Osteoblastic proliferation is shown in panel **A**, and osteoblastic apoptosis is shown in panel **B**. The determination of cell proliferation was performed by the Alarmablue™ fluorometric cell viability assay (A). Osteoblastic apoptosis was measured by TUNEL assay (B). Data are expressed as mean ± SEM; n = 5-6 animals for each group. $^{*}p < .05$ compared with the ND group. $^{\#}p < .05$ compared with the non-insulin-stimulation group. ND, normal diet group; HFD, high-fat-diet group; -, non-insulin stimulation; +, insulin-stimulation.

The bone apposition rate was measured as described by Parfitt *et al.* (1987). The distance between the tetracycline lines from the first dose of labeling to the subsequent ones (second and third doses) was measured. Then, each distance was divided by the labeling intervals of 7 days and presented as μm/day. The distance between the tetracycline lines of each mandible was measured at 10 positions in each mandible. Each bone apposition rate (μm/day) was normalized with body weight in each rat. The average of 10 apposition rates in each animal (μM/day/bodyweight) represented the bone apposition rate of that animal. We used a confocal laser scanning microscope (FLUOVIEW FV10i, Olympus, Tokyo, Japan) to detect the tetracycline-labeled lines. The frontal sections of the lower second molar area were evaluated for the mineral apposition rate (MAR), as described in a previous study (Abbassy *et al.*, 2010).

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S.N. Pramojanee[1], M. Phimphilai[3], S. Kumphune[2,5], N. Chattipakorn[2,4], and S.C. Chattipakorn[1,2]* [AQ: 1]

[1]Department of Oral Biology and Diagnostic Science, Faculty of Dentistry; [2]Neurophysiology Unit, Cardiac Electrophysiology Research and Training Center, Faculty of Medicine; [3]Department of Medicine, Faculty of Medicine; [4]Biomedical Engineering Center, Chiang Mai University, Chiang Mai 50200, Thailand; and [5]Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand; *corresponding author, s.chat@chiangmai.ac.th

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Decreased Jaw Bone Density and Osteoblastic Insulin Signaling in a Model of Obesity

ABSTRACT

Previous studies have demonstrated that decreased bone mass results from either the impairment of osteoblastic insulin signaling or obesity. Our previous study revealed that 12-week high-fat-diet (HFD) consumption caused obesity as well as peripheral and brain insulin resistance. However, the osteoblastic insulin resistance induced by HFD has not been elucidated. Therefore, we hypothesized that 12-week HFD rats exhibited not only peripheral insulin resistance but also osteoblastic insulin resistance, which leads to decreased jawbone quality. We found that the jawbones of rats fed a 12-week HFD exhibited increased osteoporosis. The osteoblastic cells isolated from HFD-fed rats exhibited the impairment of osteoblastic insulin signaling as well as reduction of cell proliferation and survival. In conclusion, this study demonstrated that insulin resistance induced by 12-week HFD impaired osteoblastic insulin signaling, osteoblast proliferation, and osteoblast survival and resulted in osteoporosis in the jawbone.

KEY WORDS: obesity, insulin resistance, osteoblast, mandible, cell proliferation. [AQ: 2]

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INTRODUCTION

High-fat food consumption contributes to obesity (Pratchayasakul *et al.*, 2011). Fat accumulation causes changes in bone health (Chen *et al.*, 2010; Pollock *et al.*, 2011). Some studies have shown a positive correlation between body mass and bone density (Felson *et al.*, 1993; Lee *et al.*, 2012), suggesting that obesity protects against osteoporosis. However, other clinical and preclinical studies have shown an inverse relationship between bone quality and obesity (Chen *et al.*, 2010; Pollock *et al.*, 2011). The effect of obesity on bone density and bone quality remains controversial. Furthermore, the relationship between obesity and jawbone loss and the underlying mechanisms involved have not been investigated.

Several studies and our previous study have shown that rats fed with a 10- to 12-week high-fat diet (HFD) developed obesity, insulin resistance, and the impairment of insulin receptor function in several organs: brain (Pratchayasakul *et al.*, 2011), heart (Apaijai *et al.*, 2012), and skeletal muscles (Bernard *et al.*, 2006). Osteoblasts have been known as insulin target cells, since they express insulin receptor (Ferron *et al.*, 2010; Fulzele *et al.*, 2010). However, defects of insulin signaling in osteoblasts isolated from insulin-resistant subjects have not been intensively investigated.

Insulin plays important roles in bone development and physiology, as has been shown in previous *in vitro* and *in vivo* studies (Kream *et al.*, 1985; Pun *et al.*, 1989; Ogata *et al.*, 2000; Kawamura *et al.*, 2007; Mukherjee and Rotwein, 2009; Fulzele *et al.*, 2010; Yang *et al.*, 2010). Insulin increases bone anabolic markers, including collagen synthesis, and alkaline phosphatase production and promotes osteoblast proliferation and differentiation (Kream *et al.*, 1985; Pun *et al.*, 1989; Yang *et al.*, 2010). Several studies have revealed that insulin receptor signaling molecules, including IR, IRS-1, and Akt, are required for osteoblast proliferation and differentiation (Ogata *et al.*, 2000; Kawamura *et al.*, 2007; Mukherjee and Rotwein, 2009; Fulzele *et al.*, 2010). Osteoblasts isolated from osteoblastic-specific insulin receptor knock-out mice exhibited a decrease in osteoblast proliferation as well as an increase in osteoblast apoptosis, which resulted in the reduction of bone acquisition (Fulzele *et al.*, 2010). In addition, osteoblasts isolated from IRS-1 knockout mice showed a lack of tyrosine phosphorylation activation as well as a reduction in proliferation and differentiation, which may entail the osteopenic phenotype as shown in that animal model (Ogata *et al.*, 2000). Moreover, the deletion of *Akt* in osteoblasts resulted in an increase in osteoblast apoptosis (Kawamura *et al.*, 2007). Those findings suggest that an impairment of osteoblastic

insulin receptor signaling could lead to the negative effect on bone quality. However, the effects of obesity-induced insulin resistance on bone quality as well as osteoblastic insulin signaling have not yet been investigated. Therefore, the aims of this study were (1) to investigate changes of jawbone quality induced by 12-week HFD consumption in insulin-resistant obese rats, (2) to investigate whether the impairment of insulin signaling in osteoblasts contributes to insulin resistance in those obese rats, and (3) to determine the proliferation and survival of osteoblasts in those obese rats.

MATERIALS & METHODS

Animals

Fifty male Wistar rats (180-200 g) were obtained from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand. All experiments in animals were performed in accordance with a protocol approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Rats were randomly assigned to two different dietary groups (n = 25/group): normal diet (ND) and high-fat diet (HFD). Rats in the ND group and HFD group were given 12 wks of standard chow (19.77% fat) and high-fat chow (59.28% fat), respectively, as previously described in our study (Pratchayasakul *et al*., 2011). At the end of week 12, all animals were deeply anesthetized and sacrificed after fasting for at least 5 hrs. Blood samples were collected. Plasma was separated and stored at -80°C for subsequent biochemical analysis, including plasma glucose, cholesterol, insulin, and osteocalcin. The calvariae from 20 rats in each dietary group were rapidly removed for the preparation of osteoblastic culture, and the mandibles were removed for micro-CT analysis. The other 5 rats in each dietary group were used for bone formation assessment. (See details on plasma analysis and preparation of primary osteoblastic culture in the Appendix.)

Immunoprecipitation and Immunoblotting

Osteoblastic cells from ND and HFD groups were cultured in osteogenic culture medium for 7 days. Cells were starved in 0.1% FBS for 24 hrs to reduce cellular activity to quiescent levels before the addition of insulin (500 nM) for 5 min. Then, the cells underwent lysis with buffer containing protease and phosphatase inhibitors. The protein samples were subjected to immunoprecipitation with IR, IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) conjugated with protein A-Sepharose beads, followed by immunoblotting with antibodies against the IR, IRS-1, and tyrosine phosphorylation. The cell-extracted protein was subjected to Western blot analysis with antibodies against the Akt, phosphorylated Akt (S473), Bax, caspase-8, Bcl-2, and cyclin D1, as described in previous studies (Perrini *et al*., 2008; Pramojanee *et al*., 2011).

Cell Proliferation and Apoptosis Assay

The osteoblasts isolated from ND and HFD groups were plated in 96-well plates at a density of 1 x 10^3 cells/well and cultured in DMEM supplemented with 10% FBS for 24 hrs. Cells were starved by DMEM supplemented with 1% FBS for 24 hrs before being stimulated with 10 nM insulin for 24 hrs. Cell proliferation was measured by Alamarblue™ fluorometric cell viability assay (Biosource, Carlsbad, CA, USA) (see details in the Appendix). The TUNEL assay was used to determine cellular apoptosis (see details in the Appendix). Cell proliferation/apoptosis was given as percentage of control (non-insulin-stimulated cells).

Micro-computed Tomography (micro-CT) of the Mandible

Mandibles (n = 5 in each group) were embedded in polymethyl methacrylate (PMMA) and imaged at the molar region, as described previously (Abbassy *et al*., 2010) by micro-CT (Skyscan 1072 microCT, Skyscan, Aartselaar, Belgium). (See details in the Appendix.)

