สัญญาเลขที่ TRG5680018

รายงานวิจัยฉบับสมบูรณ์

ผลร่วมของการขาดเอสโตรเจนกับภาวะอ้วนจากการเหนี่ยวนำโดยการบริโภคอาหารไขมันสูงต่อภาวะดื้ออินซูลินในสมอง, การปรับเปลี่ยนที่จุดประสานประสาท, ความจดจำ, ความเครียดออกซิเดชั่นในสมองและการทำงานของไมโตครอนเดรียในสมอง

Effects of the combined estrogen deprivation with obesity induced by high-fat diet consumption on neuronal insulin resistance, synaptic plasticity, cognition, brain oxidative stress and brain mitochondrial function

โดย

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สนับสนุนโดย สำนักงานกองทุนสนับสนุนการวิจัย และ
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Wasana Pratchayasakul

บทคัดย่อ

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ชื่อโครงการ: **ผลร่วมของการขาดเอสโตรเจนกับภาวะอ้วนจากการเหนี่ยวนำโดยการบริโภคอาหารไขมันสูงต่อภาวะดื้ออินซูลินในสมอง, การปรับเปลี่ยนที่จุดประสานประสาท, ความจดจำ, ความเครียดออกซิเดชั่นในสมองและการทำงานของไมโตครอนเดรียในสมอง**

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บทคัดย่อ:
วัตถุประสงค์/สมมุติฐาน: การศึกษาที่ผ่านมาของทีมผู้วิจัยพบว่าภาวะอ้วนจากการเหนี่ยวนำด้วยอาหารไขมันสูงเป็นสาเหตุของภาวะดื้อต่ออินซูลินในเนื้อเยื่อส่วนปลายและในสมอง ภาวะการสูญเสียการทำงานของไมโตคอนเดรียในสมอง และการสูญเสียการเรียนรู้จดจำ หลายๆการศึกษาที่ผ่านมาพบว่าไม่ว่าจะเป็นภาวะอ้วนอย่างเดียวหรือการตัดรังไข่เพียงอย่างเดียวมีผลให้เกิดการสูญเสียการเรียนรู้จดจำผ่านทางการปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัส อย่างไรก็ตามผลของภาวะอ้วนร่วมกับการตัดรังไข่ต่อความไวของการทำงานของอินซูลินในเนื้อเยื่อส่วนปลายและในสมอง การทำงานของไมโตคอนเดรียในสมอง การปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัส และการเรียนรู้จดจำ ยังไม่มีการศึกษามาก่อน จึงมีสมมุติฐานของงานวิจัยที่ว่าภาวะอ้วนจะเร่งการเกิดภาวะดื้อต่ออินซูลินในสมอง ภาวะการสูญเสียการทำงานของไมโตคอนเดรียในสมอง ภาวะการสูญเสียการปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัส และการสูญเสียการเรียนรู้จดจำในกรณีที่มีการตัดรังไข่
วิธีการทดลอง: หนูเพศเมียจำนวน **72** ตัวถูกแบ่งออกเป็นกลุ่มผ่าตัดหลอกกับกลุ่มผ่าตัดรังไข่ หนูแต่ละกลุ่มจะถูกแบ่งออกเป็น **2** กลุ่มย่อยแต่ละกลุ่มย่อยจะถูกให้อาหารปกติหรืออาหารไขมันสูงเป็นเวลา **4, 8, 12** สัปดาห์ เมื่อสิ้นสุดแต่ละช่วงเวลาการทดลอง ตัวอย่างเลือดจะถูกเก็บเพื่อตรวจตัวชี้วัดเมตาโบลิค การเรียนรู้จดจำจะถูกทดสอบก่อนการเก็บตัวอย่างสมอง ตัวอย่างสมองจะถูกนำไปตรวจภาวะเครียดออกซิเดชั่น ความไวของการทำงานของอินซูลินในสมอง การทำงานของไมโตคอนเดรียในสมอง การปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัส

ผลการทดลอง: จากผลการทดลองพบว่า 1) ภาวะดื้อต่ออินซูลินในเนื้อเยื่อส่วนปลาย ภาวะเครียดออกซิเดชั่น การสูญเสียการปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัสจะถูกตรวจพบก่อนในสัปดาหที่ **8** ในกลุ่มผ่าตัดรังไข่อย่างเดียว กลุ่มอ้วนอย่างเดียว และกลุ่มที่มีภาวะอ้วนร่วมกับการตัดรังไข่ แต่ผลเสียดังกล่าวจะแย่มากที่สุดในกลุ่มที่มีภาวะอ้วนร่วมกับการตัดรังไข่ 2) ภาวะดื้อต่ออินซูลินในสมอง ภาวะการสูญเสียการทำงานของไมโตคอนเดรียในสมอง และการสูญเสียการเรียนรู้จดจำจะถูกตรวจพบก่อนในสัปดาห์ที่ **8** ในกลุ่มที่มีภาวะอ้วนร่วมกับการตัดรังไข่ อย่างไรก็ตามภาวะดังกล่าวจะถูกตรวจพบตามมาในสัปดาห์ที่ **12** ในกลุ่มผ่าตัดรังไข่อย่างเดียว หรือ กลุ่มอ้วนอย่างเดียว

สรุปผลการทดลอง: จากผลการทดลองทั้งหมดสามารถสรุปได้ว่า ภาวะอ้วนจะสามารถเร่งการสูญเสียการเรียนรู้จดจำในสภาวะขาดเอสโตรเจนได้ โดยการทำให้ภาวะเครียดออกซิเดชั่น ภาวะการสูญเสียการทำงานของไมโตคอนเดรียในสมอง ภาวะดื้อต่ออินซูลินในสมอง และการสูญเสียการปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัสมีความรุนแรงขึ้น

คำหลัก: อาหารไขมันสูง, การผ่าตัดรังไข่, ความไวของการทำงานของอินซูลินในสมอง, การทำงานของไมโตคอนเดรียในสมอง, การปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัส

Abstract

Project Code: TRG5680018

Project Title: Effects of the combined estrogen deprivation with obesity induced by high-fat diet consumption on neuronal insulin resistance, synaptic plasticity, cognition, brain oxidative stress and brain mitochondrial function

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Project Period: 24 months

Abstract:

Aims/hypothesis: Our previous studies showed that obese induced by high-fat diet (HFD) caused peripheral and brain insulin resistance, brain mitochondrial dysfunction and cognitive impairment. Several studies also demonstrated that either ovariectomy alone or obese alone lead to cognitive impairment through hippocampal synaptic dysfunction. However, the effect of obese on top of ovariectomized condition on peripheral and brain insulin sensitivity, brain mitochondrial function, hippocampal synaptic plasticity and cognition has never been investigated. We hypothesized that obese accelerates the occurrence of brain insulin resistance, brain mitochondrial dysfunction, hippocampal synaptic dysfunction and cognitive impairment in ovarietomized condition.

Methods: Seventy-two female rats were divided into sham and ovariectomized (OVX) groups. Rats in each group were divided into 2 subgroups and each subgroup was fed with either normal diet or HFD for 4, 8 and 12 weeks. At the end of each period, blood was collected for the metabolic analysis. The cognition was tested before brain was removed for investigating oxidative stress, insulin sensitivity, mitochondrial function and hippocampal synaptic function.

Results: We found that 1) peripheral insulin resistance, brain oxidative stress and hippocampal synaptic dysfunction were observed earlier (week 8) in OVX alone, obese alone and obese OVX-condition, but those conditions were worsen in obese OVX-condition, and 2) brain insulin resistance, brain mitochondria dysfunction and cognitive impairment appeared earlier (week 8) in

obese OVX-condition, however, those conditions demonstrated later (week 12) in OVX alone and obese alone.

Conclusions: All of these findings suggest that obese accelerates the cognitive impairment in estrogen deprivation by aggravating brain oxidative stress, brain mitochondrial dysfunction, brain insulin resistance and hippocampal synaptic dysfunction.

Keywords: High-fat diet, Ovariectomy, Brain insulin sensitivity, Brain mitochondrial function, Hippocampal synaptic function

เนื้อหางานวิจัย (Executive summary)

บทนำ (Introduction)

Several studies showed that either estrogen deprivation alone or obesity alone increased oxidative stress level, leading to the development of insulin resistance and cognitive impairment (1-4). Interestingly, our previous studies demonstrated that obesity induced by a high-fat diet (HFD) increased brain oxidative stress, as indicated by increased brain corticosterone level, brain malondialdehyde (MDA) level and brain mitochondrial reactive oxygen species (ROS) production, which lead to impair brain insulin sensitivity, indicated by the impairment of insulin-induced long term depression (LTD) and impaired brain insulin signaling (3, 5-7). In addition, those HFD rats demonstrated the cognitive impairment in the Morris Water Maze test (3, 7).

Moreover, several studies also showed that either estrogen deprivation alone or obesity alone could cause cognitive decline through impaired hippocampal synaptic function (3, 8-10). The studies in estrogen deprivation model showed that ovariectomized (OVX) rats caused the impairment of hippocampal synaptic function as indicated by decreased the long term potentiation (LTP) amplitude, dendritic spine density and synaptic proteins as well as the impairment of learning and memory process (11, 12). Those impairments following OVX were alleviated by estrogen administration (8, 9, 13). In addition, consumption of a high calorie diet exhibited impaired hippocampal synaptic plasticity indicated by reduced the LTP induction in hippocampus and impaired spatial learning ability (10, 14, 15). Although several studies have shown that either estrogen deprivation alone or obesity has adverse effects on hippocampal synaptic function and cognitive function, but 1) the effects of estrogen deprivation alone on brain insulin sensitivity, brain mitochondrial function have not been determined, and 2) the combination of estrogen deprivation and obesity on brain oxidative stress, brain insulin sensitivity, brain mitochondria function, hippocampal synaptic function and cognitive behaviors have never been investigated. Therefore, in the present study, we hypothesized that 1) estrogen deprivation alone causes the impairment of brain insulin sensitivity, brain mitochondria function, hippocampal synaptic function and cognitive behaviors, and 2) obesity accelerates those impairments in estrogen-deprived condition.

วิธีการทดลอง (Methods)

Animal models and experimental protocols

All experimental protocols were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Seventy two female Wistar rats weighting 200-220 g (aged ~ 6-weeks old) were achieved from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were housed individually in a temperature-controlled environment under a light-dark cycle of 12:2 h. One week after arrival, rats were divided into sham-operated (Sham;S) and ovariectomized (OVX;O) groups. Dietary protocol was started after one week of surgery. Rats were randomly assigned as a normal diet (ND; 19.77% E fat) group, which received a standard laboratory pellet diet (Mouse Feed Food No. 082, C.P.Company, Bangkok, Thailand) or a high-fat diet (HFD; 59.28 % E fat) group, which were fed with a diet containing 59.3% total energy from fat with the major composition of fat saturated fatty acid from lard as described in our previous study (6). All animals were given ad libitum access to food and water. At the end of week 4, 8 and 12, the oral glucose tolerance test, locomotor activities test and the spatial learning and memory behaviors test were completed in each animal. Then, animals were deeply anesthetized with isoflurane after fasting for at least 5 hours and killed by decapitation. Blood samples were collected for determining metabolic parameters such as plasma glucose, HDL, LDL, total cholesterol, triglyceride, insulin and estrogen levels. Total visceral fat, including peritoneal, periovarian and perirenal fat pads were removed and weighed. The brain was rapidly removed for electrophysiological study, including insulin-induced LTD and electrical induced LTP, and biochemical analyses, including the expression of insulin receptor (IR), IR phosphorylation Akt/PKB and Akt/PKB phosphorylation, brain mitochondrial function, brain oxidative stress and hippocampal dendritic spine density.

Ovariectomy Procedure

Female rats were anesthetized with xylazine (LBS Laboratories, Bangkok, Thailand; 0.15 ml/kg) and Zoletil (VirbacLabolatories, Carros, France; 50 mg/kg). The bilateral ovariectomy was performed through a midline dorsal skin incision. The incision was centered between the bottom of the rib cage and the front of the hind limb. The skin was separated from the underlying muscle. The connections between the uterine tubes and uterine horn inside peritoneal cavity were transected, the ovaries were removed and the incision was closed.

Chemical analysis for metabolic parameters

Fasting plasma glucose, HDL, LDL, cholesterol and triglyceride levels were determined by enzymatic colorimetric assay using commercially available kits (ERBA diagnostic, Mannheim, Germany). The fasting plasma insulin levels were investigated by Sandwich ELISA kits (LINCO Research, Missouri, USA). Serum estrogen levels were determined using an enzyme immunoassay kit (Cayman chemical, Ann Arbor, Michigan, USA).

Determination of insulin resistance

Insulin resistance was evaluated by the Homeostasis Model Assessment (HOMA) index (16) and the total area under the curve of oral glucose tolerance test (OGTT). Fasting plasma insulin and fasting plasma glucose levels were used to calculate HOMA index. A higher HOMA index showed a higher degree of insulin resistance. OGTT was tested after fasting for 12 hours, and then rats were given a gavage of 2 g/kg body weight of glucose. Blood was collected at 0, 15, 30, 60 and 120 min after glucose loading for plasma glucose measurement.

Determination of malondialdehyde (MDA) levels

Malondialdehyde (MDA) level, an indicator of oxidative stress, was determined by a high performance liquid chromatography (HPLC) method following previous study protocol (17). MDA levels were measured by the HPLC system at the absorbance of 532 nm, and were calculated directly from the standard curve.

Brain slice preparation

At the end of each experimental period, brain slice preparation was performed following our previous study guidelines (18). Briefly, brain slices were cut in ice-cold “high sucrose” aCSF solution using a vibratome (Vibratome Company, St. Louis, Missouri, USA). After that, brain slices were transferred to a standard aCSF for an additional 30 minutes before extracellular recording.

Extracellular recordings of hippocampal slices for insulin-induced long-term depression (LTD)

Field excitatory postsynaptic potentials (fEPSPs) were recorded according to our previous protocol (6, 19). The initial slopes of the fEPSP were measured and plotted against time. For baseline condition, hippocampal slices were perfused with aCSF for 10 minutes. After that, insulin-induced LTD condition was performed by perfused with aCSF plus 500 nM insulin for an additional 10 minutes and then the slices were perfused with aCSF again for 50 minutes further.

Extracellular recordings of hippocampal slices for electrical-induced long term potentiation (LTP)

To examine electrical-induced long term potentiation (LTP), fEPSPs were recorded according to our previous protocol (6, 19). The initial slopes of the fEPSPs were measured and plotted against time. After baseline condition for 20 minutes, LTP was induced by delivering high-frequency stimulation (HFS; four trains at 100 Hz; 0.5 s duration; 20 s interval). Experiments were performed for at least 40 minutes after HFS. The amount of potentiation was calculated at 40 minutes after tetanus.

Preparation of brain homogenates for immunoblotting

The subsequent brain homogenates for immunoblotting was performed as described in our previous (6). Briefly, brain homogenates for immunoblotting were prepared by incubated whole brain slices into aCSF or aCSF plus 500 nM insulin (Humulin R, Eli Lilly, Giessen, Germany) for 5 minutes. After that, whole brain slices in each conditioned group were homogenized with ice-cold brain slice lysis buffer and centrifuged at 15,000 g for 30 minutes at 4^{o}C. Cytosol fraction was separated and used for further biochemical analysis of the IR protein expression, Akt /PKB protein expression, IR tyrosine phosphorylation and Akt/PKB serine phosphorylation.

Immunoblotting

IR protein, Akt/PKB protein, IR tyrosine phosphorylation and Akt/PKB at serine 473 kinases phosphorylation were electrophoresed and immunoblotted with rabbit antibodies for IR (1:1000; Santa Cruz Biotechnology, CA, USA)), Akt/PKB (1:1000; Cell Signaling Technology, MA, USA), IR at tyrosine (1:1000; Santa Cruz Biotechnology, CA, USA) and Akt/PKB at serine 473(1:1000; Cell Signaling Technology, MA, USA), respectively. All blots were incubated with a horseradish peroxidase conjugated anti-rabbit secondary antibody (1:2000; Cell Signaling

Technology, MA, USA). The protein bands were developed with Amersham hyperfilm ECL (GE Healthcare, Buckinghamshire, UK). Intensities of bands were quantified using Scion image software.

The isolation of brain mitochondria

After decapitation, the brain tissue was transferred into 10 ml of ice-cold MSE-nagarse solution and homogenized at 600 rpm/min using a homogenizer. The subsequent brain mitochondrial isolation was prepared as described in our previous study (5).

Brain mitochondrial reactive oxygen species (ROS) assay

Brain mitochondrial ROS production was measured by a fluorescent probe and dichlorohydrofluoresceindiacetate (DCFHDA) as described in our previous study (5). Brain mitochondrial protein (0.4 mg/ml) was incubated with 2 μM DCFDA at 25°C for 20 minutes. ROS was evaluated by a fluorescent microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA) at the excited wavelength of 485 nm and emission wavelength at 530 nm. The increased of fluorescent intensity was represented as increased brain mitochondrial ROS.

The mitochondrial membrane potential ($\Delta\Psi_m$) assay

The measurement of brain mitochondrial membrane potential ($\Delta\Psi_m$) change was determined by fluorescent dye 5, 5′,6,6′-tetrachloro-1, 1′,3,3′-tetraethyl benzimidazolcarbocyanine iodide (JC-1) as described in our previous studies (5). The brain mitochondrial protein (0.4 mg/ml) was incubated with JC-1 dye at 37 °C for 15 minutes. The mitochondrial membrane potential was determined as fluorescence intensity by a fluorescent microplate reader at the excited wavelength of 485 nm and the emission wavelength of 530 nm for JC-1 monomer form (green fluorescent), and the excited wavelength of 485 nm and the emission wavelength of 590 nm for JC-1 aggregate form (red fluorescent). Red to green fluorescence intensity ratio was used to calculate mitochondrial membrane potential change.

Brain mitochondria swelling assay

Brain mitochondrial swelling was determined following our previous protocol (5). The brain mitochondrial protein (0.4 mg/ml) was incubated in a 2 ml of respiration buffer. The brain mitochondrial suspension was read at 540 nm by using a microplate reader. The mitochondrial swelling was indicated by a decrease in the absorbance.

Golgi impregnation and analysis

After decapitation, brains were removed and rinsed with double distilled water, then was processed for Golgi staining method by using a commercially available kit (FD Neurotechnologies kit, PK 401, Ellicott City, U.S.A.). For analysis of dendritic spine density, the secondary and tertiary dendrites of 3 neuron at CA1 hippocampus area. Dendritic segments were viewed through inverted microscope (IX-81, Olympus, Tokyo, Japan).

The open-field test

The open-field test was developed to determine the locomotor activity and was modified from the method of Arakawa (20). The open field was made of black platform and consisted of a floor (75 cm×75 cm) with40-cm walls. The box floor was draw with white line (6 mm) to form 25 equal squares. The animals were placed into the box and observation for 2 minutes. The numbers of line which the animals crossed were counted as activity. This parameter was used to exclude the different locomotor activity among rats.

Morris water maze test (MWM)

In the present study, the protocol of MWM was modified from Vorhees et al, 2006 (21). This experiment was determined learning and memory ability with 2 different types of MWM, acquisition test (hidden platform) and probe trial test (removal of the platform from the water pool) as described in our previous study (3).

Statistical analysis

All of data were presented as mean ± SEM. The significance of the difference in peripheral metabolic parameters was calculated by Mann-Whitney U test. The comparison of the percentage of electrical induced-LTP, insulin-induced LTD, dendritic spine density, brain insulin signaling, brain oxidative stress and brain mitochondrial function between groups were calculated using one-way ANOVA followed by post-hoc LSD. Comparisons among groups in MWM tests for learning task (time to reach platform) were performed using two-way ANOVA followed by post-hoc LSD. For all comparison, a value of $P < 0.05$ was considered to be statistically significant.

ผลการทดลอง (Results)

The addition of obesity onto an ovariectomized condition aggravated the impairment of peripheral insulin sensitivity.

Initial animal body weight, plasma glucose and plasma insulin levels were not significantly different among the experimental groups. In addition, we confirmed the effect of ovariectomy (OVX) by determining uterine weight and serum estrogen levels at four, eight and 12-weeks of dietary periods. We found that OVX resulted in decreased uterine weight and serum estrogen levels in both ND- and HFD-fed rats ($p<0.05$; Table 1-3).

At week 4, sham HFD-fed rats (HFS) and ovariectomized HFD-fed rats (HFO) significantly increased in body weight and visceral fat compared to sham ND-fed rats (NDS) and ovariectomized ND-fed rats (NDO) ($p<0.05$; Table 1). However, the levels of glucose, cholesterol, triglyceride, HDL, LDL, insulin, HOMA index and the total area under the glucose curve (AUCg) from the OGTT were not significantly different among the experimental groups (Table 1).

At week 8 and 12, NDO, HFS and HFO rats developed the impairment of peripheral insulin sensitivity, which were showed by the increasing of body weight, visceral fat, plasma insulin level, HOMA index and AUCg compared with NDS rats ($p<0.05$; Table 2-3). In addition, plasma LDL and cholesterol levels of HFS and HFO rats were significantly increased compared to those of NDS rats ($p<0.05$; Table 2-3). Interestingly, we demonstrated that body weight, visceral fat weight, plasma glucose level, plasma total cholesterol level, plasma LDL level, plasma insulin level, HOMA index and AUCg were highest in HFO rats ($p<0.05$; Table 2-3). These findings of week 8 and 12 suggest that the obesity added onto ovariectomy significantly increase the severity of impaired peripheral insulin sensitivity.

Table 1. The metabolic parameters in both sham and OVX rats receiving either ND or HFD feeding for 4 weeks

Parameter	NDS	NDO	HFS	HFO
Body weight (g)	255.00 ± 4.28	285.83 ± 6.88	292.14 ± 9.62*	356.87 ± 16.76*,†,‡
Visceral fat (g)	11.01 ± 1.31	9.33 ± 0.84	20.72 ± 1.60*,†	22.12 ± 2.15*,†
Uterus (g)	0.35 ± 0.02	0.11 ± 0.00*,‡	0.34 ± 0.05	0.10 ± 0.01*,‡
Food intake (g/day)	16.65± 0.98	16.10 ± 1.13	17.57 ± 4.39	18.54 ± 0.96
Glucose (mg%)	128.43 ± 6.68	125.61 ± 8.12	128.07 ± 10.56	128.31 ± 11.39
Cholesterol (mg/dl)	93.23 ± 6.38	104.00 ± 8.60	103.89 ± 9.90	101.08 ± 12.69
Triglyceride (mg/dl)	34.02 ± 3.64	32.22 ± 3.49	33.68 ± 3.64	31.60 ± 3.70
HDL(mg/dl)	5.85 ± 0.32	6.04 ± 0.13	5.56 ± 0.47	5.68 ± 0.41
LDL (mg/dl)	76.23 ± 3.79	82.70 ± 6.55	75.90 ± 8.51	70.37 ± 8.85
Insulin (ng/ml)	0.83 ± 0.08	0.83 ± 0.11	0.92 ± 0.15	1.01 ± 0.09
HOMA index	6.28 ± 1.56	6.10 ± 0.11	7.11 ± 1.35	7.75 ± 1.32
Serum estrogen (pg/ml)	167.76 ± 39.27	58.73 ± 17.47*,‡	151.87 ± 21.20†	63.17 ± 20.60*,‡
Plasma glucose AUC (mg/dl×min×10^4)	4.10 ± 0.29	4.45 ± 0.41	4.53 ± 0.37	4.05 ± 0.11

*, $p<0.05$ compared with NDS, †, $p<0.05$ compared with NDO, ‡, $p<0.05$ compared with HFS; n=5-6/group; NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats, HFO: ovariectomized HFD-fed rats

Table 2. The metabolic parameters in both sham and OVX rats receiving either ND or HFD feeding for 8 weeks

Parameter	NDS	NDO	HFS	HFO
Body weight (g)	275.00 ± 6.75	311.38 ± 3.80*	310.00 ± 7.52*	384.00 ± 10.74*,†,‡
Visceral fat (g)	10.49 ± 1.07	14.01 ± 0.91*	23.28 ± 5.44*,†	28.56 ± 1.94*,†
Uterus (g)	0.35 ± 0.03	0.09 ± 0.01*,‡	0.49 ± 0.14	0.09 ± 0.01*,‡
Food intake (g/day)	15.72 ± 1.14	15.20 ± 1.14	16.19 ± 0.75	16.55 ± 0.65
Glucose (mg%)	122.76 ± 7.34	125.44 ± 7.73	138.90 ± 7.97	148.82 ± 7.16*
Cholesterol (mg/dl)	98.89 ± 3.93	107.86 ± 2.77	124.04 ± 14.70	137.15 ± 12.31*,†
Triglyceride (mg/dl)	45.78 ± 4.02	41.43 ± 2.96	44.93 ± 3.42	40.92 ± 4.14
HDL(mg/dl)	6.30 ± 0.10	6.86 ± 0.05	6.13 ± 0.33	7.11 ± 0.43
LDL (mg/dl)	70.70 ± 1.17	89.52 ± 3.39	90.37 ± 4.59*	120.50 ± 2.85*,†,‡
Insulin (ng/ml)	0.78 ± 0.12	1.24 ± 0.15*	1.44 ± 0.21*	1.58 ± 0.16*
HOMA index	5.90 ± 1.01	10.69 ± 1.55*	12.49 ± 1.82*	13.18 ± 1.45*
Serum estrogen (pg/ml)	111.02 ± 10.83	51.88 ± 2.22*,‡	112.84 ± 15.24†	56.71 ± 3.70*,‡
Plasma glucose AUC (mg/dl×min×10^4)	3.55 ± 0.09	3.84 ± 0.20*	3.80 ± 0.93*	4.30 ± 0.25*

*, $p<0.05$ compared with NDS, †, $p<0.05$ compared with NDO, ‡, $p<0.05$ compared with HFS; n=5-6/group; NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats, HFO: ovariectomized HFD-fed rats

Table 3. The metabolic parameters in both sham and OVX rats receiving either ND or HFD feeding for 12 weeks

Parameter	NDS	NDO	HFS	HFO
Body weight (g)	289.23 ± 7.63	330.31 ± 6.60*	337.00 ± 15.19*	412.22 ± 6.72*,†,‡
Visceral fat (g)	12.04 ± 0.88	16.06 ± 1.54*	26.88 ± 8.40*,†	32.90 ± 4.83*,†,‡
Uterus (g)	0.41 ± 0.02	0.11 ± 0.01*,‡	0.41 ± 0.04†	0.10 ± 0.01*,‡
Food intake (g/day)	13.15 ± 1.12	13.93 ± 0.79	15.18 ± 0.40	15.00 ± 0.43
Glucose (mg%)	120.54 ± 5.25	132.22 ± 8.50	124.58 ± 4.74	148.03 ± 7.30*,†,‡
Cholesterol (mg/dl)	87.90 ± 6.46	109.40 ± 11.61	136.15 ± 11.30*,†	141.32 ± 12.20*,†
Triglyceride (mg/dl)	34.15 ± 3.80	36.63 ± 3.23	38.21 ± 3.56	34.08 ± 4.53
HDL(mg/dl)	5.77 ± 0.18	6.08 ± 0.22	5.69 ± 0.21	6.02 ± 0.33
LDL (mg/dl)	65.90 ± 5.65	74.84 ± 3.19	114.69 ± 16.42*,†	124.84 ± 15.57*,†
Insulin (ng/ml)	0.87 ± 0.14	1.41 ± 0.25*	1.35 ± 0.20*	1.32 ± 0.14*
HOMA index	6.75 ± 1.57	12.36 ± 2.32*	11.52 ± 2.14*	11.56 ± 1.71*
Serum estrogen (pg/ml)	119.64 ± 18.24	60.12 ± 6.85*,‡	124.25 ± 7.15†	75.51 ± 7.35*,†
Plasma glucose AUC (mg/dl×min×10^{4})	3.60 ± 0.18	4.40 ± 0.27*	4.11 ± 0.18*	4.56 ± 0.32*

*, $p<0.05$ compared with NDS, †, $p<0.05$ compared with NDO, ‡, $p<0.05$ compared with HFS; n=5-6/group; NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats, HFO: ovariectomized HFD-fed rats

The addition of obesity onto an ovariectomized condition accelerated the impairment of brain insulin receptor function.

At week 4, we found that 500 nM insulin applied to hippocampal slices caused insulin-induced LTD, indicated by a reduced size of the fEPSP responses in NDS, NDO, HFS and HFO rats. In addition, the degree of insulin-induced LTD detected from brain slices of NDS, NDO, HFS and HFO rats were not significantly different (n=5 independent slices/group, n=6 animals/group, Fig. 1a). At week 8, the degree of insulin-induced LTD was significantly reduced in HFO rats compared to that of NDS, NDO and HFS rats ($p<0.05$, n=5 independent slices/group, n=6 animals/group, Fig. 1b). At 30-minutes post-insulin stimulation, the percentage reduction in normalized fEPSP slopes compared with the baseline levels of NDS, NDO, HFS and HFO rats were 88.98 ± 5.00%, 70.72 ± 15.50%, 82.12 ± 9.15% and 34.55 ± 19.80%, respectively. At week 12, insulin-induced LTD was diminished in NDO, HFS and HFO rats as showed by the disappearance of insulin-induced LTD phenomenon ($p<0.05$, n=5 independent slices/group, n=6 animals/group, Fig. 1c). At 30-minutes after the application of 500 nM insulin, the percentage reduction of the normalized fEPSP slopes compared with the baseline levels of NDS, NDO, HFS and HFO rats were 95.08 ± 9.35%, 4.01 ± 0.98%, 5.28 ± 5.72% and 2.82 ± 3.07%, respectively. These findings suggest that either ovariectomy alone or obesity alone can impair brain insulin receptor function, and obesity accelerated that impairment in an ovariectomized condition.