Assessment of Bone Formation Rate with Tetracycline

Rats were injected intra-peritoneally with tetracycline (7.5 mg/kg BW) on the 1st, 7th, and 14th days after the end of 12 wks of either diet (n = 5/group). At the end of the treatment, all animals were sacrificed. The mandible of each rat was used to determine the bone apposition rate, which was measured as described by Parfitt *et al.* (1987). (See details in the Appendix.)

Data Analysis

All data were expressed as means ± SEM. Comparisons between groups were determined by the independent-sample *t* test. A p value $< .05$ was considered statistically significant.

RESULTS

Twelve-week HFD consumption caused a decline in bone formation and an increase in jawbone porosity.

Rats fed a 12-week HFD developed peripheral insulin resistance, as indicated by significantly increased body weight, visceral fat, plasma insulin level, plasma cholesterol level, and HOMA index, compared with those of ND-fed rats ($p < .05$, Table). We also investigated the plasma level of osteocalcin, a marker for osteoblastic formation (Brown *et al*., 1984), comparing the 12-week ND-fed rats and the 12-week HFD-fed rats. Our results demonstrated that the 12-week HFD-fed rats had reduced plasma osteocalcin levels compared with 12-week ND-fed rats ($p < .05$, Table). These findings suggest that the osteoblastic formation of HFD-fed rats was decreased compared with that of ND-fed rats.

The alveolar bones at the molar area of 12-week HFD rats revealed increases in porosity in both 2D and 3D micro-CT images, compared with those of 12-week ND rats (Fig. 1A). The quantitative changes in trabecular bone in the mandibles of ND and HFD groups are shown in Fig. 1B. In the HFD group, trabecular thickness and the BT/TV were significantly decreased compared with those of the ND group ($p < .05$, Fig. 1B). The trabecular separation and percentage of porosity were significantly higher in the HFD group ($p < .05$, Fig. 1B).

Table. Metabolic Parameters, Osteoblastic Proliferation, and Osteoblastic Apoptosis of Normal-diet-fed (ND) Rats and of High-fat-diet-fed (HFD) Rats

	Dietary	
Measurement Parameters	12-week ND	12-week HFD
Body weight (g)	460.18 ± 8.37	546.25 ± 8.32*
Visceral fat (g)	25.19 ± 1.96	45.47 ± 2.01*
Plasma glucose (mg/dL)	149.41 ± 4.39	153.42 ± 2.95
Plasma insulin (ng/mL)	2.14 ± 0.54	4.23 ± 0.59*
Plasma cholesterol (mg/dL)	90.80 ± 4.33	136.02 ± 7.36*
Plasma osteocalcin (ng/mL)	37.44 ± 3.08	24.89 ± 1.71*
HOMA index	20.05 ± 5.57	39.69 ± 5.69*
Percentage of cell proliferation (%) (with Alamarblue™ cell viability assay)	245.6 ± 27.62	173.8 ± 14.88*
Percentage of cell apoptosis (%)	4.75 ± 0.25	8.20 ± 0.58*

Reduction of the Mineral Apposition Rate in Mandibles of Insulin-resistant Obese Rats Induced by 12-week HFD Consumption

Green fluorescent lines labeled with a tetracycline fluorescent marker at 3 different time-points (Fig. 1C) indicated that bone growth occurred on days 1, 7, and 14 after 12-week diet consumption in both ND and HFD groups. The HFD group represented a significant reduction of mineral apposition rate in alveolar bone after normalizing for body weight compared with the ND group ($p < .05$, Fig. 1D).

Determination of Osteoblastic Phenotypes of Isolated Bone Cells

The cultured bone-derived cells had positive Alizarin Red Staining at 14 days in the presence of osteogenic medium (OM) in both ND and HFD groups (Fig. 2A), indicating that these cultured cells were osteoblasts. In addition, the ALP activities at day 7 between the ND (3.03 ± 0.77 mM of p-nitrophenol/mg protein) and HFD groups (3.92 ± 0.58 mM of p-nitrophenol/mg protein) were not significantly different.

The Impairment of Osteoblastic Insulin Signaling in Insulin-resistant Obese Rats Induced by 12-week HFD Consumption

Figs. 2B-2D demonstrate the phosphorylation of IR, IRS-1, and Akt. The baseline expression levels (without insulin stimulation) of p-IR, p-IRS-1, and p-Akt were

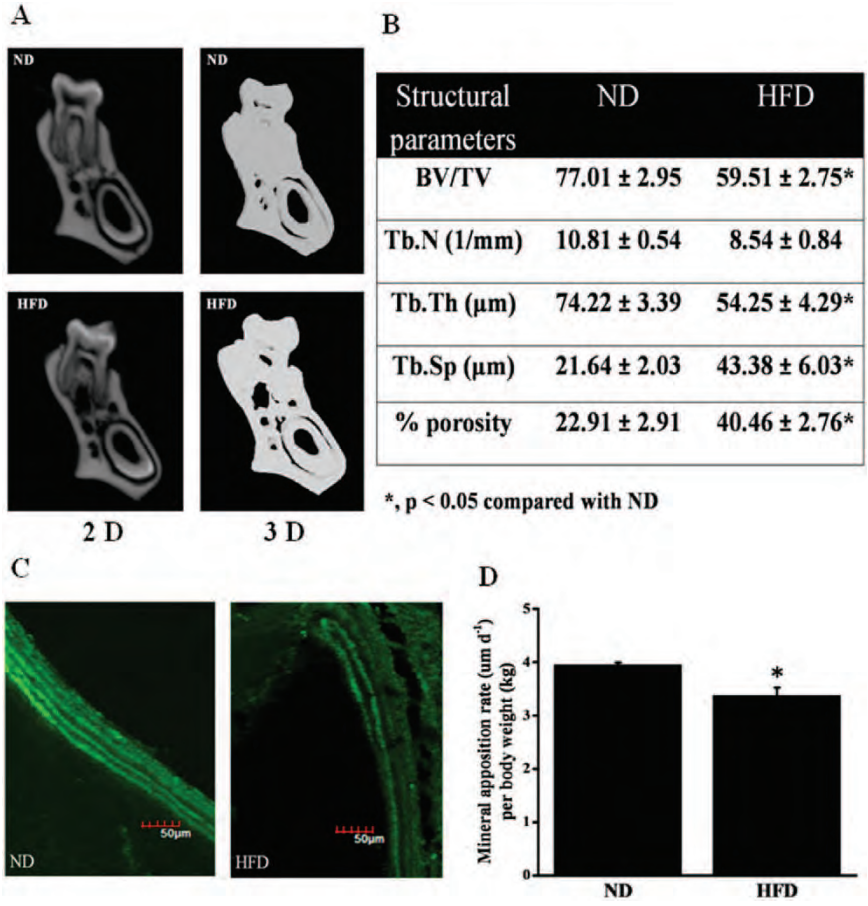

Figure 1. The changes of skeletal phenotype in jawbones extracted from ND and HFD rats. **(A)** A representative micro-CT of mandibular trabecular bone in the second molar region of ND and HFD rats. Note increased porosity demonstrated in both 2D and 3D images of trabecular bone in HFD rats. **(B)** Structural properties of alveolar bones of mandibles from ND rats and HFD rats. **(C)** Fluorescent labeling on the periosteal surface indicates new alveolar bone formation in ND and HFD rats. **(D)** Changes in the mineral apposition rate (MAR) of the alveolar bone between the ND group and the HFD group after normalizing for body weight. The data are expressed as mean ± SEM; n = 5 for each group. *$p < .05$ compared with the ND group. ND, normal diet group; HFD, high-fat-diet group; 2D, two-dimensional image; 3D, three-dimensional images; (BV/TV), percentage of bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; and % porosity, percentage of porosity.