The addition of obesity accelerated the impairment of brain insulin signaling in an ovariectomized condition.

To determine the underlying mechanism in the impairment of brain insulin receptor function of either obesity alone or ovariectomy alone or the combination of obesity and ovariectomy, we investigated brain insulin signaling, particularly the phosphorylation of IR, Akt/PKB in the brain. Our results revealed that the IR and Akt/PKB proteins levels were not significantly different in both sham and OVX rats receiving either ND or HFD feeding for 4, 8 and 12-weeks (Fig. 2a-c, 3a-c). However, the levels of tyrosine phosphorylation of IR and Akt/PKB phosphorylation at the serine 473 site were not significantly different in both sham and OVX rats receiving either ND or HFD feeding for four weeks (Fig. 2d, 3d). At week 8, we found that the levels of tyrosine phosphorylation of IR and Akt/PKB phosphorylation at the serine 473 site were significantly decreased in HFO rats compared with those of NDS, NDO and HFS rats ($p<0.05$, Fig. 2e, 3e). At week12, we found that the levels of tyrosine phosphorylation of IR and Akt/PKB phosphorylation at the serine 473 site of NDO, HFS and HFO rats were significantly reduced compared with those of NDS rats ($p<0.05$, Fig. 2f, 3f). These findings

suggest that either ovariectomy alone or obesity alone can impair brain insulin signaling, and the addition of obesity accelerated that impairment in an ovariectomized condition.

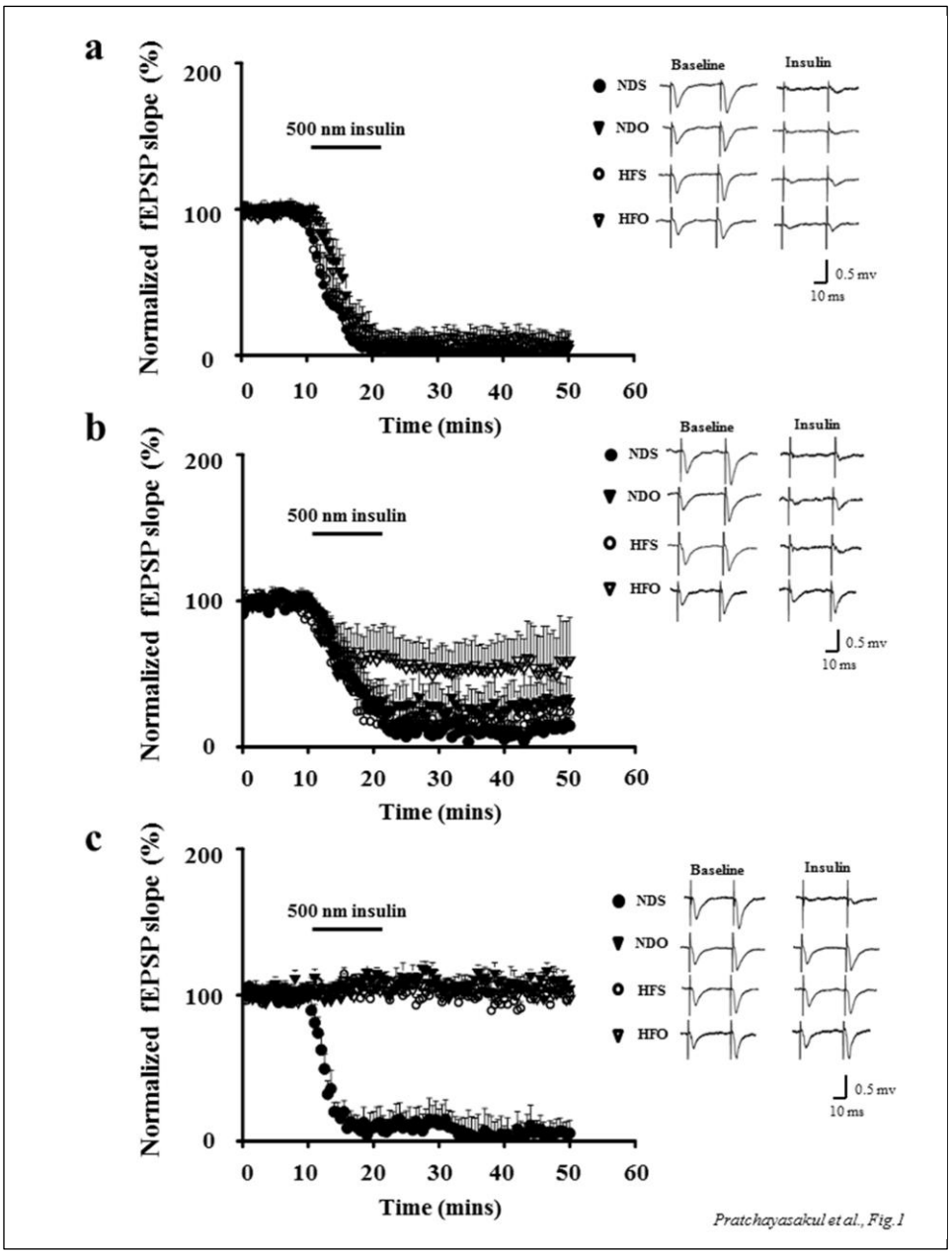

Fig. 1 The combination of obesity and ovariectomy aggravate the impairment of brain insulin receptor function or insulin-induced LTD. Panels a-c: A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from NDS, NDO, HFS and HFO rats at 4weeks (a), 8weeks (b) and 12 weeks (c) of dietary periods; (n=5 independent slices, n=6 animals/group); NDS, sham ND-fed rats; NDO, ovariectomized ND-fed rats; HFS, sham HFD-fed rats; HFO: ovariectomized HFD-fed rat

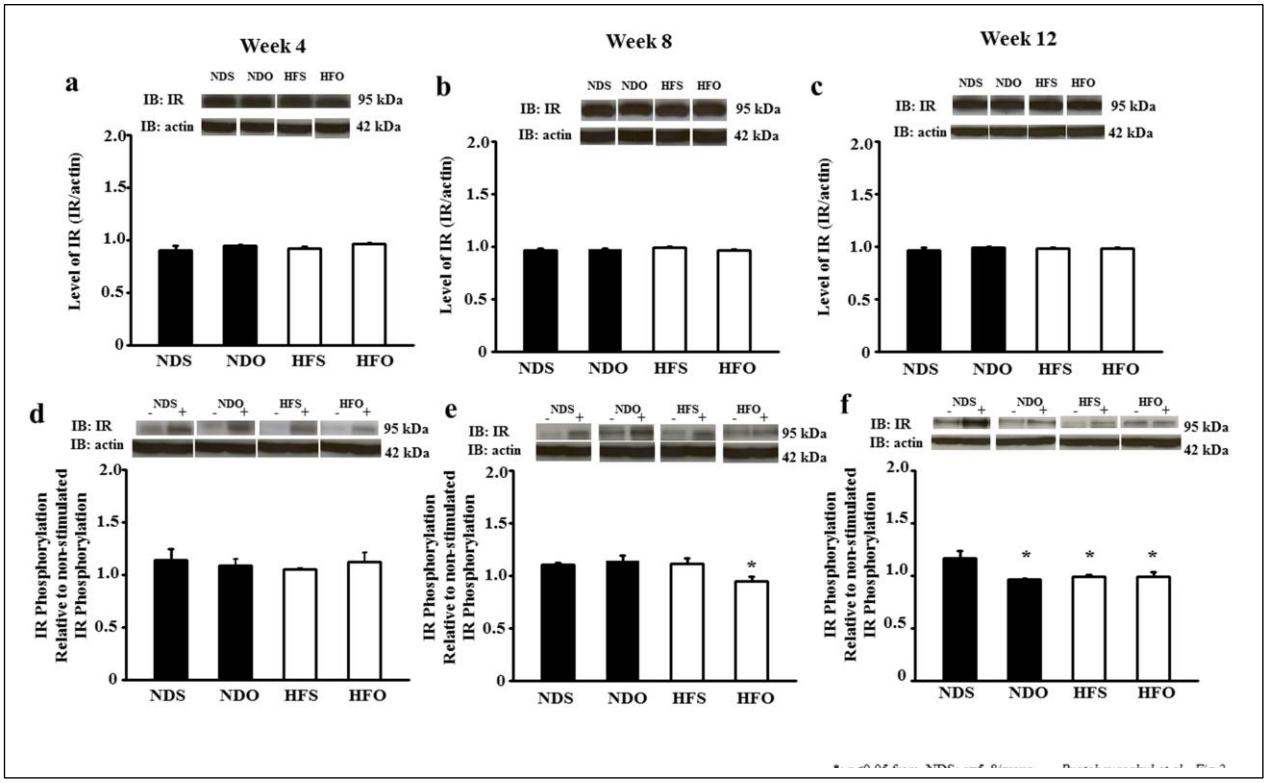

*Fig. 2 The combination of obesity and ovariectomy aggravated the impairment of brain IR tyrosine phosphorylation. Panels a-c: Representative blots of protein level of IR in brain slices harvested from the NDS, NDO, HFS and HFO rats at 4weeks (a), 8weeks (b) and 12 weeks (c) of dietary periods. The densitometric quantitation of blots from all groups was not different. Panels d-f: Representative blots of IR tyrosine phosphorylation in brain slices harvested from NDS, NDO, HFS and HFO rats at four week (d), eight week (e) and 12 week (f) of dietary periods. All immunoblotting lanes were loaded with equal amounts of protein (30μg/lane); *, p<0.05 compared with NDS; n=5-6/group; NDS, sham ND-fed rats; NDO, ovariectomized ND-fed rats; HFS, sham HFD-fed rats; HFO, ovariectomized HFD-fed rats; -, no insulin stimulation; +, insulin stimulation.*

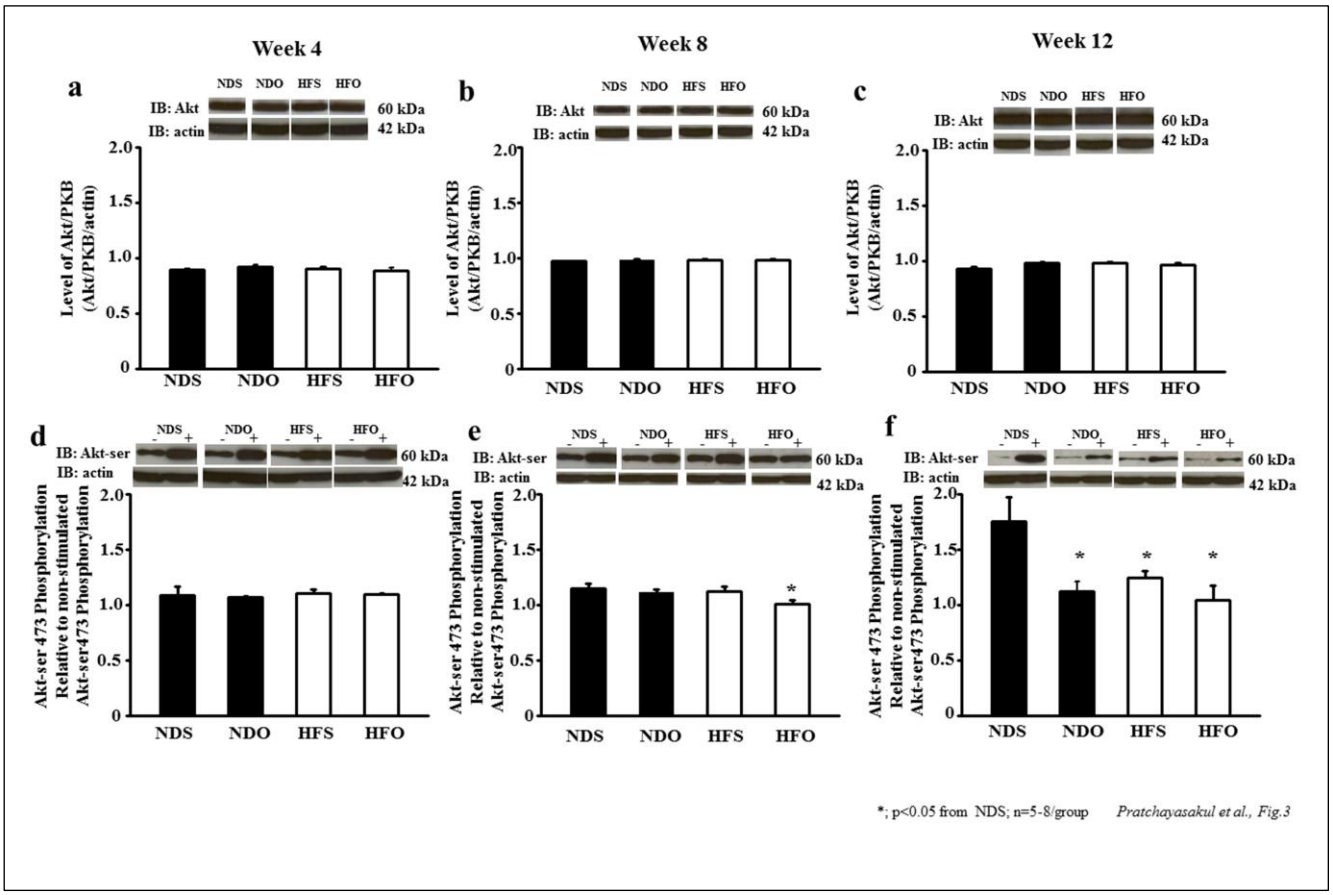

*Fig. 3 The combination of obesity and ovariectomy aggravate the impairment of brain Akt/PKB phosphorylation at the serine 473 site. Panels a-c: Representative blots of protein level of Akt/PKB in brain slices harvested from the NDS, NDO, HFS and HFO rats at 4weeks (a), 8weeks (b) and 12 weeks (c) of dietary periods. The densitometric quantitation of blots from all groups was not different. Panels d-f: Representative blots of Akt/PKB phosphorylation at the serine 473 site in brain slices harvested from NDS, NDO, HFS and HFO rats at 4weeks (d), 8weeks (e) and 12 weeks (f) of dietary periods. All immunoblotting lanes were loaded with equal amounts of protein (30μg/lane); *, p<0.05 compared with NDS; n=5-6/group; NDS, sham ND-fed rats; NDO, ovariectomized ND-fed rats; HFS, sham HFD-fed rats; HFO, ovariectomized HFD-fed rats; -, no insulin stimulation; +, insulin stimulation*

The addition of obesity accelerated the impairment of brain mitochondrial function in an ovariectomized condition.

The effects of the combination of the obesity and ovariectomy on brain mitochondrial function were determined by measuring the brain mitochondrial ROS production, brain mitochondrial membrane potential changes and brain mitochondrial swelling. At 4 weeks, we found that the brain mitochondrial functions were not significantly different among the experimental groups (Fig. 4a, d, g). At week 8, brain mitochondrial dysfunction was occurred in HFO rats, as indicated by increased brain mitochondrial ROS production, brain mitochondrial membrane depolarization and brain mitochondrial swelling compared with the NDS rats ($p<0.05$; Fig. 4b, e, h). At week 12, we found that brain mitochondrial dysfunctions were observed in NDO, HFS and HFO rats ($p<0.05$; Fig. 4c, f, i). In addition, we found that the severity of brain mitochondria dysfunction of HFO rats as indicated by increased the degree of brain mitochondrial swelling, was significantly greater than that of NDS, NDO and HFS rats ($p<0.05$; Fig. 4i). Moreover, we found that the disruption of brain mitochondrial morphology, as indicated by unfolding and swelling, was observed earlier (at week 8) in HFO rats, and occurred later at week 12 in NDO and HFS rats (Fig. 4h, i). These findings suggest that either ovariectomy alone or obesity alone causes the impairment of brain mitochondria function, and the addition of obesity accelerates that impairment in an ovariectomized condition.

The addition of obesity aggravated serum and brain oxidative stress in an ovariectomized condition.

According to the result of brain mitochondrial ROS production, we investigated serum and brain oxidative stress concentrations by determining MDA levels. At week 4, we found that serum and brain MDA levels were not significantly different between groups. At week 8 and 12, serum and brain MDA levels in NDO, HFS and HFO rats were significantly increased when compare with NDS rats ($p<0.05$; Table1). Interestingly, the serum and brain MDA levels in HFO group were significantly higher than NDO and HFS groups ($p<0.05$; Table1). This result suggests that either ovariectomy alone or obesity alone lead to increase brain oxidative stress, and the addition of obesity aggravates the severity of brain oxidative stress in an ovariectomized condition.

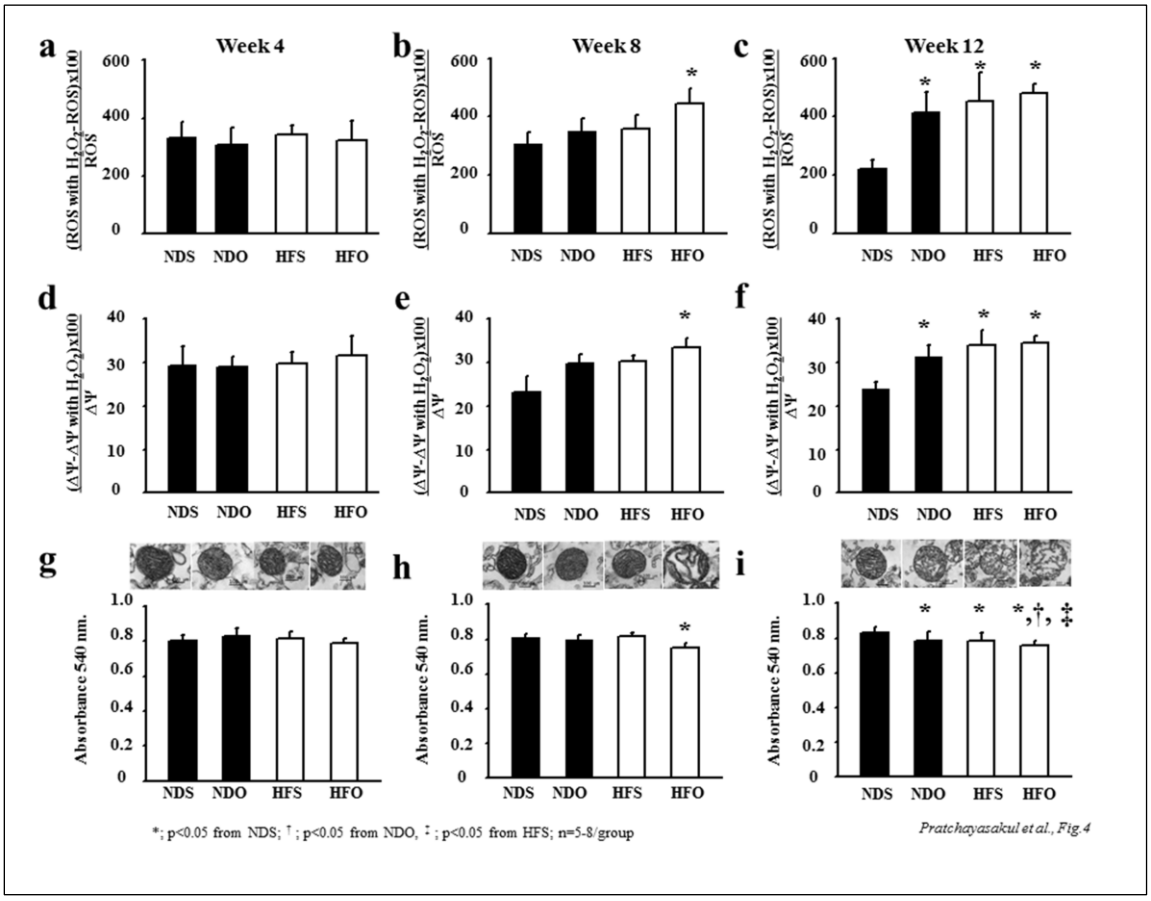

*Fig. 4 The combination of obesity and ovariectomy aggravate the impairment of brain mitochondrial functions. Panels a-c: Brain mitochondrial ROS production during 2mM H2O2 application to brain mitochondria from the NDS, NDO, HFS and HFO rats at four weeks (a), eight weeks (b) and 12 weeks (c) of dietary periods; Panels d-f: Brain mitochondrial membrane potential change ($\Delta\psi$m) during two mM H2O2 application to brain mitochondria from the NDS, NDO, HFS and HFO rats at 4weeks (d), 8 weeks (e) and 12 weeks (f) of dietary periods; Panels g-i: Brain mitochondrial swelling and transmission electron microscopy (original magnification x 25,000) shows the ultrastructure of brain mitochondria; n=5-6/group; from the NDS, NDO, HFS and HFO rats at 4weeks (g), 8weeks (h) and 12 weeks (i) of dietary periods; *, p<0.05 compared with NDS, †, p<0.05 compared with NDO, ‡, p<0.05 compared with HFS; n=5-6/group; NDS, sham ND-fed rats; NDO, ovariectomized ND-fed rats; HFS, sham HFD-fed rats; HFO, ovariectomized HFD-fed rats.*

The addition of obesity aggravated the impairment of hippocampal synaptic long term potentiation in an ovariectomized condition.

Several studies demonstrated that the impairment of synaptic plasticity was influenced by oxidative stress (22, 23). Therefore, the synaptic plasticity or electrical-induced long term potentiation (LTP) was determined by extracellular recording of hippocampal slices in the present study. At week 4, we found that high-frequency stimulation increased the size of the fEPSP responses in NDS, NDO, HFS and HFO rats. Moreover, the degree of electrical-mediated LTP observed from slices of NDS, NDO, HFS and HFO groups were not significantly different (n=5 independent slices/subgroup, n=6 animals/subgroup, Fig. 5a). At 30-minute after high-frequency stimulation, the percentage increment of the normalized fEPSP slope from NDS, NDO, HFS and HFO rats were 32.70 ± 7.33%, 31.74 ± 3.00%, 33.28 ± 10.33 % and 21.67 ± 10.84%, respectively.

At week 8, we found that the percentage increment of the normalized fEPSP slope following the high-frequency stimulations from NDO, HFS and HFO groups significantly decreased when compared with that of NDS group ($p<0.05$; Fig. 5b). In addition, this percentage increment in HFO rats was significantly lower than in NDO and HFS groups ($p<0.05$; Fig. 5b). At 30-minute after high-frequency stimulations, the percentage increment of the normalized fEPSP slope from NDS, NDO, HFS and HFO groups were 41.29 ± 4.82 %, 20.88 ± 1.77%, 20.35 ± 2.13% and 9.71 ± 2.71%, respectively. These findings suggest that either ovariectomy alone or obesity alone impairs the hippocampal synaptic plasticity, and the addition of obesity aggravates the severity of this impairment in an ovariectomized condition.

At week 12, the percentage increment of the normalized fEPSP slopes following the high-frequency stimulations of NDO, HFS and HFO groups significantly decreased when compared with that of NDS group (Fig. 5c). In HFO rats the normalized fEPSP slopes significantly attenuated when compared with that of NDO and HFS group ($p<0.05$; Fig. 5c). At 30-minute after high-frequency stimulation, the percentage increment of the normalized fEPSP slopes of NDS, NDO, HFS and HFO groups were 42.52 ± 4.12%, 23.34 ± 8.23%, 21.90 ± 4.09%, 5.67 ± 1.28%, respectively. These findings suggest that either ovariectomy alone or obesity alone causes the impairment of hippocampal synaptic function, and the addition of obesity aggravates that impairment in an ovariectomized condition.

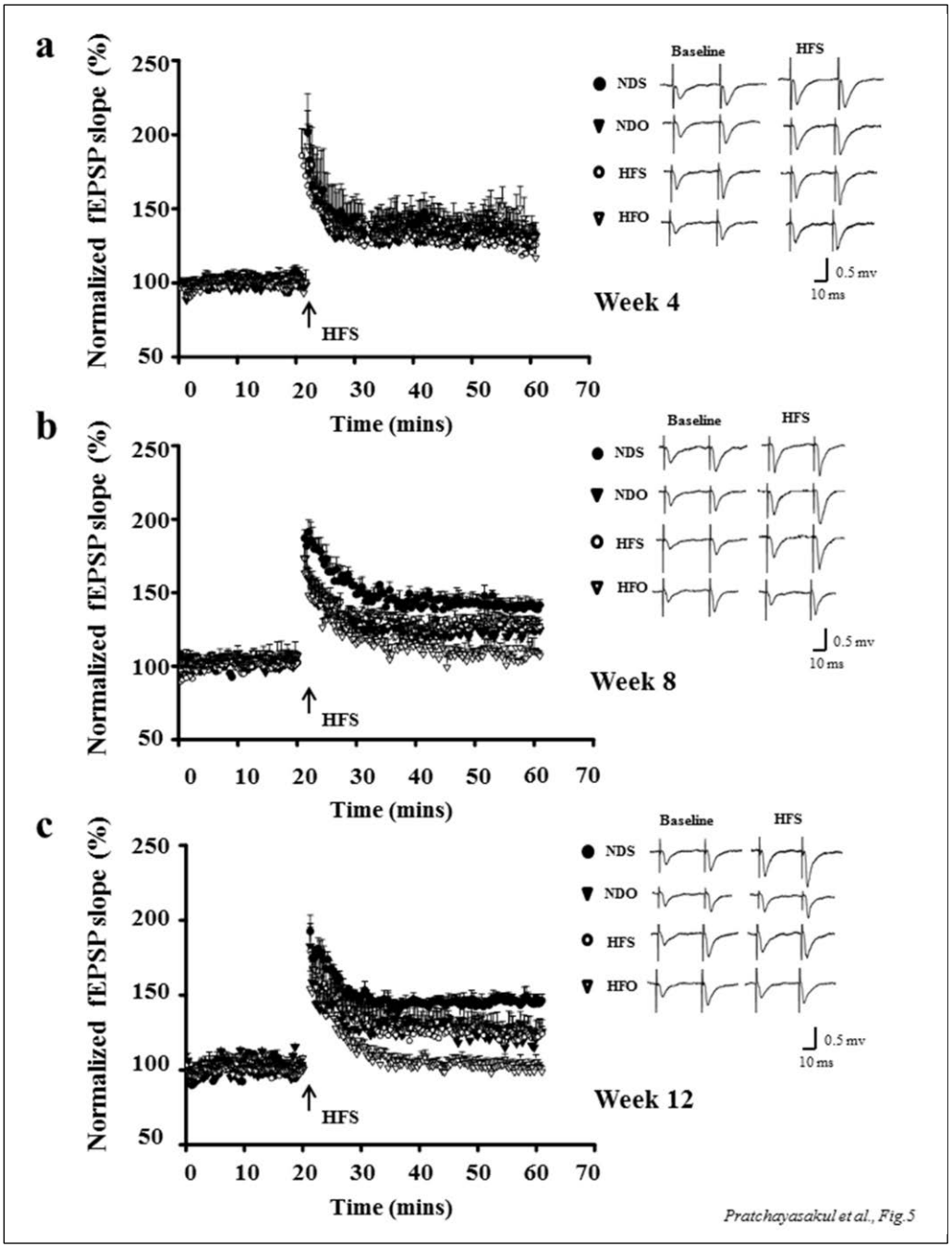

Fig. 5 The combination of obesity and ovariectomy aggravate the impairment of hippocampal synaptic long term potentiation. Panels a-c: A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from NDS, NDO, HFS and HFO rats at 4weeks (a), 8weeks (b) and 12 weeks (c) of dietary periods; (n=5 independent slices, n=6 animals/group); NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats and HFO: ovariectomized HFD-fed rats

The addition of obesity aggravated the impairment of hippocampal dendritic spine density in an ovariectomized condition.

Several studies found that the synaptic long term potentiation caused dendritic spine genesis in hippocampus; an important brain region in the learning and memory process (24, 25). At week 4, we found that the number of dendritic spines on secondary and tertiary dendrites in apical dendrite of CA1 hippocampus were not significantly different between groups. At week 8 and 12, the number of dendritic spines on secondary and tertiary dendrites in apical dendrite of CA1 hippocampus in NDO, HFS and HFO groups significantly decreased when compared with that of NDS group ($p<0.05$; Fig. 6b-c). Interestingly, the number of dendritic spines in HFO group was significantly lower than that of NDO and HFS groups ($p<0.05$; Fig. 6b-c). This result suggests that either ovariectomy alone or obesity alone leads to decrease in the number of hippocampal dendritic spines, and the addition of obesity aggravates that impairment in an ovariectomized condition.

The addition of obesity accelerated the impairment of cognitive function in an ovariectomized condition.

Before the MWM test at 4, 8, 12-weeks, the locomotor activity of all rats was determined by the open-field test. We found that the number of lines that the rats crossed during the test was not significantly different among the experimental groups. This finding suggested that the locomotor activity of all rats were not significantly different.