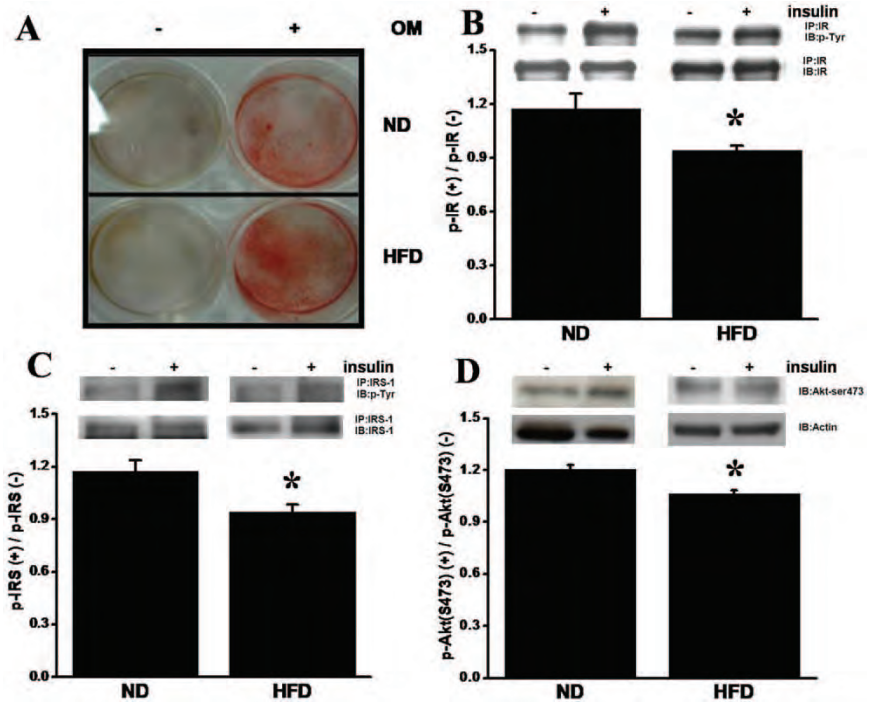

Figure 2. [AQ: 7] **(A)** Mineralization properties of bone-derived cells. Osteoblasts isolated from ND and HFD groups were grown in standard medium and osteogenic medium (OM). The cultured bone-derived cells had positive Alizarin Red Staining at 14 days in the presence of osteogenic medium (OM). The representative blots of tyrosine phosphorylation (upper traces) illustrate a decrease in the ability of insulin to stimulate IR **(B)**, IRS-1 **(C)**, and serine 473 kinase of Akt **(D)** in osteoblasts of HFD-fed rats, compared with those of ND-fed rats. Fold phosphorylation of insulin-stimulated p-IR, p-IRS-1, and p-Akt (S473) was significantly greater in ND osteoblasts than in HFD osteoblasts. Data are expressed as mean ± SEM; n = 4 for each group. *$p < .05$ compared with the ND group. ND, normal diet group; HFD, high-fat-diet group; -, non-insulin stimulation; +, insulin-stimulation; p-IR, tyrosine phosphorylation of insulin receptor; p-IRS, tyrosine phosphorylation of insulin receptor substrate 1; p-Akt(S473), phosphorylation of serine 473 kinase of Akt.

not significantly different between both dietary groups (Figs. 2B, 2C, 2D). These Figs. also demonstrate that the HFD osteoblasts exhibited a significant decrease in insulin-stimulated p-IR, p-IRS, and p-Akt (S473), compared with those in the ND osteoblasts, accounting for ~24% reduction ($p < .05$), indicating the impairment of osteoblastic insulin signaling in the HFD group.

Insulin Resistance Induced by 12-week HFD Consumption Caused a Reduction in Osteoblastic Proliferation and an Increase in Osteoblastic Apoptosis

Since the impairment of insulin signaling was found in osteoblasts isolated from HFD rats, the effects of HFD-induced insulin resistance on osteoblastic survival as well as on osteoblastic proliferation were further investigated by measurement of the basal expression of the apoptotic indicators Bax/Bcl-2 ratio and caspase-8, and proliferative protein cyclin D1 (Fig. 3). Pro-apoptotic protein expression was significantly increased in HFD osteoblasts, as indicated by an increased Bax and caspase-8 expression ($p < .05$, Figs. 3B, 3C), whereas the expression of the anti-apoptotic protein Bcl-2 was not significantly different between ND osteoblasts and HFD osteoblasts (Fig. 3D). However, the Bax/Bcl-2 ratio in HFD osteoblasts (2.087 ± 0.123) was significantly increased, compared with that of ND osteoblasts (1.519 ± 0.196; $p < .05$). Furthermore, the expression of cyclin D1 was significantly decreased in HFD osteoblasts, compared with that in the ND group ($p < .05$, Fig. 3E). In addition, the Alamarblue™ cell viability assay, which was used to determine the effects of insulin on osteoblast proliferation, demonstrated that the percentages of insulin-induced cell proliferation of HFD osteoblastic cells (173.8 ± 14.88%) were significantly lower than those isolated from the ND group (245.6 ± 27.62%, n = 6/group; $p < .05$ as shown in the Table; details in the Appendix Fig.). A TUNEL assay demonstrated that HFD osteoblasts exhibited higher percentages of cell apoptosis than those of ND osteoblasts (8.20 ± 0.58% *vs.* 4.75 ± 0.25%, n = 5/group; $p = .0017$) (Table; details in the Appendix Fig.). These findings indicated that the physiological effect of insulin in HFD osteoblasts was impaired.

DISCUSSION

The present study revealed for the first time that HFD-induced obesity impaired jaw-bone density. Consistent with our study, previous studies in skeletal bones demonstrated that HFD-induced obesity caused adverse effects on bone mass by decreasing bone density and micro-architecture (Cao *et al.*, 2009; Chen *et al.*, 2010). Those previous studies proposed several mechanisms linking impaired bone quality and obesity, such as hyperlipidemia-induced osteoclastogenesis (Graham *et al.*, 2010) and free fatty acid (FFA)-induced adipogenesis instead of osteoblastogenesis (Chen *et al.*, 2010). However, no such studies illustrated the adverse effects of obesity on bone in terms of the impairment of osteoblastic insulin signaling and the reduction of osteoblastic proliferation and survival.

It is well-known that obesity is a major cause of insulin resistance, a condition in which insulin signaling in target tissues is impaired. Several previous studies demonstrated that 10- to 12-week HFD consumption caused an impairment of insulin receptor signaling in skeletal muscle (Bernard *et al.*, 2006), fat (Boyd *et al.*, 1990), liver (Watarai *et al.*, 1988), and brain (Pratchayasakul *et al.*, 2011). The osteoblast is one of the insulin target organs, and insulin signaling is required for bone growth and development (Fulzele *et al.*, 2010). However, changes in the osteoblastic insulin signaling of HFD-induced insulin-resistant animals have not been elucidated.

In the present study, rats with 12-week HFD-induced obesity developed not only peripheral insulin resistance, but also impairment of osteoblastic insulin signaling, as demonstrated by the

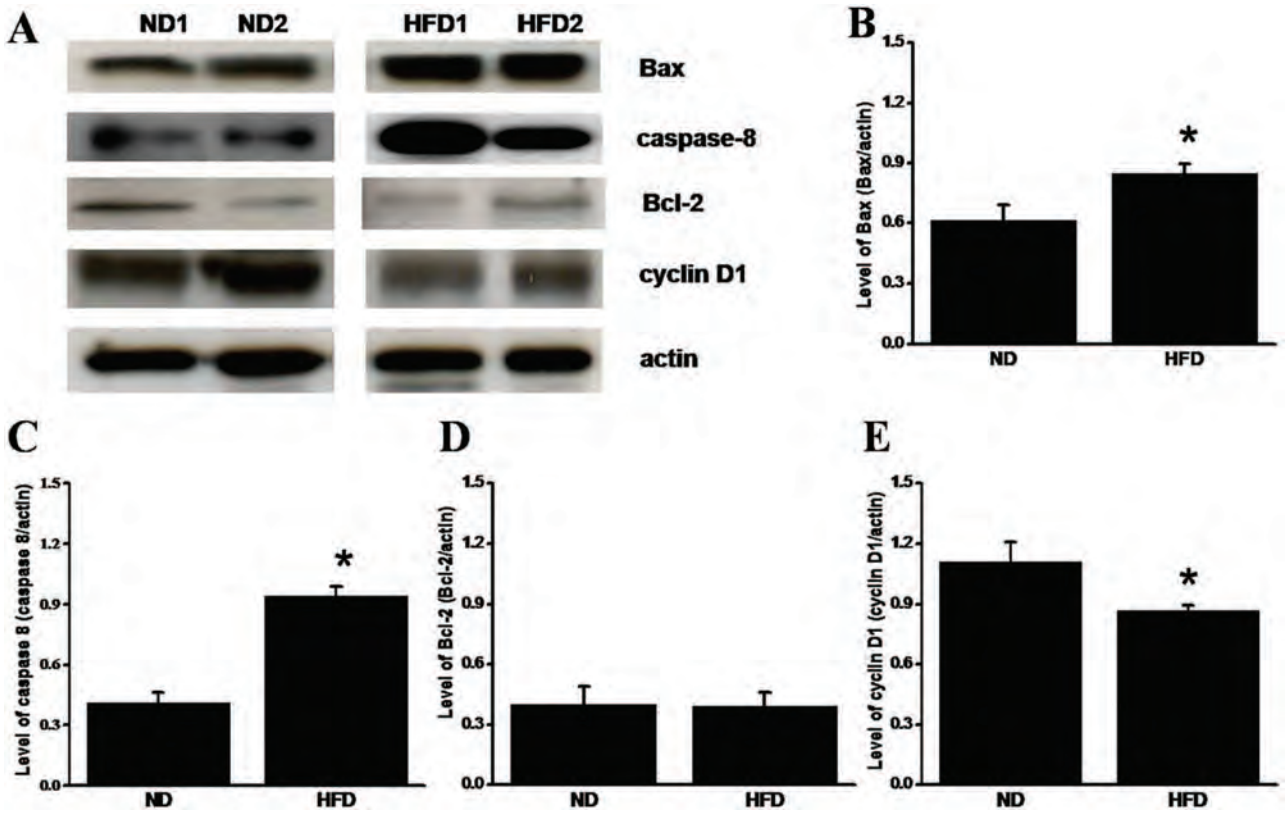

Figure 3. [AQ: 8] (**A**) The expression of Bax, caspase-8, Bcl-2, and cyclin D1 at basal levels (non-insulin stimulation) in ND and HFD osteoblasts after 12-week feeding. The two representative immunoblots of ND osteoblasts (ND1 and ND2) and of HFD osteoblasts (HFD1 and HFD2) are illustrated. The representative blots of Bax and caspase-8 demonstrate an increase in Bax and caspase-8 expression in HFD osteoblasts compared with that in ND osteoblasts, whereas the representative blots of cyclin D1 show a decrease in cyclin D1 expression in HFD osteoblasts compared with that in ND osteoblasts. (**B**) Densitometric quantitation of Bax in the ND osteoblasts was significantly lower than that in the HFD osteoblasts. (**C**) Densitometric quantitation of caspase-8 in the HFD osteoblasts was significantly higher than that in the ND osteoblasts. (**D**) Densitometric quantitation of Bcl-2 was not significantly different between ND osteoblasts and HFD osteoblasts. (**E**) Densitometric quantitation of cyclin D1 in the HFD osteoblasts was significantly lower than that in the ND osteoblasts. All immunoblotting lanes were loaded with equal amounts of protein (40 μg/lane). Data are expressed as mean ± SEM; n = 4 for each group. *$p < .05$ compared with the ND group. ND, normal diet group; HFD, high-fat-diet group.