In present study, learning and memory behaviors were determine by MWM test at 4, 8, 12-weeks of dietary period. At week 4, we found that the learning and memory were not significantly different among experimental groups (Fig. 7a, b). At week 8, the mean of time to reach the platform during the acquisition test was not significantly different among NDS, NDO and HFS rats. Interestingly, the mean of time to reach the platform during the acquisition test significantly increased in HFO group when compared with that of NDS group (Fig. 7c). In addition, the mean of time to spend in the target quadrant during the probe test significantly decreased in HFO group compared with that of NDS group (Fig. 7d). These findings indicate that 8-week of HFD consumption after ovariectomy accelerates the impairment of learning and memory behaviors. At 12-week period, learning and memory behaviors in NDO, HFS and HFO groups were impaired when compared with that in NDS group (Fig. 7e, f), indicating by the increased the mean of time to reach the platform during the acquisition test and decreased the

mean of time to spend in the target quadrant during probe test. These findings indicate that 12-week of ovariectomy alone or 12-week HFD consumption alone impairs the learning and memory behaviors, but obesity accelerates that cognitive impairment in ovariectomized condition at earlier time-course (8-week period of HFD consumption).

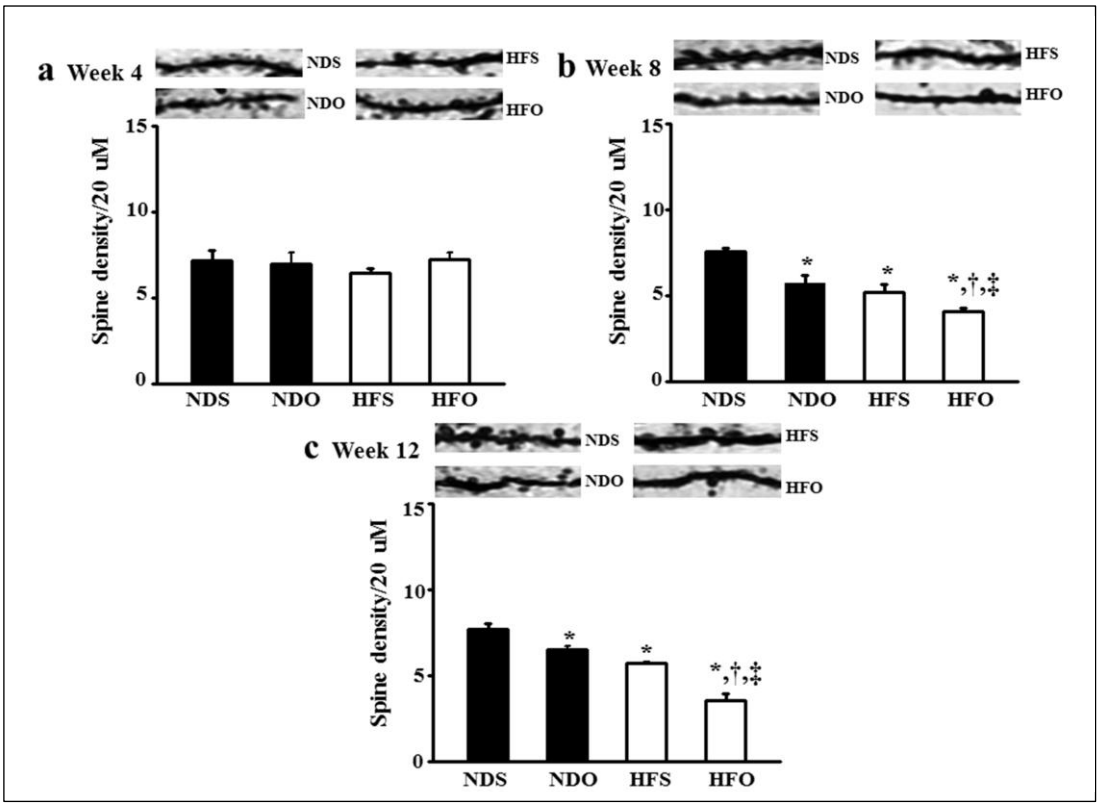

*Fig. 6 The combination of obesity and ovariectomy aggravate the impairment of dendritic spine density. The number of dendritic spines on secondary and tertiary dendrites in apical dendrite from the NDS, NDO, HFS and HFO rats at 4weeks (a), 8 weeks (b) and 12 weeks (c) of dietary periods; *, p<0.05 compared with NDS, †, p<0.05 compared with NDO, ‡, p<0.05 compared with HFS; n=5-6/group; NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats, HFO: ovariectomized HFD-fed rats.*

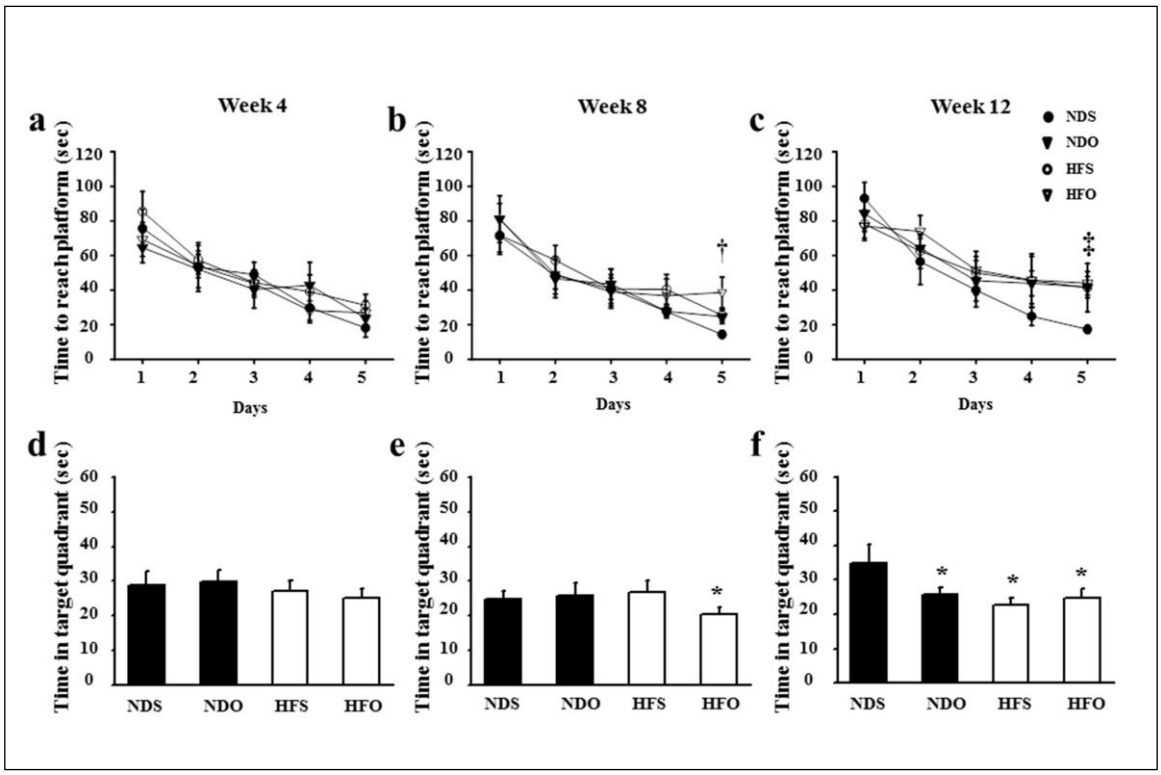

*Fig. 7 The effect of the combination of obesity and estrogen deprivation on Morris Water Maze (MWM) test at 4, 8, 12-week period. At week 8, HFO rats decreased learning and memory, indicated by increased time to reach platform in acquisition test (c) and decreased time spent in target quadrant in probe test (d). At week 12, NDO, HFS, and HFO decreased learning and memory, which indicated by increased time to reach platform in acquisition test (e), and decrease time spent in target quadrant in probe test (f). *, p<0.05 compared with NDS, †, p<0.05 compared with NDO, ‡, p<0.05 compared with HFS; n=5-6/group; NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats, HFO: ovariectomized HFD-fed rats.*

บทวิจารณ์ (Discussion)

Major findings of the present study are 1) impaired peripheral insulin sensitivity was initially observed at week 8 in ovariectomized ND-fed rats (NDO), sham HFD-fed rats (HFS), and ovariectomized HFD-fed rats (HFO); 2) the impairment of brain insulin sensitivity, indicated by the reduction of insulin-induced LTD and brain insulin signaling, as well as brain mitochondrial dysfunction occurred earlier (at week 8) in HFO rats, and was found later at week 12 in NDO, HFS and HFO rats; 4) the occurrence of oxidative stress and hippocampal synaptic dysfunction were observed at week 8 in NDO, HFS and HFO rats; 5) the impaired cognitive function developed earlier (at week 8) in HFO rats, and was found later at week 12 in NDO and HFS rats; and 6) obesity significantly accelerated and aggravated the severity of the impairment of peripheral insulin sensitivity, brain insulin sensitivity, brain mitochondrial function, hippocampal synaptic function and cognition in ovariectomized rats.

Previous studies demonstrated that either obesity alone or estrogen deficit alone could impair peripheral insulin sensitivity (6, 26, 27). Our previous studies (5, 6) and the present study also confirmed that obesity-induced by HFD consumption impaired peripheral insulin sensitivity as indicated by increased body weight, visceral fat, plasma glucose level, plasma insulin level, liver triglyceride, HOMA index and the total area under the curve (AUCg). Moreover, several evidences demonstrated that estrogen deficit alone caused obesity, leading to the development of peripheral insulin resistance (28, 29). Consistent with those studies, the present study showed that 8-week of estrogen deprivation alone caused increased body weight and visceral fats the, leading to reduced peripheral insulin sensitivity. Interestingly, the severity of impaired peripheral insulin sensitivity in obese estrogen deprived rats was significantly greater than obesity alone or estrogen deprivation alone. These findings suggest that obesity aggravates the severity in impaired peripheral insulin sensitivity in estrogen deprived models, thus obesity on top of estrogen deprivation may lead to the development of Type II diabetes, indicated by plasma glucose level in obese estrogen deprived rats.

The present study showed that the peripheral insulin resistance occurred earlier (at week 8), but the impairment of brain insulin receptor function and brain mitochondrial function appeared later (at week 12) in both estrogen deprived group or obese group. However, obese estrogen deprived group caused greater the peripheral insulin resistance, leading the appearance of the impairment of brain insulin receptor and brain mitochondrial dysfunction in same time of the peripheral insulin resistant condition. It has been shown that estrogen deprivation caused increased obese insulin resistant condition (29, 30). Several studies and

our studies also showed that insulin resistance leads to increased oxidative stress and brain mitochondrial dysfunction (3, 5, 6, 31). In addition, another study found that ATP content and rate of ATP synthesis in the hippocampal mitochondria were decreased following an ovariectomy (32) and estrogen administration suppressed brain mitochondrial oxidative stress as well as decreased brain mitochondrial ROS production in female and male rats (33). It has been known that mitochondrial dysfunction affected the activation of several serine kinase pathways and lead to the defective insulin signal transduction (34, 35). Our previous study also demonstrated that brain mitochondrial dysfunction caused the impairment of brain insulin receptor function (5). Therefore, more severe peripheral insulin resistance caused brain mitochondrial dysfunction and finally caused brain insulin resistance. Interestingly, the present study demonstrated for the first time that the impairment of brain insulin receptors was observed at the earlier stage in only 8-week obese estrogen deprived rats. Rats in this group had the severest impairment of peripheral insulin sensitivity as well as the severest brain mitochondrial dysfunction compared with obese alone or estrogen deprivation alone at the same time period. These findings indicate that obesity aggravates the impairment of brain insulin sensitivity in estrogen deprived condition.

Several evidence showed that the impaired insulin sensitivity was caused by several mechanisms such as lipotoxicity, adipocyte derived hormone, sympathetic over-activity and increased oxidative stress (31, 36-38). According to that evidence, our data emphasized the role of oxidative stress caused by either HFD-induced obesity or estrogen deprivation, lead to the reduction of peripheral and brain insulin sensitivity as observed in the present study. We found that significantly increased serum and brain MDA levels has been found in groups of either estrogen deprivation alone, obese alone or obese estrogen deprivation at week 8 and 12. In consistent with the present study, previous studies demonstrated that obese rats induced by HFD consumption increased serum and brain oxidative stress, as indicated by increased MDA levels (3, 39). Several evidence also showed that loss of estrogen leads to increase brain oxidative stress levels, as indicated by increased brain lipid peroxidation level and brain MDA levels (40, 41). Furthermore, the present study showed that the serum and brain MDA levels in obese estrogen-deprived group was significantly greater than groups of estrogen-deprivation alone and of obese alone. An increase of oxidative stress of obese estrogen-deprived group was consistent with occurrence of peripheral insulin resistance, brain insulin resistance and brain mitochondrial dysfunction.

In addition, increased oxidative stress plays an important role in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease (42) as well as dementia in aging

(43). Those pathological conditions have shown by increased neuronal apoptosis and impaired hippocampal synaptic plasticity as indicated by decreased the LTP amplitude and the reduction of dendritic spine density (44, 45). According to those findings, the present study also demonstrated that either estrogen deprivation alone or obese alone impaired hippocampal synaptic plasticity as indicated by decreased LTP and decreased the density of apical dendrite spines of CA1 hippocampus. Obesity exacerbated those impairments in estrogen deprived group. Therefore, the present study supports that either obese alone or estrogen deprivation alone leads to increase oxidative stress levels in brain and finally causes the impairment of hippocampal synaptic plasticity. However, this is the first time to demonstrate that obesity aggravates that impairment in the estrogen deprived condition.

Previous study demonstrated that rats fed with HFD, high glucose for 8 months impaired spatial learning ability by decreased dendritic spine density and reduced LTP at schaffer-CA1 synapse (10, 14). In addition, our previous study showed that 12-week HFD consumption in male rats caused the impairment of the learning and memory via increased brain MDA levels and mitochondrial ROS production which lead to impair brain insulin sensitivity (3). Moreover, ovariectomized rats showed obvious spatial learning and memory deficits in the Morris water maze test with significant loss of neurons and impaired synaptic function in the hippocampus (11, 12). Interestingly, the present study demonstrated for the first time that obesity accelerated the cognitive impairment in estrogen-deprived condition, because the cognitive impairment occurred earlier at week 8 in obese estrogen-deprived group. The possible explanation of the worsening cognitive impairment in obese estrogen-deprived group may be that this group had significantly increased peripheral insulin resistance and oxidative stress level, leading to the impairment of brain insulin receptor function, brain mitochondrial dysfunction and reduced hippocampal synaptic plasticity.

In conclusion, the present study demonstrated that either obesity alone or an estrogen deprivation alone caused the impaired peripheral insulin sensitivity, the reduction of brain insulin sensitivity, brain mitochondrial dysfunction, hippocampal synaptic dysfunction and cognitive impairment. Interestingly, obesity adding on the estrogen deprived condition accelerates the appearance of those deleterious effects at the earlier stage.

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Output **จากโครงการวิจัยที่ได้รับทุนจาก สกว.**

1. **ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)**

1. **Pratchayasakul W**, Chattipakorn N, Chattipakorn S. Estrogen restores brain insulin sensitivity in ovariectomized non-obese rats, but not in ovariectomized obese rats. Metabolism. 2014; 63:851-9. (IF=3.611) **(**เอกสารแนบหมายเลข **1)**

2. Sivasinprasasn S, Sa-Nguanmoo P, **Pratchayasakul W**, Kumfu S, Chattipakorn SC, Chattipakorn N. Obese-insulin resistance accelerates and aggravates cardiometabolic disorders and cardiac mitochondrial dysfunction in estrogen-deprived female rats. Age (Dordr). 2015;37(2):28. (IF=3.445) **(**เอกสารแนบหมายเลข **2)**

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2. **การนำผลงานวิจัยไปใช้ประโยชน์**

2.1 เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

ได้มีการนำเสนอผลงานวิจัยในงานประชุมทั้งในระดับชาติ และนานาชาติ โดยได้เผยแพร่ความรู้เกี่ยวกับผลเสียจากภาวะอ้วนจะสามารถเร่งการสูญเสียการเรียนรู้จดจำในสภาวะขาดเอสโตรเจนได้ โดยการทำให้ภาวะเครียดออกซิเดชั่น ภาวะการสูญเสียการทำงานของไมโตคอนเดรียในสมอง ภาวะดื้อต่ออินซูลินในสมอง และการสูญเสียการปรับเปลี่ยนที่จุดประสานประสาทในสมองส่วนฮิบโปแคมพัสมีความรุนแรงขึ้น ซึ่งในปัจจุบันถือว่าเป็นภาวะที่มีความสำคัญอย่างมากเนื่องจากมีจำนวนประชากรที่ภาวะอ้วนร่วมกับสภาวะขาดเอสโตรเจนเพิ่มขึ้นในทุกปี

2.2 เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
โดยมีการตีพิมพ์เผยแพร่ในวารสารทางวิชาการในระดับนานาชาติแล้ว ดังต่อไปนี้

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3. **Pratchayasakul W**, Sa-Nguanmoo P, Sivasinprasasn S, Pintana H, Tawinvisan R, Sripetchwandee J, Kumfu S, Chattipakorn N, Chattipakorn SC. Obesity accelerates cognitive decline by aggravating mitochondrial dysfunction, insulin resistance and synaptic dysfunction under estrogen-deprived conditions. Hormone and Behavior. 2015;72:68-77

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3. **อื่น ๆ (เช่น หนังสือ การจดสิทธิบัตร)**

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Estrogen restores brain insulin sensitivity in ovariectomized non-obese rats, but not in ovariectomized obese rats

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ABSTRACT

Objective. We previously demonstrated that obesity caused the reduction of peripheral and brain insulin sensitivity and that estrogen therapy improved these defects. However, the beneficial effect of estrogen on brain insulin sensitivity and oxidative stress in either ovariectomy alone or ovariectomy with obesity models has not been determined. We hypothesized that ovariectomy alone or ovariectomy with obesity reduces brain insulin sensitivity and increases brain oxidative stress, which are reversed by estrogen treatment.

Materials/Methods. Thirty female rats were assigned as either sham-operated or ovariectomized. After the surgery, each group was fed either a normal diet or high-fat diet for 12 weeks. At week 13, rats in each group received either the vehicle or estradiol for 30 days. At week 16, blood and brain were collected for determining the peripheral and brain insulin sensitivity as well as brain oxidative stress.

Results. We found that ovariectomized rats and high-fat diet fed rats incurred obesity, reduced peripheral and brain insulin sensitivity, and increased brain oxidative stress. Estrogen ameliorated peripheral insulin sensitivity in these rats. However, the beneficial effect of estrogen on brain insulin sensitivity and brain oxidative stress was observed only in ovariectomized normal diet-fed rats, but not in ovariectomized high fat diet-fed rats.

Conclusions. Our results suggested that reduced brain insulin sensitivity and increased brain oxidative stress occurred after either ovariectomy or obesity. However, the reduced brain insulin sensitivity and the increased brain oxidative stress in ovariectomy with obesity could not be ameliorated by estrogen treatment.

Abbreviations: HFD, high-fat diet; OVX, ovariectomy; sham, sham-operated; HFOE, ovariectomized HFD-fed rats with estradiol administration; HFOV, ovariectomized HFD-fed rats with vehicle treatment; HFSV, sham HFD-fed rats with vehicle treatment; ND, normal diet; NDOE, ovariectomized ND-fed rats with estradiol administration; NDOV, ovariectomized ND-fed rats with vehicle administration; NDSV, sham ND-fed rats with vehicle administration; OVX, ovariectomized; LTD, long term depression; Akt/PKB, serine/threonine protein kinase/protein kinase B; IR, insulin receptor; MDA, malondialdehyde; ROS, reactive oxygen species; TBA, thiobabituric acid solution; TCA, trichloroacetic acid; aCSF, artificial cerebrospinal fluid; BHT, butylated hydroxytoluene.

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1. Introduction

The loss of ovarian function associated with menopause or ovariectomy (OVX) has been shown to be related to increased oxidative stress levels in several organs [1–3]. It is known that oxidative stress plays a role in the impairment of insulin sensitivity via the inactivation of insulin signaling [4,5]. In addition, several studies showed that OVX caused the reduction of insulin sensitivity, which may progress to peripheral insulin resistance, obesity, and the development of Type II diabetes [6,7]. Several studies showed that estrogen therapy reduced peripheral insulin resistance, indicated by improved insulin sensitivity, reduced obesity, lowered lipid and cholesterol levels and decreased oxidative stress levels [8–11].

Our previous studies have shown that obesity induced by a high-fat diet (HFD) led to not only peripheral insulin resistance, but also brain insulin resistance, indicated by the impairment of insulin-induced long term depression (LTD) [12–14]. We also demonstrated that obesity increased brain oxidative stress, as indicated by increased brain malondialdehyde (MDA) levels and brain mitochondrial reactive oxygen species (ROS) production [12,15]. Furthermore, we previously demonstrated that chronic (30-day) administration of estrogen attenuated the occurrence of obesity-induced brain insulin resistance in female rats with intact ovary [13]. However, the effects of OVX alone on the brain insulin sensitivity and brain oxidative stress, and the effect of estrogen replacement on brain insulin sensitivity and brain oxidative stress in this model have not been investigated. Furthermore, the effects of estrogen administration on peripheral and brain insulin sensitivity as well as brain oxidative stress in the model of OVX combined with obesity induced by HFD have never been determined. In the present study, we tested the hypotheses that 1) OVX alone or OVX combined with obesity leads to reduced peripheral and brain insulin sensitivity and increased brain oxidative stress and 2) estrogen administration ameliorates the peripheral and brain insulin sensitivity as well as decreased brain oxidative stress that occurred following OVX alone or OVX combined with obesity.

2. Materials and methods

2.1. Animal models and experimental protocols

All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Thirty female Wistar rats weighing 180–200 g (~ 6–7 weeks old) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were individually housed in a temperature and light-controlled environment (12 h of light and 12 h of dark). Rats were randomly assigned as either sham-operated (sham; S; n = 10) or ovariectomized (OVX; O; n = 20). One week after the OVX surgery, rats were fed either a normal diet (ND; 19.7% E fat) or a high-fat diet (HFD; 59.3 % E fat) for 12 weeks. Animals in the ND group were fed with a standard laboratory pellet diet (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand). Animals in the HFD group were fed a diet containing 59.3% total energy from fat, the major composition of fat was saturated fatty acid from lard as described in our previous study [14]. During week 13, rats in each dietary group were divided to receive subcutaneous administration of either vehicle (V; 90% sesame oil + 10% ethanol) or estrogen (E; 50 μg/kg estradiol velerate; Progynon, Schering, Berlin, Germany) for 30 consecutive days, as described in our previous study [13]; rats were still presented either an ND or HFD feeding during the treatment period. Thus, there was a total of six experimental subgroups (n = 5/subgroup) in this study that included sham ND-fed rats with vehicle administration (NDSV), ovariectomized ND-fed rats with vehicle administration (NDOV), ovariectomized ND-fed rats with estradiol administration (NDOE), sham HFD-fed rats with vehicle treatment (HFSV), ovariectomized HFD-fed rats with vehicle treatment (HFOV) and ovariectomized HFD-fed rats with estradiol administration (HFOE). The animals were housed in individual cages with free access to food and water. Body weight and food intake were recorded daily. At the end of the experimental periods (Week 16), animals were deeply anesthetized with isoflurane after fasting for at least five hours and decapitated. Blood samples were collected, the plasma was separated and stored at −80 °C for subsequent biochemical analyses including plasma glucose, total cholesterol, triglyceride, insulin and estrogen assay. Total visceral fat, including peritoneal, periovarian and perirenal fat pads, was removed and weighed. The brain was rapidly removed for electrophysiological and biochemical analyses including insulin-induced LTD, IR and Akt/PKB phosphorylation and MDA level.

2.2. Ovariectomy procedure

Female rats were anesthetized with xylazine (LBS Laboratories, Bangkok, Thailand; 0.15 ml/kg) and Zoletil (Virbac Laboratories, Carros, France; 50 mg/kg). The bilateral ovariectomy was performed through a midline dorsal skin incision. The incision was centered between the bottom of the rib cage and the front of the hind limb. The skin was separated from the underlying muscle. The connections between the uterine tubes and uterine horn inside peritoneal cavity were transected, the ovaries were removed and the incision was closed.

2.3. Chemical analyses for glucose, total cholesterol, triglyceride, insulin and estrogen levels

Fasting plasma glucose, total cholesterol and triglyceride concentrations were determined by colorimetric assay using commercially available kits (Biotech, Bangkok, Thailand). The fasting plasma insulin levels were measured by Sandwich ELISA kits (LINCO Research, Missouri, USA). Serum estrogen levels were measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan, USA).

2.4. Determination of insulin resistance (HOMA index)

Insulin resistance was assessed by the Homeostasis Model Assessment (HOMA) index [16], as a mathematical model describing the degree of insulin resistance, calculated from the fasting plasma insulin and the fasting plasma glucose concentration. A higher HOMA index indicated a higher degree of insulin resistance.

2.5. Brain slice preparation

At the end of experimental period, the animals were anesthetized with isoflurane after fasting for at least five hours. Brain slice preparation followed our previous study guidelines [17]. After decapitation, the brain was removed and immersed in ice-cold "high sucrose" aCSF containing (mmol/L): NaCl 85; KCl 2.5; $MgSO_4$ 4; $CaCl_2$ 0.5; NaH_2PO_4 1.25; $NaHCO_3$ 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95% O_2/5% CO_2 (pH 7.4). Hippocampal slices were cut using a vibratome (Vibratome, St. Louis, Missouri, USA). Following a 30-min post-slice incubation in high sucrose aCSF, slices were transferred to a standard aCSF solution containing (mmol/L): NaCl 119; KCl 2.5; $CaCl_2$ 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1; $NaHCO_3$ 26; and glucose 10, saturated with 95% O_2/5% CO_2 (pH 7.4) for an additional 30 min, before being used for the extracellular recordings.

2.6. Extracellular recording of hippocampal slices

To investigate insulin-induced long-term depression (LTD), the hippocampal slices were transferred to a submersion recording chamber and continuously perfused at three to four ml/min with standard aCSF warmed to 25–28 °C). Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collateral–commissural pathway with a bipolar tungsten electrode, while recordings were gathered from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 Mohm) filled with 2 mol/L NaCl. The stimulus frequency was 0.033 Hz. The stimulus intensity was adjusted to yield an fEPSP of 0.8–1.0 mV in amplitude. Hippocampal slices were perfused with aCSF (as baseline condition) for ten minutes and then perfused with aCSF plus 500 nmol/L insulin (as insulin stimulation) for ten minutes, after which the hippocampal slices were perfused with aCSF again (wash out) and recorded for the next 30 min. All data were filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using pClamp 9.2 software (Axon Instruments, Foster City, CA, USA). The initial slope of the fEPSPs was measured and plotted versus time using Origin 8.0 software.

2.7. Preparation of brain homogenates for immunoblotting

To examine the alteration of brain insulin-mediated phosphorylation of the IR and Akt/PKB, eight whole brain slices per animal were placed into either aCSF or aCSF plus 500 nmol/L insulin (Humulin R, Eli Lilly, Giessen, Germany) for five minutes. Then, four whole brain slices in each conditioned group were homogenized in 500 μl of ice-cold brain slice lysis buffer [1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 1% Triton X-100 and supplemented with a protease inhibitor cocktail (Roche complete mini-tablets, Roche Molecular Biochemicals, Indianapolis, Indiana, USA)]. Next, the homogenates were centrifuged at 15,000 *g* for 30 min at 4 °C and the protein concentration was measured from cytosol fraction by using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Hercules, California, USA). These cytosol fractions were then stored at −80 °C for further biochemical analysis of the tyrosine phosphorylation of IR and the serine phosphorylation of Akt/PKB. To determine the level of IR and Akt/PKB protein expression in the brain, another set of four whole brain slices in aCSF was homogenized over ice in non-ionizing lysis buffer containing: 100 mmol/L NaCl, 25 mmol/L EDTA, 10 mmol/L Tris, 1% Triton X-100, 1% NP-40 supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals).

2.8. Immunoblotting

IR tyrosine phosphorylation and Akt/PKB at serine 473 kinases phosphorylation were electrophoresed and immunoblotted with rabbit antibodies for IR at tyrosine and Akt/PKB at serine 473, respectively. Examination of the level of IR and Akt/PKB protein expression was conducted with homogenates prepared from another set of four whole brain slices. These proteins were resolved by the immunoblot assay conducted with rabbit anti-IR at tyrosine phosphorylation, IR, Akt/PKB at serine 473 kinases phosphorylation and Akt/PKB (Santa Cruz Biotechnology, California, USA). For loading control, immunoblotting for each membrane was incubated with anti-β-actin (1:400; rabbit polyclonal; Sigma, St. Louis, Missouri, USA). All membranes for visualizing the phosphorylation and the protein levels of IR and Akt/PKB expression were incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (1:8000 in TBST, Bio-Rad Laboratories). The protein bands were visualized on Amersham hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) using Amersham ECL western blotting detection reagents (GE Healthcare). Band intensities were quantified by Scion Image and the results were shown in average signal intensity (arbitrary) units.