failure of insulin-induced tyrosine phosphorylation of the insulin receptor (IR), of insulin receptor substrate-1 (IRS-1), and of serine473 phosphorylation of Akt. In addition, our findings demonstrated that the impairment of insulin signaling initially occurred at all levels of the signaling pathway, from the post-insulin receptor level down to Akt.

Previous studies have demonstrated that osteoblastic insulin receptor signaling is indispensable for osteoblast proliferation, osteoblast differentiation, and osteoblast survival (Ogata *et al.*, 2000; Kawamura *et al.*, 2007; Fulzele *et al.*, 2010). Therefore, the impairment of osteoblastic insulin signaling in rats with HFD-induced obesity can affect the osteoblastic proliferation and survival of osteoblasts isolated from HFD rats. This hypothesis was confirmed by the present findings that the percentage of insulin-induced osteoblastic proliferation in HFD group was significantly lower than that of the ND group, whereas cellular apoptosis was greater in the HFD than ND osteoblasts, indicating that the physiological osteoblastic proliferation in response to insulin of osteoblasts isolated from the HFD group is impaired (as shown in the Table). Among insulin signaling molecules, Akt, a downstream signal in the insulin signaling cascade, is a key molecule regulating both cellular proliferation and cell survival (Lawlor and Alessi, 2001). Akt promotes cell proliferation by stimulating proliferative regulatory proteins, such as cyclin D1 (Fatrai *et al.*, 2006), and prevents cellular apoptosis by suppressing pro-apoptotic proteins, such as Bax (Yamaguchi and Wang, 2001) and caspase-8 (Numata *et al.*, 2011). Our results demonstrated that the impairment of osteoblastic insulin signaling in rats can lead to the impairment of cellular proliferation and of cellular survival, as shown by down-regulation of cyclin D1 and up-regulation of Bax and caspase-8, without the alteration of Bcl-2 expression level. In addition, 12-week HFD-fed rats had decreased osteoblastic formation, as demonstrated by the reduction of plasma osteocalcin levels. Therefore, our findings indicated that HFD-induced obesity can lead to not only the impairment of osteoblastic insulin signaling, but also the impairment of osteoblastic formation and abnormalities in osteoblast growth and survival, all of which can lead to impaired jawbone quality.

In conclusion, this study demonstrated that HFD consumption induced not only peripheral insulin resistance, but also

osteoblastic insulin resistance. The impairment of osteoblastic insulin signaling may lead to decreased osteoblastic proliferation and decreased osteoblastic survival, and could cause the reduction of jawbone quality.

Although we demonstrated the osteoblast insulin resistance in osteoblastic cells isolated from insulin-resistant rats, the condition might not specifically represent the physiologic condition of osteoblast insulin resistance *in vivo*. In addition, decreased osteoblastic proliferation in HFD-fed rats has been demonstrated with the Alamarblue™ fluorometric cell viability assay, which is still the indirect method for the measurement of osteoblastic proliferation. Future studies for direct measurement are needed to investigate the role of HFD.

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Inhibition of p38 MAPK activation protects cardiac mitochondria from ischemia/reperfusion injury

Short title: p38-MAPK inhibition protects cardiac mitochondria

Sarawut Kumphune, PhD [1,2], Sirirat Surinkaew, PhD,[1]

Siriporn Chattipakorn, DDS, PhD,[1,3], Nipon Chattipakorn, MD, PhD[1,4*]

[1]Cardiac Electrophysiology Research and Training Center,

Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand,

[2]Biomedical Research Unit in Cardiovascular Research (BRUCS) and Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000 Thailand, [3]Faculty of Dentistry, Chiang Mai University, Chiang Mai, 50200, Thailand,

[4]Cardiac Electrophysiology Unit, Department of Physiology, Faculty of Medicine,

Chiang Mai University, Chiang Mai, Thailand

Correspondence: Nipon Chattipakorn, MD, PhD

Cardiac Electrophysiology Research and Training Center

Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand

Tel: +66-53-945329, Fax: +66-53-945368

E-mail: nchattip@gmail.com

*SK and SS contributed equally in this work

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Abstract

Inhibition of p38 MAPK has been shown to reduce cardiac cell death, improve left ventricular function, and decrease the incidence of fatal arrhythmia during myocardial ischemia/reperfusion (I/R), suggesting its promising therapeutic benefits. Although cardiac mitochondrial dysfunction is known as a key underlying cause of I/R injury, the *in vivo* effects of p38 MAPK inhibition on cardiac mitochondrial function have not been directly investigated. In the present study, adult male Wistar rats were subject to a 30-min left anterior descending coronary artery (LAD) occlusion and 120-min reperfusion in the presence of 2 mg/Kg body weight of SB203580 given at one of the 3 different time points: at 15 min before LAD occlusion, or at 15 min after the onset of ischemia (during ischemia), or at the onset of reperfusion. At the end of the experiment, the mitochondrial function, ultrastructure, and Western blot analysis were performed. Administration of SB203580, a p38 MAPK inhibitor, at any time point of I/R injury significantly attenuated the ROS generation and cardiac mitochondrial swelling. SB203580 attenuated mitochondrial membrane depolarization only when given prior to the LAD occlusion or during ischemia. Nevertheless, administration of SB203580 at any time point effectively protected mitochondrial ultrastructure rupture caused by I/R. In addition, pre-treatment of SB203580 significantly reduced the phosphorylation of p38 MAPK, HSP27, p53, CREB, and α-B-crystalline, decreased Bax expression without any changes in Bcl2 expression, and reduced cytochrome c and cleaved caspase 3 levels. However, SB203580 given at the onset of reperfusion could only inhibit the phosphorylation of CREB and α-B-crystalline, without an effect on apoptotic regulatory proteins. These findings suggest that timing of p38 MAPK inhibition with respect to onset of ischemia is an important determinant of cardiac mitochondria protection through the attenuation of p53-mediate mitochondrial trigger cell death pathway.

Highlight:

- p38 MAPK inhibitor SB203580 preserves I/R induced cardiac mitochondrial dysfunction
- SB203580 preserves I/R induced cardiac mitochondrial ultrastructure rupture
- The beneficial effect of SB203580 was mediated by inhibition of p53 phosphorylation
- Administration of p38 MAPK reduced the level of Bax, caspase 3, and cytochrome c

Keywords:p38 MAPK; SB203580; ischemia/reperfusion injury; mitochondrial functions

1. Introduction

Acute myocardial infarction has been a serious health burden in most countries around the world for many decades [1]. Although myocardial reperfusion has been shown to be the effective way of myocardial salvage, reperfusion itself is known to cause cellular injury [2]. One of the major underlying causes of myocardial ischemia/reperfusion (I/R) injury is cardiac mitochondrial dysfunction. In particular, I/R injury has been shown to lead to mitochondrial swelling, increased mitochondrial reactive oxygen species (ROS) production, and mitochondrial membrane depolarization [3].

Previous studies demonstrated that the activation of the 38kDa mitogen activated protein kinase (p38 MAPK) during myocardial ischemia aggravates lethal injury and has been found to be worsen in ischemia followed by reperfusion[4-6]. The pharmacological catalytic site inhibitor SB203580 has been shown to prevent p38 MAPK activation and activity and also reduce cardiomyocytes death and the infarct size, as well as improve left ventricular function [5-9]. However, prevention of p38 MAPK activation by using inhibitor prior to ischemia seems to be impractical in real clinical setting, as myocardial ischemia is an unpredictable condition. Therefore, it will be more clinically useful to know if inhibition of p38 MAPK activation at postischemic state, such as at reperfusion period, could provide cardioprotection. Recently, we reported the cardioprotective effect of SB203580 against myocardial infarction and the incidence of fatal arrhythmia in I/R rat model and also suggested that timing of drug administration is critical related to its therapeutic outcomes [9].