2.9. Brain malondialdehyde (MDA) level

Brain malondialdehyde (MDA) level, an indicator of oxidative stress, was determined by a high performance liquid chromatography (HPLC) method [18]. Briefly, the brain homogenates were mixed with 10% trichloroacetic acid (TCA) containing butylated hydroxytoluene (BHT), incubated at 90 °C for 30 min, and centrifuged at 6000 rpm for 10 min. The supernatant was mixed with H_3PO_4 and thiobabituric acid solution (TBA), incubated at 90 °C for 30 min. MDA levels were measured via the absorbance detected at 532 nm by the HPLC system, and were determined directly from the standard curve, and reported as the MDA equivalent concentration.

2.10. Statistical analysis

Data are presented as mean ± SEM. All statistical analyses were performed using the program SPSS (version 16; SPSS, Chicago, Ill., USA). For all comparisons, the significance of the difference between the mean were calculated by a three-way ANOVA and post-hoc analysis with Fisher's test with $p < 0.05$.

3. Results

3.1. Estradiol administration improved peripheral insulin sensitivity in both ovariectomized ND-fed rats and ovariectomized HFD-fed rats

Rats exposed to an HFD for 12 weeks increased body weight and reduced insulin sensitivity as evidenced by increased fasting

Table 1 – Estrogen administration improved the body weight, visceral fat, uterine weight and plasma estrogen level in both ovariectomized rats and HFD-fed rats.

Group	Food intake (g/wk)	Body weight (g)	Visceral fat (g)	Uterine weight (g)	Plasma Estrogen (pg/ml)
NDSV	97.00 ± 2.00	271.00 ± 6.40	14.97 ± 1.04	0.51 ± 0.05	135.20 ± 12.69
NDOV	89.00 ± 6.40	332.00 ± 19.85 †	18.27 ± 1.46	0.14 ± 0.02 †	29.53 ± 8.60 †
NDOE	102.00 ± 4.63	275.00 ± 14.32 ‡	12.86 ± 2.01	0.83 ± 0.18 ‡	226.92 ± 19.23 ‡
HFSV	106.00 ± 5.34	422.00 ± 27.46 *	46.43 ± 3.42 *	0.53 ± 0.07	135.03 ± 16.84
HFOV	103.00 ± 7.18	490.00 ± 33.42 *,†	52.46 ± 6.55 *	0.41 ± 0.08 *	78.63 ± 23.67 †
HFOE	105.00 ± 4.47	350.00 ± 20.98 *,‡	27.75 ± 3.80 *,‡	0.85 ± 0.05 ‡	264.45 ± 22.95 ‡

*$p < 0.05$ compared with normal diet group in the same experimental subgroups, †$p < 0.05$ compared with sham-operated group in the same experimental subgroup, ‡$p < 0.05$ compared with vehicle-treated group in the same experimental subgroup; n = 4–5/group; NDSV = sham ND-fed rats with vehicle administration, NDOV = ovariectomized ND-fed rats with vehicle administration, NDOE = ovariectomized ND-fed rats with estradiol administration, HFSV = sham HFD-fed rats with vehicle treatment, HFOV = ovariectomized HFD-fed rats with vehicle treatment, HFOE = ovariectomized HFD-fed rats with estradiol administration.

plasma total cholesterol, fasting plasma insulin and HOMA index (Table 1, Fig. 1). In addition, ovariectomy (OVX) reduced insulin sensitivity in ND feeding, as evidenced by increased body weight, fasting plasma glucose levels, fasting plasma insulin levels and HOMA index (Table 1, Fig. 1). Interestingly, OVX increased the severity of reduced insulin sensitivity in the obese group as shown by significantly increased plasma total cholesterol, plasma insulin levels and HOMA index, compared with sham HF-fed rats and ovariectomized ND-fed rats (Fig. 1). However, the estrogen therapy for 30 days had significantly positive effects on improving insulin sensitivity in both ovariectomized ND-fed rats and ovariectomized HFD-fed rats, as indicated by decreased body weight, visceral fat, fasting plasma total cholesterol levels, fasting plasma glucose levels, fasting plasma insulin levels and HOMA index (Table 1, Fig. 1). Moreover, we found that the fasting plasma triglyceride was not significantly different between experimental groups (NDSV: 273.80 ± 16.82 mg%; NDOV: 299.00 ± 9.91 mg%; NDOE: 301.40 ± 6.81 mg%; HFSV: 301.80 ± 22.93 mg%; HFOV: 301.40 ± 17.16 mg%; and HFOE : 299.60 ± 7.95 mg %).

Furthermore, we confirmed the effect of OVX by determining uterine weight and serum estrogen levels. We found that OVX resulted in decreased uterine weight and serum estrogen levels in both ND- and HFD-fed rats (Table 1). However, one month of estrogen therapy significantly attenuated these effects, compared to the vehicle-treated ND rats or vehicle-treated HFD rats ($p < 0.05$; Table 1). In addition, our study demonstrated that there was no significant difference of serum estradiol level between both dietary groups; this result was consistent with previous studies [19,20].

3.2. *Estradiol administration prevented the impairment of brain insulin receptor function only in ovariectomized ND-fed rats, but not in ovariectomized HFD-fed rats*

Brain insulin receptor function in hippocampus was determined by insulin-induced LTD. In ND-fed rats, we found that 500 nmol/L insulin applied to hippocampal slices reduced the size of the fEPSP responses or insulin-induced LTD in the sham-operated group. In contrast, insulin-induced LTD was impaired in ovariectomized ND-fed rats. However, one month of estrogen therapy reversed that impairment of insulin-induced LTD in ovariectomized ND-fed rats. In addition, the percentage of insulin-mediated LTD observed from slices of sham ND-fed rats with vehicle administration and ovariectomized ND-fed rats with estrogen administration was not significantly different (n = 8–9 independent slices per group, n = 4–5 animals/group, Fig. 2A). These findings suggest that the OVX alone in non-obese rats can lead to the impairment of brain insulin receptor function, and estrogen therapy could reverse that impairment.

In HFD-fed rats, insulin-induced LTD was impaired following obesity as indicated by the disappearance of insulin-induced LTD in the hippocampus of sham HFD-fed rats (n = 8–9 independent slices per group, n = 4–5 animals/group, Fig. 2B). In addition, OVX impaired insulin-induced LTD to the same percentages as sham HFD-fed rats. Interestingly, estradiol administration did not reverse that impairment effect in ovariectomized HFD-fed rats (Fig. 2B). These findings suggest that either obesity or OVX can lead to the impairment of brain insulin receptor function; however, estrogen therapy could not reverse that impairment in obese rats with OVX.

3.3. *Estradiol administration improved brain insulin signaling only in ovariectomized ND-fed rats, but not in ovariectomized HFD-fed rats*

To investigate mechanisms associated with the beneficial effects of estradiol administration on brain insulin receptor function, we determined whether estradiol improves brain insulin signaling, particularly IR and Akt/PKB signaling in the brain. Our results demonstrated that the level of IR and Akt/PKB proteins was not different in both sham and ovariectomized (OVX) rats receiving either ND or HFD. Furthermore, estradiol administration did not alter IR and Akt/PKB protein expressions in the brain of all treatment groups (Figs. 3A, 4A). However, the levels of tyrosine phosphorylation of IR and Akt/PKB phosphorylation at the serine 473 site were significantly decreased following either obesity or OVX. In ND-fed rats, we found that IR and Akt/PKB phosphorylation were impaired following an OVX (Figs. 3B, 4B). Similar to the insulin-induced LTD data, we found that 30 days of estrogen therapy reversed the impairment of insulin-mediated IR and Akt/PKB phosphorylation (Figs. 3B, 4B). These findings suggest that OVX in non-obese rats can lead to the impairment of brain insulin receptor function possibly via decreased IR and Akt/PKB phosphorylation, and that estrogen therapy could reverse this impairment. In HFD-fed rats, IR and Akt/PKB phosphorylation

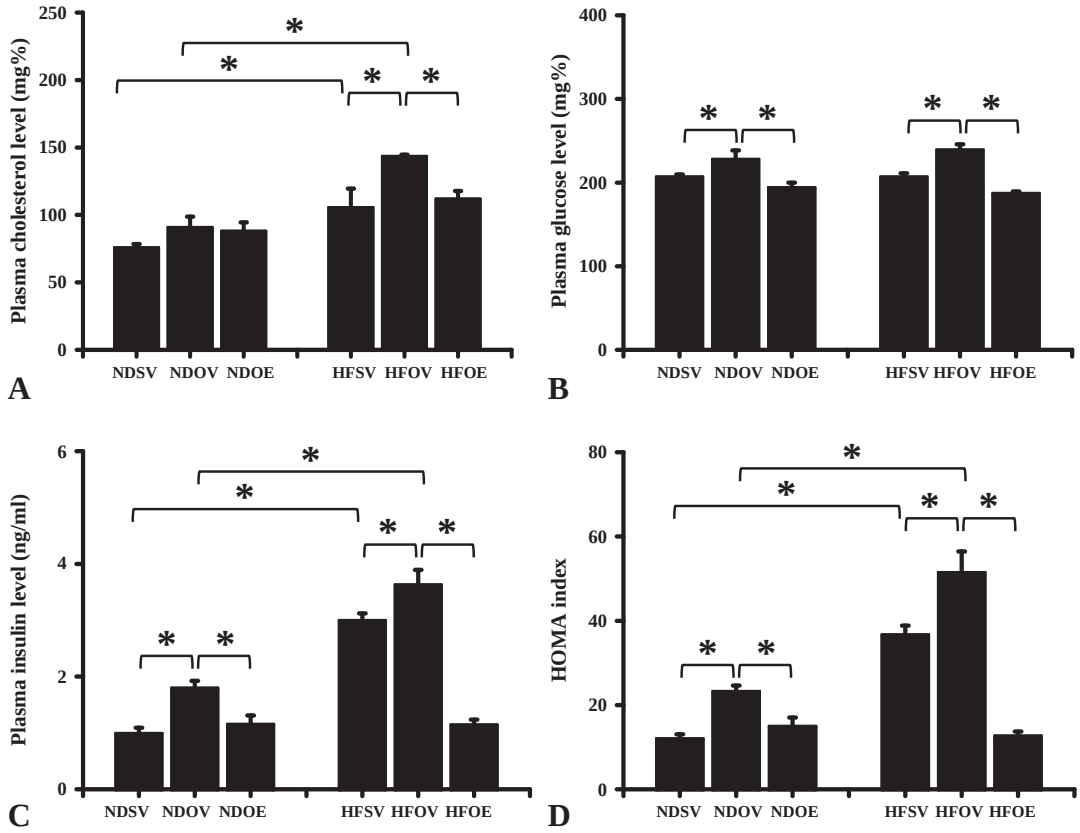

Fig. 1 – Estradiol administration improved peripheral insulin sensitivity in ovariectomized rats. (A) Plasma cholesterol level (mg%), (B) plasma glucose level (mg%), (C) plasma insulin level (ng/ml) and (D) HOMA index from the NDSV, NDOV, NDOE, HFSV, HFOV, and HFOE groups. *p < 0.05 compared with the same experimental subgroups; n = 4–5/group; NDSV = sham ND-fed rats with vehicle administration, NDOV = ovariectomized ND-fed rats with vehicle administration, NDOE = ovariectomized ND-fed rats with estradiol administration, HFSV = sham HFD-fed rats with vehicle treatment, HFOV = ovariectomized HFD-fed rats with vehicle treatment, HFOE = ovariectomized HFD-fed rats with estradiol administration.

were impaired following obesity (Figs. 3B, 4B). In addition, the ovariectomized HFD-fed rats had impaired IR and Akt/PKB phosphorylation to the same degree as in the sham HFD-fed rats. Interestingly, estrogen therapy prevented the impairment of IR and Akt/PKB phosphorylation in the sham HFD-fed rats, but not in the ovariectomized HFD-fed rats (Figs. 3B, 4B).

3.4. *The combination of high-fat diet consumption and estrogen deprivation increased the severity of brain oxidative stress levels*

Several studies demonstrated that the oxidative stress plays a role on the impairment of insulin signaling and synaptic plasticity [4,21]. Moreover, either the obesity alone or OVX alone caused the increment of oxidative stress in the brain [15,22]. In the present study, brain oxidative stress was determined by MDA level. We found that brain MDA level significantly increased following either obese induced by HFD or OVX, compared with sham ND-fed rats (Fig. 5). Interestingly, the brain MDA level in ovariectomized HFD-fed rats was significantly higher than the other treatment groups ($p < 0.05$, Fig. 5). This result suggests that the combination of obesity and OVX enhances the severity of oxidative damage in the brain. In addition, one month of estradiol administration significantly decreased the brain MDA level in ovariectomized ND-fed rats, but not in ovariectomized HFD-fed rats ($p < 0.05$, Fig. 5).

4. Discussion

The major findings of the present study are 1) non-obese rats with OVX caused the impairment of peripheral and brain insulin sensitivity as well as increased brain oxidative stress, 2) obese rats induced by HFD consumption with or without OVX also caused the impairment of peripheral and brain insulin sensitivity as well as increased brain oxidative stress, 3) estrogen therapy improved the peripheral insulin sensitivity in non-obese rats with OVX as well as obese rats with and without OVX, and 4) estrogen therapy improved the brain insulin sensitivity and brain oxidative stress only in non-obese rats with OVX, but not in obese rats with OVX.