Since inhibition of p38MAPK provides benefit on infarct size reduction in I/R hearts, the role of p38 MAPK inhibition could directly involve cardiac mitochondria. Moreover, since p53 [11-13], CREB [14, 15], and α-B-crystalline [16, 17] could be activated downstream of p38 MAPK, they could play an important role in cardiac mitochondrial responses to I/R, particularly on the cellular necro-apoptotic regulatory molecules such as

Bax, Bcl2, caspase 3, and cytochrome c. However, the effects of SB203580 on these markers, in term of linkage between cardiac mitochondrial dysfunction and cell death, have not been investigated. Therefore, in the present study, we determined the effects of p38 MAPK inhibitor, SB203580, on cardiac mitochondrial function, including mitochondrial swelling, ROS production, mitochondrial membrane potential changes ($\Delta\Psi m$), as well as cardiac mitochondrial ultrastructure, in an *in vivo* I/R rat model. The signaling cascades involved in cardiac mitochondrial protection downstream of p38 MAPK activation such as p53, CREB, and α -B-crystalline as well as necro-apoptotic regulatory molecules such as Bax, Bcl2, caspase 3, and cytochrome c were also determined.

2. Materials and Methods

2.1 Animal model

Adult male Wistar rats weighing 350-400 g were obtained from the National Animal center, Salaya campus, Mahidol University, Bangkok, Thailand, and were fed with normal food and water. All animal experiments were approved by the Institutional Animal Care and Use Committees and conform to the Guide for the Care and Use of Animals of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

2.2 Ischemia and reperfusion

Myocardial ischemia/reperfusion was induced for 30-min by ligation of the left anterior descending coronary artery (LAD), followed by reperfusion for 120-min as described previously[18]. Ischemic heart was confirmed by the change in the myocardial tissue color and ST elevation on the electrocardiogram that was recorded throughout the experiment. The left femoral vein was cannulated for administration of either SB203580, p38 inhibitor (2

mg/kg) [19] or normal saline as a vehicle at 0.33 ml/min for 3 min[18]. Rats were divided into 3 treatment groups to receive SB203580 at 15-min before LAD occlusion (pretreatment group), 15-min after the onset of ischemia (during ischemia group), or at the onset of reperfusion (reperfusion group) (Figure 1). Rats in the control group received normal saline. At the end of the experiment, the heart was excised and washed in cold NSS. The ventricles were used for mitochondrial study or heart protein collection.

2.3 Mitochondrial isolation

Mitochondria were freshly isolated from myocardial tissue by differential centrifugation as describe previously [20]. Briefly, ventricular tissue were homogenized in ice-cold isolated buffer (300 mM sucrose, 0.2 mM EGTA, 5 mM TES, pH 7.2) and centrifuged at 800 x g, 4°C for 5 min. Then, the supernatant were collected and re-centrifuged at 8,800 x g, 4°C for 5 min. The mitochondrial pellet was washed by resuspending in ice-cold isolation buffer and re-centrifuged at 8,800 x g, 4°C for 5 min. Mitochondrial protein concentration was determined by the Bicinchoninic Acid (BCA) method, using bovine serum albumin (BSA) as a standard. The isolated cardiac mitochondria that harvested from I/R rat hearts with different time point of SB203580 administration was used to examine mitochondrial swelling, mitochondrial ROS production, and alteration of mitochondrial membrane potential ($\Delta\Psi m$).

2.4 Determination of mitochondrial swelling

To determine the mitochondrial sensitivity to mitochondrial membrane permeability transition (mPT), the mitochondrial swelling was measured. The isolated cardiac mitochondria was re-suspended in respiration buffer that consisting of 100 mM KCl, 50 mM sucrose, 10 mM HEPES, 5 mM KH_2PO_4. The permeability transition-induced swelling of

mitochondria was measured by rapid loss of the absorbance at λ540 nm by spectrophotometric method. The isolated cardiac mitochondria (0.4 mg/ml) were incubated with 1.5 ml of respiration buffer, and then measured the decreasing in the absorbance for 30 min at room temperature. The data were represented in arbitrary units of absorbance.

2.5 Determination of mitochondrial ROS production

It has been known that mitochondrial ROS production increased in many pathological conditions such as myocardial ischemia/reperfusion and aging [21, 22]. Generation of mitochondrial ROS caused oxidative damage to the cell and finally resulting in cell death. We tested the hypothesis that SB203580 could protect mitochondrial functions by reducing the mitochondrial ROS generation. The mitochondrial ROS production was assessed by measuring the intensity of the fluorescent signal of fluorescent 2′,7′-dichlorohydrofluorescein (DCF), which is converted from non-fluorescent 2',7' - dichlorofluorescein – diacetate (DCFH-DA) in the presence of ROS[23]. Isolated cardiac mitochondria (0.4 mg/ml) were incubated with 2 μM DCFH-DA for 30 min at room temperature. The fluorescence intensity was determined by fluorescence microplate ready with the excitation at λ485 nm and emission at λ530 nm. The ROS level was expressed in arbitrary units of fluorescence intensity of DCF.

2.6 Determination of mitochondrial membrane potential changes

One of the molecular responses of mitochondria occurring in myocardial ischemia/reperfusion injury is an opening of the mitochondrial permeability transition pore (MPTP), which consequently results in the change of mitochondrial membrane potential (ΔΨm), and finally leading to cell death. We tested the hypothesis that treatment of SB203580 can protect the loss of ΔΨm. The JC-1 or 5,5',6,6'-tetrachloro-1,1',3,3'-

tetraethylbenzimidazolcarbocyanine iodide is a lipophilic cationic dye that capable of entering the mitochondrial membrane. Monomeric form of JC-1 could fluoresce in green. Increasing of mitochondrial membrane potential changes ($\Delta\Psi m$) causes aggregation of the dye, which appeared in red fluorescein. Alteration of $\Delta\Psi m$ causes changing in red: green ratio. Isolated cardiac mitochondria (0.4 mg/ml) were incubated with 5 μM JC-1 at 37°C for 30 min. The fluorescence intensity for monomeric green fluorescein was determined by fluorescence microplate ready with the excitation at λ485 nm and emission at λ530 nm, while the aggregate red fluorescein was determined by fluorescence microplate ready with the excitation at λ485 nm and emission at λ590 nm. The ratio of red/green fluorescence intensity ratio was determined. The decreased red/green fluorescent intensity ratio indicated mitochondrial membrane depolarization.

2.7 Identification of cardiac mitochondrial ultrastructure

The mitochondrial pellet was fixed in 2.5% glutaraldehyde at 4°C overnight. Then, the pellet was rinsed in 0.1 M phosphate buffer (PO_4) for 15 min twice and post-fixed in 1% cacodylate-buffer osmium tetroxide for 2 hr at room temperature. The mitochondrial pellet was rinsed in 0.1M phosphate buffer (PO_4) 5 min twice and was dehydrated in a graded series of ethanol, 50% ethanol for 5 min twice, 70% ethanol for 5 min twice, 85% ethanol for 5 min twice, 95% ethanol for 5 min twice and 100% ethanol for 5 min twice, respectively. After that, the pellet was infiltrated with propylene oxide (PO) for 10 min twice, followed by the cocktail between resin and PO in 1:2 ratio for 30 min, resin and PO in 1:1 ratio for 60 min and resin for overnight, respectively. On the next day, the pellet was embedded in EM-bed 812 resin [24]. Ultrathin sections were cut with diamond knife, placed in copper grids and stained with uranyl acetate and lead citrate. The cardiac mitochondria were identified with transmission electron microscope (TEM).

2.8 Western blot analysis

The heart tissue was homogenated in extraction buffer (20 mmol/L Tris HCl, 1 mmol/L Na_3VO_4, 5 mmol/L NaF). The heart protein was collected and subjected to 10% or 15% SDS-Polyacrylamide gel electrophoresis; transferred to polyvinylidene difluoride membranes, which were blocked for 2 h with 5% non-fat milk in Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100. The membranes were probed overnight at 4°C with the appropriate primary antibody as follows:total-p38, phospho-p38, and phospho-HSP27 (Cell Signaling Technology, Danvers, MA, USA), total-p53, phospho-p53, total-CREB, phosphor-CREB, total-alpha B-crystalline, phospho-alpha B-crystalline, Bax, Bcl2, Cytochrome c, caspase-3 (Santa Cruz Biotechnology, Inc, California, USA). After washing and exposure to horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, antibody-antigen complexes were visualized by enhanced chemiluminescence assay. Bands corresponding to the protein of interest were appeared as dark regions on the developed film. The film images of the western blots were scanned and were analyzed using Image J (NIH image) analysis software[25]. For quantitation of the proteins of interest, phosphorylated proteins were normalized to total protein expression.

2.9 Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) and LSD. All procedures were performed with an SPSS statistical program (Version 15.0). A p-values < 0.05 was accepted as statistically significant.

3. Results

3.1 p38 MAPK inhibition by SB203580 improved cardiac mitochondrial function

I/R injury caused cardiac mitochondrial swelling by decreasing the absorbance at 540 nm, increased mitochondrial ROS production, and mitochondrial membrane depolarization (Figure 2). Although administration of SB203580 at any time points of I/R protocol significantly attenuated cardiac mitochondrial swelling pretreatment of SB203580 was found to be the most effective timing to prevent cardiac mitochondrial swelling, compared to drug treatment during ischemia or at the onset of reperfusion (Figure 2A). Administration of SB203580 at any time points in I/R could also significantly reduce ROS production in cardiac mitochondria caused by I/R injury, compared to the vehicle control group (Figure 2B). For cardiac mitochondrial membrane potential alteration, we found that administration of SB203580 prior to ischemia or during ischemia significantly prevented the change of $\Delta\Psi m$ caused by I/R injury, when compared to the vehicle control group (Figure 2C). However, SB203580 administered at the onset of reperfusion failed to prevent mitochondrial depolarization caused by I/R (Figure 2C).

3.2 Inhibition of p38 MAPK protected I/R-induced cardiac mitochondrial ultrastructure disruption.

Our results demonstrated that I/R injury not only caused cardiac mitochondrial dysfunction, but also distorted the cardiac mitochondrial ultrastructure by increasing matrix space and disorganization of cristae (Figure 3A). Administration of SB203580 at any time points could preserve the cardiac mitochondrial ultrastructure from the disruption caused by I/R (Figure 3B-C). Interestingly, administration of SB203580 prior to ischemia gave the

most effective treatment to protect I/R induced-cardiac mitochondria ultrastructure rupture (Figure 3D).

3.3 p38 MAPK inhibitor, SB203580, protected cardiac mitochondria by attenuation of apoptotic regulatory molecules activation

Myocardial I/R has been shown to cause activation of p38 MAPK and its activities by phosphorylation of downstream signaling molecules HSP27. Administration of p38 MAPK inhibitor SB203580, prior to or during ischemia, significantly reduced p38 MAPK phosphorylation as well as its activity to phosphorylate downstream substrate HSP27 (Figure 4A-C). However, SB203580 given at the onset of reperfusion could not reduce the p38 MAPK phosphorylation as well a s the level of phosphorylated HSP27 (Figure 4A-C). Moreover, we found that pretreatment of SB203580 significantly inhibited the phosphorylation of p53 and CREB, while SB203580 given during ischemia failed to inhibit the activation of these two downstream molecules (Figure 4). In addition, SB203580 given at the onset of reperfusion could significantly inhibit phosphorylation of CREB, but not p53

Since mitochondria are the key machinery driven cell death, especially the necro-apoptosis in I/R injury, the effects of SB203580 on apoptotic regulatory molecules such as Bax, Bcl2, caspase 3, and cytochrome c were also determined. Our results showed that administration of SB203580 prior to or during ischemia, but not at the onset of reperfusion, significantly decreased Bax expression without any changes in Bcl2 expression level (Figure 5). Administration of SB203580 significantly reduced the cytochrome c level only when it was given prior to the onset of ischemia, but failed to change the cytochrome c level when treated during ischemia or at the onset of reperfusion. Moreover, pretreatment of SB203580

as well as given during ischemia significantly reduced the level of cleaved caspase 3. However, SB203580 given at the onset of reperfusion failed to prevent caspase 3 cleavages.

4. Discussion

In the present study, we demonstrated *for the first time* the direct effect of p38 MAPK inhibitor, SB203580, on cardiac mitochondrial function in the heart underwent I/R injury, and provided molecular mechanistic insights of cardiac mitochondrial protection by p38 MAPK inhibition through the p53-Bax-cytochrome c-caspase 3 cascades. The major findings of this study are that in the heart underwent I/R injury, administration of SB203580 before or during ischemia attenuated cardiac mitochondrial dysfunction caused by I/R as indicated by preventing mitochondrial swelling, reducing mitochondrial ROS generation, and attenuating mitochondrial membrane potential depolarization. However, given SB203580 at the onset of reperfusion could attenuate only mitochondrial swelling and ROS production, but failed to prevent the loss of mitochondrial membrane potential. In addition, inhibition of p38 MAPK activation as well as its activity reduced the activation of p53 and CREB, and decreased the apoptotic regulatory protein Bax, cytochrome c release, and caspase 3.

Mitochondria are vital organelles orchestrate in cellular activities by generating ATP to energize cells. In the heart, cardiac mitochondria play an important role not only generating the energy, but also involving in cell death regulation. Cardiac mitochondrial dysfunction is known as a key mechanism involved in I/R injury and cardiac cell death[3, 26-30]. During I/R injury, cardiac mitochondria loss their function and could contribute to electrical and contractile dysfunction of the cardiac cell and the whole heart[31]. The excessive formation of ROS during I/R injury has been shown to induce prolonged-opening of mitochondria permeability transition pore (MPTP), thus dissipating the proton electrochemical gradient or $\Delta\Psi m$, consequently causing ATP insufficiency, leading to further ROS generation and the loss of intact cardiac mitochondrial ultrastructure, which finally resulting in cardiac mitochondrial swelling and rupture[32]. This process is known to trigger the apoptotic program due to the leakage of pro-apoptotic molecules from ruptured

mitochondria[33]. Therefore, prevention or attenuation of the degree of cardiac mitochondrial dysfunction caused by I/R injury is one of the fascinating clinical targets.

In the present study, we demonstrated that inhibition of p38 MAPK by SB203580 could inhibit p38 MAPK activation and its activity. Moreover, SB203580 could reduce cardiac mitochondrial dysfunction and mitochondrial damage, and attenuate the mitochondrial stress triggering necro-apoptosis in the heart. Moreover, our data also suggest the significance of timing of drug administration on the prevention of cardiac mitochondrial dysfunction caused by I/R. Pretreatment of the inhibitor significantly protected I/R-induced cardiac mitochondrial dysfunction, and also attenuated the activation of apoptotic regulatory molecules. However, inhibition of p38 MAPK during ischemia or at the onset of reperfusion demonstrated a partial effect and be in the way that the quicker the treatment, the better the protective outcomes. Our present data show that treatment of SB203580 during ischemia seems to have more benefit to cardiac mitochondria than given at the onset of reperfusion. This is similar to our previous findings about the effect of different times of SB203580 administration on the infarct size and the incidence of fatal arrhythmia[9]. Since given drug before the onset of acute myocardial ischemia is not practical in real life because ischemia is an unpredictable episode, our findings suggested that giving drug before myocardial reperfusion could be the most effective timing to get the benefit from p38 inhibition.

In response to ROS and I/R stress, p53 protein has been known to accumulate in the mitochondrial matrix and directly mediate mitochondrial outer membrane permeabilization (MOMP) and resulting in the release of cytochrome c, which subsequently activating caspase 3[32]. This activation cascade caused programmed cell death or apoptosis. Recently, a novel role of p53 in mitochondria has been reported. p53 accumulation during I/R injury has been shown to cause MPTP opening, leading to the influx of ions, which resulting in $\Delta\Psi m$ dissipation, leading to the attenuation of the oxidative phosphorylation, and ATP

depletion[32]. The mitochondrial swelling and rupture further caused sequestered cell death factor releasing, which then orchestrated the cell death. Therefore, p53 activation in I/R is considered as a key molecule to trigger cellular necro-apoptosis[34]. It has been reported that p53 could be phosphorylated by p38 MAPK[11, 13, 35], and mediated cell death[12]. In $p38^{-/-}$ cells, phosphorylation of p53 at ser15 was decreased and contributed to down regulation of Bax protein level in cardiomyocytes[36], suggesting the role of p38 MAPK in p53-Bax regulation. Moreover, phosphorylated p53 has been shown to be less affinity for from Mouse double minute 2 homolog (Mdm2) association. None-phosphorylated p53-Mdm2 complex is an inactive and labile form, which could be subsequently degraded by ubiquitin proteolytic system[37]. Our results are consistent with those reports in which inhibition of p38 MAPK by SB203580 reduced p53 activation and Bax expression level, which in turn attenuated the release of cytochrome c level to trigger the activity of caspase 3 mediated apoptosis. The impairment of mitochondrial activity has been found to activate cAMP-responsive element-binding protein (CREB) phosphorylation at Ser133[14], which in turn activated p53 in transcriptional dependent manner. Down regulation of p53 could also affect the p53 mediate mitochondrial dysfunction and apoptosis. Therefore, inhibition of CREB phosphorylation could be a p53 regulatory target. It has been shown that, during ischemia, CREB is activated as a downstream event of p38 MAPK activation[15]. Inhibition of p38 MAPK by SB203580 could reduce I/R-induced CREB phosphorylation. However, we found that the protein expression level of p53 was not significantly different. This could possibly be due to the study protocol, which aimed to study the acute effect of p38 MAPK inhibitor. The duration of inhibitor treatment as well as I/R protocol may not be sufficient to initiate the transcriptional processes of p53. Nevertheless, our findings suggest that administration of SB203580 could inhibit p53 activation in a transcriptional independent manner.

In the present study, p38 MAPK was phosphorylated as a result of myocardial I/R injury, leading to cardiac mitochondrial dysfunction and ultrastructure rupture, which consequently resulting in cell death. Inhibition of p38 MAPK activation and its activity by SB203580 protected cardiac mitochondria from I/R injury by reducing the phosphorylated p53 in a transcription-independent manner (Figure 6). Non-phosphorylated p53 is degraded by ubiquitin proteolytic system, which is insufficient to mediate mitochondrial membrane permeabilization and reduce myocardial I/R induced cardiac mitochondrial dysfunction (Figure 6). Furthermore, the reduction of phosphorylated p53 level by SB203580 could also influence Bax expression, resulting in the decrease of mitochondrial membrane permeability, cytochrome c, and caspase 3 levels (Figure 6). These mechanistic insights of SB203580 on cardiac mitochondria explain the cardioprotective effects of p38 MAPK inhibition in myocardial I/R. Another possible explanation concerning the cardiac mitochondria protective effect of SB203580 is the attenuation of the voltage dependent anion channel (VDAC) phosphorylation, which is a porin protein involved in mitochondrial regulator of cell survival[26, 40-42]. Phosphorylation of VDAC-1 facilitates other protein binding in MPTP and mediates mitochondrial damage. Schwertz *et al*. reported that VDAC-1 was a downstream substrate of p38 MAPK during I/R injury[43]. Inhibition of p38 MAPK by PD169316 significantly reduced phosphorylation of VDAC-1, and reduced cardiac cell injury [43]. However, the effect of SB203580 on VDAC-1 phosphorylation in cardiac mitochondrial from myocardial I/R model need to be further investigated.

In summary, this is the first report to demonstrate that p38 MAPK inhibition by SB203580 could reduce cardiac mitochondrial dysfunction caused by I/R injury through the attenuation of p53-mediate mitochondrial trigger cell death. Moreover, our data suggest that the therapeutic potential of SB203580 to protect cardiac mitochondria from I/R injury could provide more clinical benefit when given prior to reperfusion.

Disclosure statement

There is no conflict of interests.

Acknowledgments

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Figure legends

Figure 1: Study protocol and arrhythmia scores. Study protocol for experimental groups and timing of SB203580 (SB) or normal saline solution (NSS) administration.

Figure 2: The effect of SB203580 on cardiac mitochondrial function. (A) mitochondrial swelling (B) mitochondrial ROS production, (C) mitochondrial membrane potential, when administration before, during ischemia, or at the onset of reperfusion in ischemia/reperfusion rats (n=4-7 animals/group). *$p<0.05$ vs. vehicle group, $^{\#}p<0.05$ vs. pretreatment group.

Figure 3: **Effect of SB203580 on mitochondrial ultrastructure**. The cardiac mitochondria were isolated from ischemia/reperfusion rats in the presence of SB203580 before (B), during ischemia (C), or at the onset of reperfusion (D) (n=4-5 animals/group). *$p<0.05$ vs. vehicle group.

Figure 4: **The effect of SB203580 on p38 MAPK activation and downstream substrates, HSP27, p53, CREB, and α-B-crystalline**. The heart homogenates were collected and subjected to Western blot analysis detecting the activation of interested proteins (A). The quantitation of fold phosphorylation is shown in panels B-F (n=3 animals/group).

Figure 5: **The effect of SB203580 on apoptotic regulatory proteins.** The heart homogenates were collected and subjected to Western blot analysis detecting the expression of Bax, Bcl2, Cytochrome c, and cleaved caspase-3 (A). The quantitation of fold phosphorylation is shown in panels B-D (n=3 animals/group).

Figure 6: **The proposed mechanistic pathway of I/R-activated p38 MAPK and downstream activation involving mitochondrial trigger cell death**. Myocardial I/R injury caused p38 MAPK activation, which consequently activated p53 phosphorylation. Phosphorylated p53 stabilized and accumulated in mitochondrial matrix during I/R injury and mediated MPTP opening. Activation of p53 also activated Bax expression, which regulated

cytochrome c release, and activation of caspase 3. p38 MAPK could also phosphorylate CREB, which in turn regulated p53 pathway in a transcription-dependent manner. Inhibition of p38 MAPK by SB203580 could reduce p53 phosphorylation, CREB phosphorylation and then protect cardiac mitochondria from I/R injury and cell death.

Figure 1.

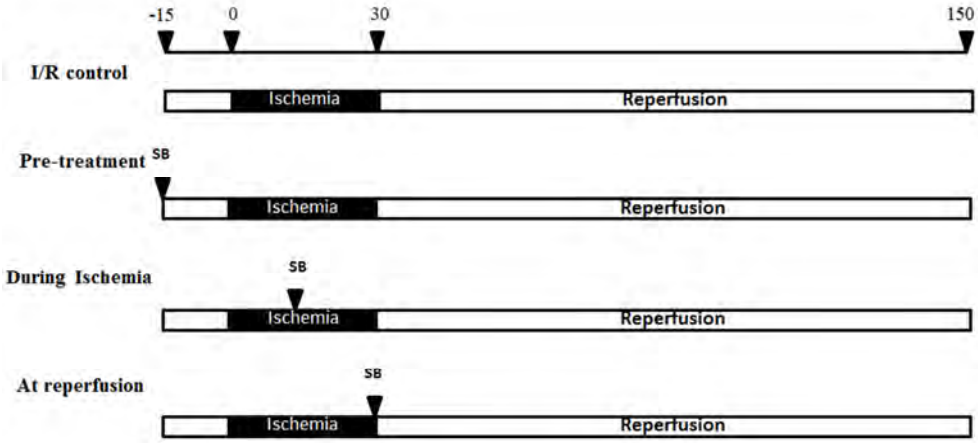


Figure 2

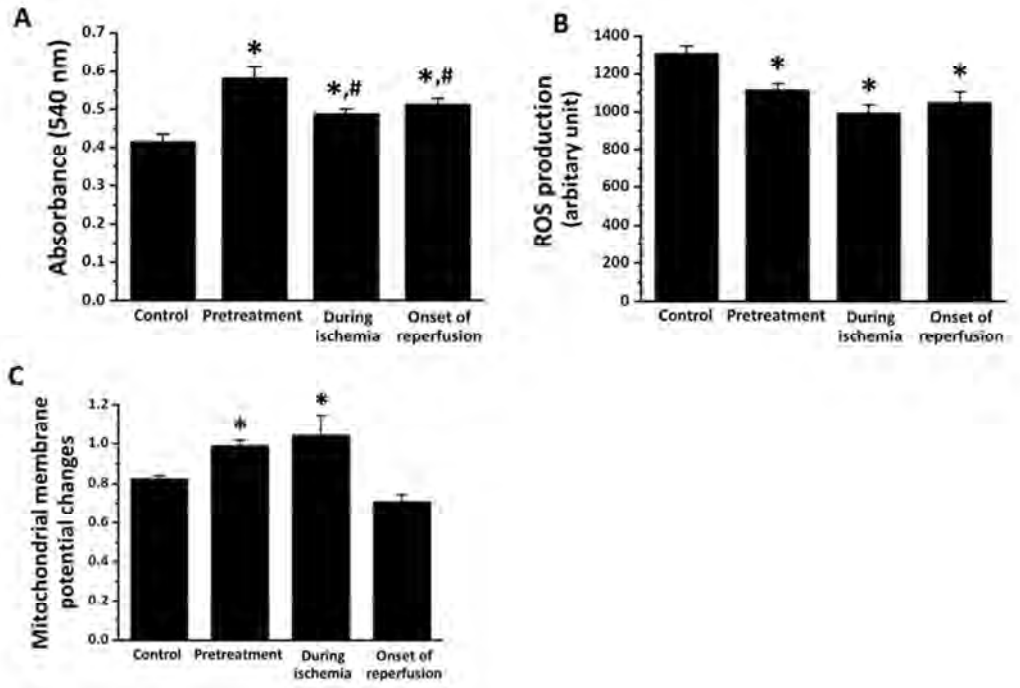


Figure 3

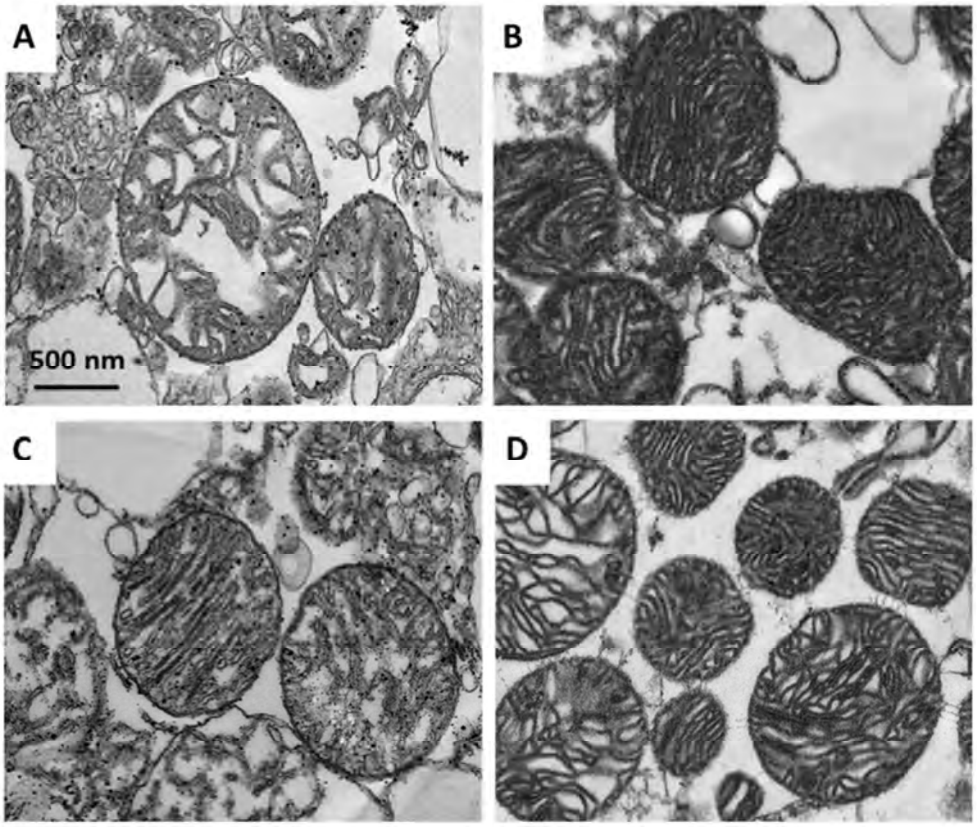
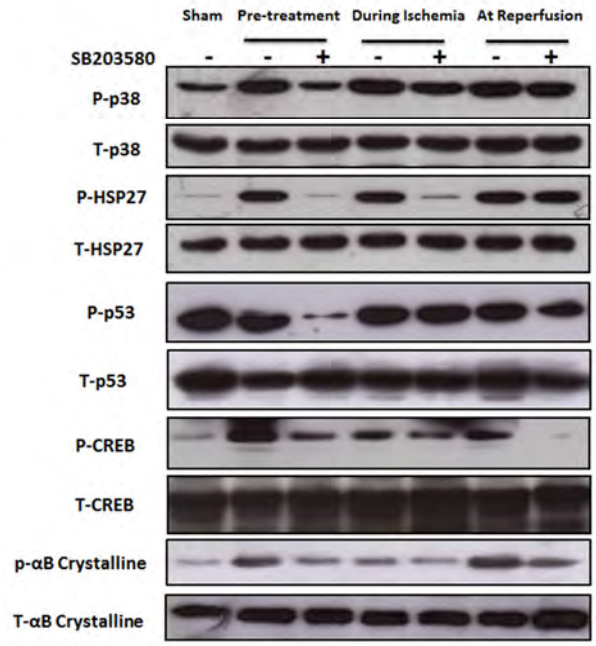


Figure 4


Sham
Pre-treatment
During Ischemia
At Reperfusion
SB203580
- - + - + - +
P-p38
T-p38
P-HSP27
T-HSP27
P-p53
T-p53
P-CREB
T-CREB
p-αB Crystalline
T-αB Crystalline

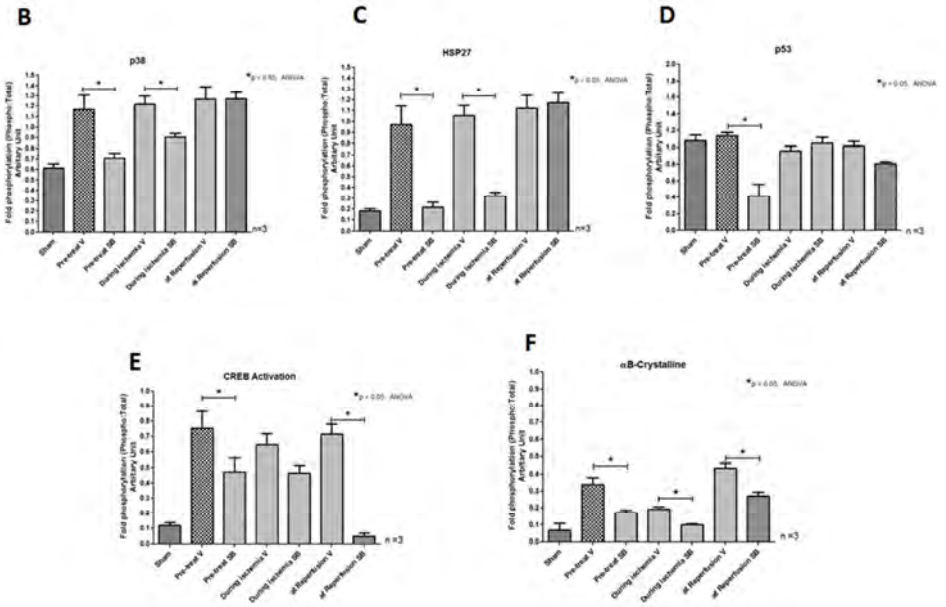

B
p38
C
HSP27
D
p53
E
CREB Activation
F
αB-Crystalline

Figure 5

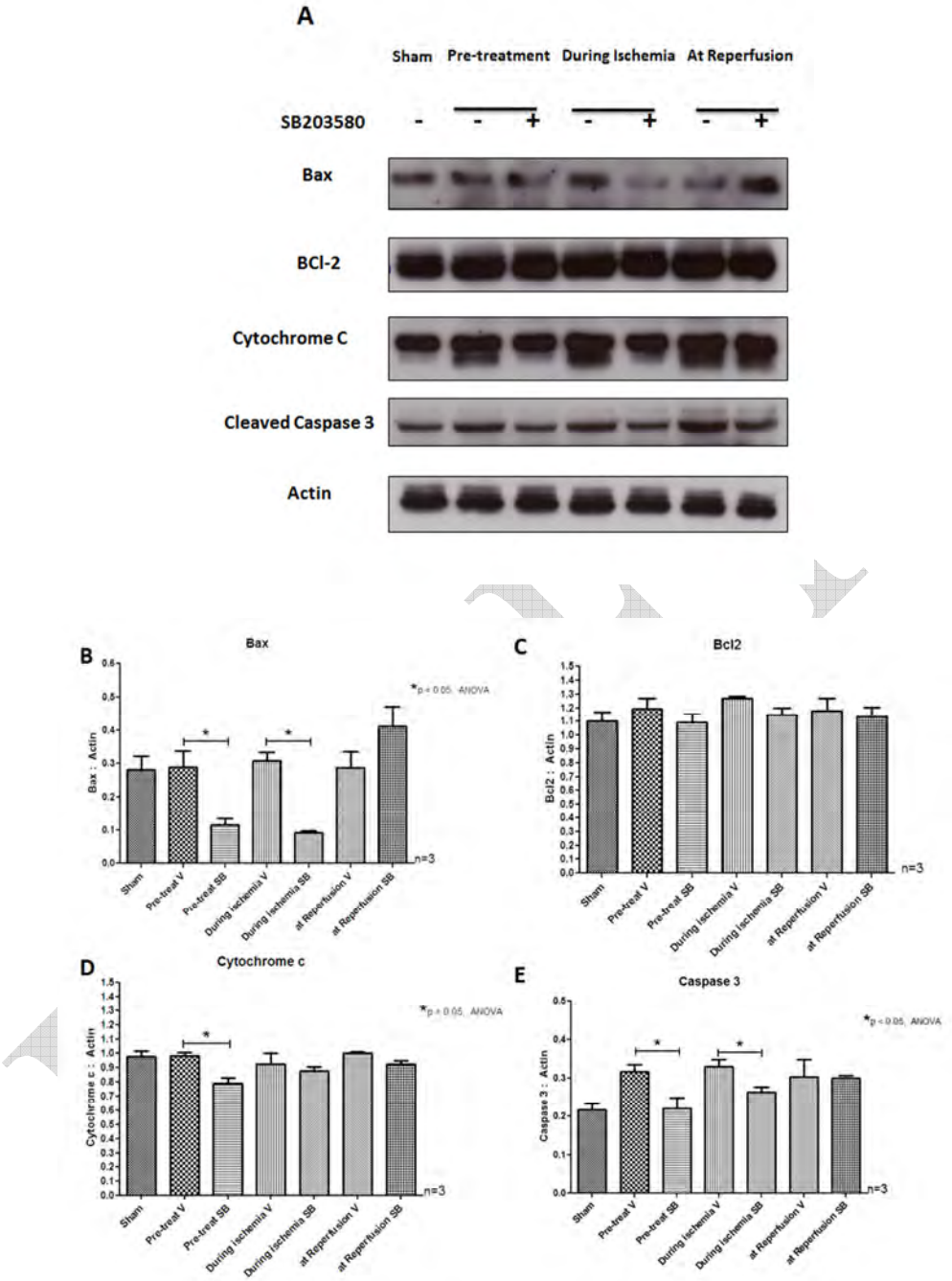

A
Sham
Pre-treatment
During Ischemia
At Reperfusion
SB203580
- - + - + - +
Bax
BCl-2
Cytochrome C
Cleaved Caspase 3
Actin
B
Bax
Bax : Actin
*p < 0.05, ANOVA
n=3
Sham
Pre-treat V
Pre-treat SB
During Ischemia V
During Ischemia SB
at Reperfusion V
at Reperfusion SB
C
Bcl2
Bcl2 : Actin
n=3
D
Cytochrome c
Cytochrome c : Actin
E
Caspase 3
Caspase 3 : Actin

Figure 6