The present study observed that obese rats without OVX caused the impairment of peripheral and brain insulin sensitivity. The excess saturated fatty acid intake from HFD led to an accumulation of saturated fatty in adipose tissues as indicated by increased visceral fat. In addition to weight gain in the HFD

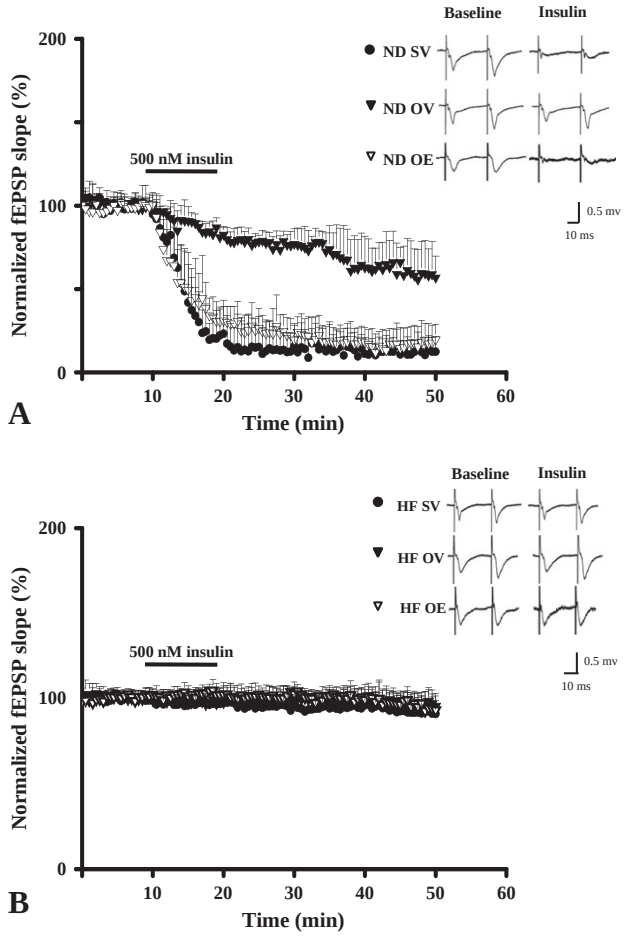

Fig. 2 – Estrogen administration for 30 days significantly improved the impairment of insulin-mediated long term depression in the CA1 hippocampus only in ovariectomized ND-fed rats, but not in ovariectomized HFD-fed rats. (A) A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from NDSV, NDOV and NDOE (n = 8–9 independent slices, n = 4–5 animals/group) brain slices; NDSV = sham ND-fed rats with vehicle administration, NDOV = ovariectomized ND-fed rats with vehicle administration, NDOE = ovariectomized ND-fed rats with estradiol administration (B) A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from HFSV, HFOV and HFOE (n = 8–9 independent slices, n = 4–5 animals/group) brain slices; HFSV = sham HFD-fed rats with vehicle treatment, HFOV = ovariectomized HFD-fed rats with vehicle treatment, HFOE = ovariectomized HFD-fed rats with estradiol administration.

group, the excess saturated fatty acids from HFD could affect the insulin signaling by stimulating several potential mediators such as diacylglycerol, inflammatory cytokines, immune cells and oxidative stress [23–25] causing the impairment of insulin signaling cascades, thus leading to decreased insulin sensitivity [12,14,23,26]. Several mechanisms have been shown to be responsible for the reduction of insulin sensitivity caused by HFD including lipotoxicity [27], adipocyte derived hormone [28], sympathetic over-activity [29] and increased oxidative stress [23]. Consistent with previous reports, our data emphasized the role of oxidative stress caused by HFD, leading to the reduction of insulin sensitivity as observed in the present study.

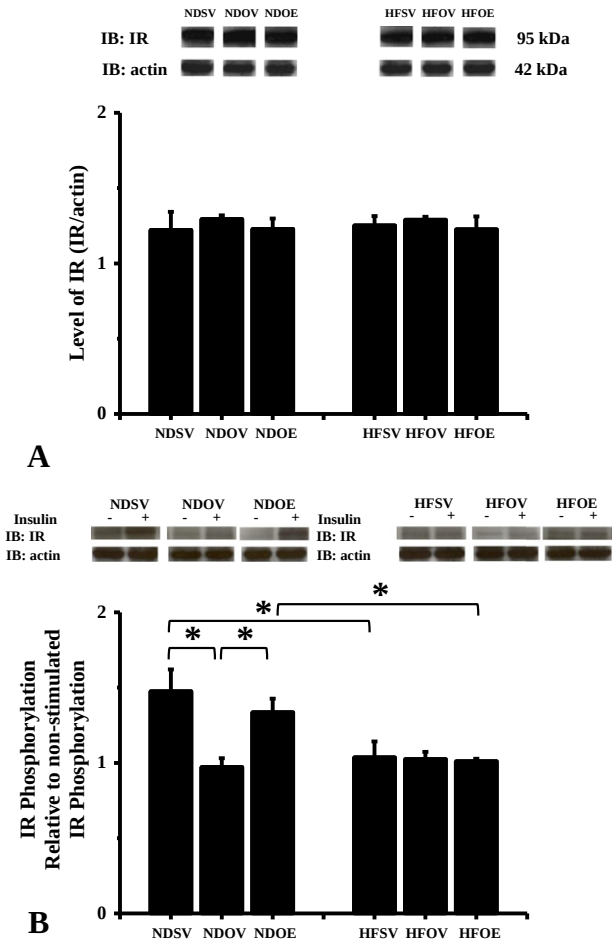

Fig. 3 – Estrogen administration for 30 days significantly improved the impairment of insulin-induced tyrosine phosphorylation of brain IR only in the ovariectomized ND-fed rat, but not in ovariectomized HFD-fed rats. (A) Representative blots of protein level of IR in brain slices harvested from the NDSV, NDOV, NDOE, HFSV, HFOV and HFOE groups. The densitometric quantitation of blots from all groups was not different. (B) Representative blots of IR tyrosine phosphorylation in brain slices harvested from the NDSV, NDOV, NDOE, HFSV, HFOV and HFOE groups. All immunoblotting lanes were loaded with equal amounts of protein (40 μg/lane). *p < 0.05 compared with the same experimental subgroups; n = 4–5/group; NDSV = sham ND-fed rats with vehicle administration, NDOV = ovariectomized ND-fed rats with vehicle administration, NDOE = ovariectomized ND-fed rats with estradiol administration, HFSV = sham HFD-fed rats with vehicle treatment, HFOV = ovariectomized HFD-fed rats with vehicle treatment, HFOE = ovariectomized HFD-fed rats with estradiol administration; –, no insulin stimulation; +, insulin stimulation.

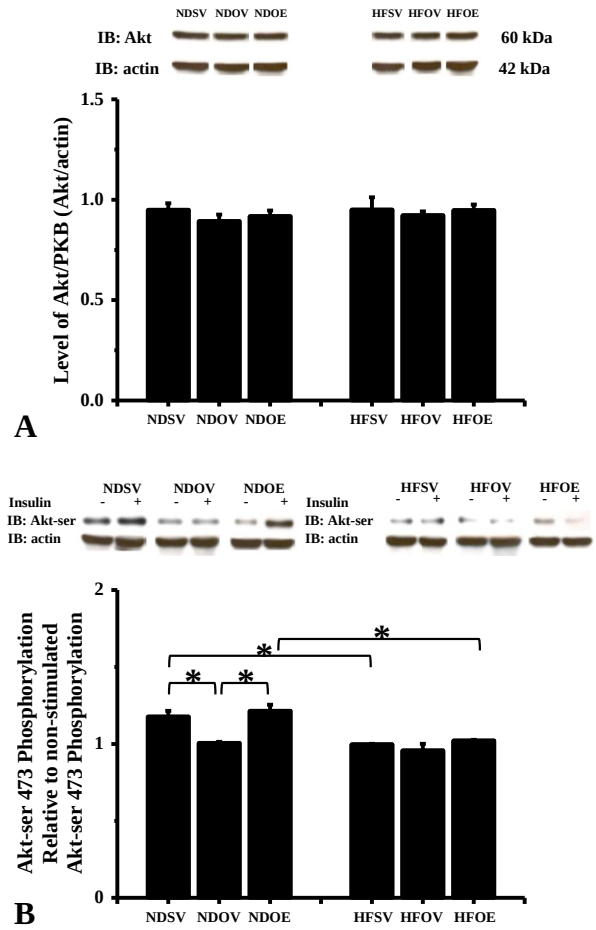

Fig. 4 – Estrogen administration for 30 days significantly improved the impairment of insulin-induced serine phosphorylation of brain Akt/PKB only in the ovariectomized ND-fed rat, but not in ovariectomized HFD-fed rats. (A) Representative blots of protein level of AKt/PKB in brain slices harvested from the NDSV, NDOV, NDOE, HFSV, HFOV and HFOE groups. The densitometric quantitation of blots from all groups was not different. (B) Representative blots of serine 473 kinase of Akt/PKB phosphorylation in brain slices harvested from the NDSV, NDOV, NDOE, HFSV, HFOV and HFOE groups. All immunoblotting lanes were loaded with equal amounts of protein (40 μg/lane). *p < 0.05 compared with the same experimental subgroups; n = 4–5/group; NDSV = sham ND-fed rats with vehicle administration, NDOV = ovariectomized ND-fed rats with vehicle administration, NDOE = ovariectomized ND-fed rats with estradiol administration, HFSV = sham HFD-fed rats with vehicle treatment, HFOV = ovariectomized HFD-fed rats with vehicle treatment, HFOE = ovariectomized HFD-fed rats with estradiol administration; –, no insulin stimulation; +, insulin stimulation.

We also found that non-obese rats with OVX had impaired peripheral and brain insulin sensitivity. This is consistent with previous studies demonstrating that OVX rats developed obesity, dyslipidemia, impaired glucose tolerance and impaired insulin-mediated glucose uptake [30,31]. Therefore, the impairment of peripheral and brain insulin sensitivity following OVX could be due to increased fat mass, increased plasma lipid profiles, hyperglycemia, elevated inflammatory markers and enhanced oxidative stress in OVX models as shown in the present study and others [32,33]. Our present study demonstrated that obese rats as well as OVX rats did not have hypertriglyceridemia, compared to lean rats. No change in hypertriglyceridemia in our study was similar to other previous studies, in which OVX rats [31,34,35] as well as HFD-fed animals did not develop hypertriglyceridemia [14,36,37]. It has been shown that excessive intake of fat initially leads to an accumulation of triglyceride in liver and adipose tissues. After both liver and adipose tissues are saturated with triglyceride, the hypertriglyceridemia will be observed [36–38]. In addition, the lack of estrogen effect on plasma triglyceride levels has been observed in the present study. These findings are similar with previous studies [31,35].

The reduction of peripheral insulin sensitivity in obese with or without OVX and non-obese rats with OVX could be reversed by estrogen therapy. Our findings confirmed the therapeutic effects of exogenous estrogen on the impairment of peripheral insulin sensitivity induced by OVX as shown in several previous studies [30,31,39]. Moreover, chronic administration of estradiol to OVX HFD-fed mice was recently shown to protect against glucose intolerance and insulin resistance, and to improve insulin signaling in skeletal muscles, when compared with vehicle-treated control mice [40].

Moreover, the present study was the first study to demonstrate that reduced brain insulin sensitivity occurs in the OVX non-obese rats and that estrogen treatment can restore brain insulin sensitivity. The beneficial effect of estrogen in brain may be due to the action of estrogen in improving peripheral insulin sensitivity and directly decreased brain oxidative stress levels as shown in the present study. Furthermore, several studies showed that estrogen may play an important role in moderating brain glucose metabolism via enhanced glucose uptake into the

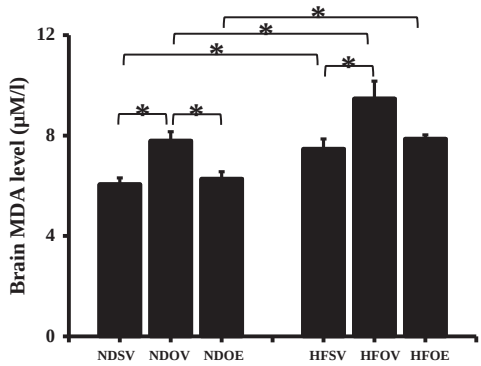

Fig. 5 – The combination of obesity and OVX could accelerate the severity of brain malondialdehyde (MDA) level. *p < 0.05 compared with the same experimental subgroups; n = 4–5/group; NDSV = sham ND-fed rats with vehicle administration, NDOV = ovariectomized ND-fed rats with vehicle administration, NDOE = ovariectomized ND-fed rats with estradiol administration, HFSV = sham HFD-fed rats with vehicle treatment, HFOV = ovariectomized HFD-fed rats with vehicle treatment, HFOE = ovariectomized HFD-fed rats with estradiol administration.

brain and increased ATP generation in neurons [41,42], in which increased brain glucose metabolism may be the other possible mechanism to restore brain insulin sensitivity in OVX models. As the limitation of the present study, this possibility has not been investigated. Therefore, further investigation about this possibility will be required.

In contrast to non-obese rats, the reduction of brain insulin sensitivity in obese rats with OVX could not be restored by estrogen therapy. The possible explanation of these findings was that the combination of obese and OVX aggravates visceral fat accumulation, leading to higher increased peripheral and brain oxidative stress levels as well as other potential mediators in the brain. This possibility is supported by the recent study [43]. Ludgero-Correia and colleagues demonstrated that the combination of obese with OVX model aggravated serum lipotoxicity, adipocyte derived hormone and adipo-cytokine levels [43]. In addition, the reduction of brain insulin sensitivity under the condition of obesity combined with OVX could also occur via the central effects including increased brain pro-inflammation and reduced brain glucose uptake. However, these possibilities were not investigated in the present study. Future study is needed to explore this issue. Therefore, the aggravation of those possible toxicities in the brain of the combined OVX with obesity may be above the efficiency of estrogen therapy.

In summary, the present study demonstrated that non-obese rats with OVX impaired peripheral and brain insulin sensitivity, and increased brain oxidative stress, and estrogen therapy could improve these impairments. Obese female rats induced by HFD with or without OVX also reduced peripheral and brain insulin sensitivity as well as increased brain oxidative stress. However, estrogen therapy improved only peripheral insulin sensitivity, but not brain insulin sensitivity and brain oxidative stress in these obese rats with OVX.

Author contributions

WP: designed the study, performed the experiments, analyzed the data, and wrote the manuscript; NC: designed the study, analyzed the data, and wrote the manuscript; SCC: designed the study, performed the experiments, analyzed the data, and wrote the manuscript.

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Disclosure statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Obese-insulin resistance accelerates and aggravates cardiometabolic disorders and cardiac mitochondrial dysfunction in estrogen-deprived female rats

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Abstract Women have a lower incidence of cardiovascular diseases (CVD) than men at a similar age but have an increased incidence of CVD and metabolic syndrome after menopause, indicating the possible protective effects of estrogen on cardiometabolic function. Although obesity is known to increase CVD risks, its impact on the heart on estrogen deprivation is still inconclusive. We investigated the effects of obese-insulin resistance on cardiometabolic function in estrogen-deprived ovariectomized rats. Adult female ovariectomized (O) or sham (S)-operated rats randomly received either normal diet (ND, 19.77 % fat) or high-fat diet (HF, 57.60 % fat) (n=6/group) for 12 weeks. The heart rate variability (HRV), left ventricular (LV) performance, cardiac autonomic balance, cardiac mitochondrial function, metabolic parameters, oxidative stress, and apoptotic markers were determined at 4, 8, and 12 weeks. Insulin resistance developed at week 8 in NDO, HFS, and HFO rats as indicated by increased plasma insulin and HOMA index. However, only HFO rats had elevated plasma cholesterol level at week 8, whereas HFS rats had showed elevation at week 12. In addition, only HFO rats had depressed HRV, impaired LV performance indicated by decreased fractional shortening (%FS) and cardiac mitochondrial dysfunction indicated by increased mitochondrial ROS level, mitochondrial depolarization and swelling, as early as week 8, whereas other groups exhibited them at week 12. Either estrogen deprivation or obesity alone may impair metabolic parameters, cardiac autonomic balance, and LV and mitochondrial function. However, an obese insulin-resistant condition further accelerated and aggravated the development of these cardiometabolic impairments in estrogen-deprived rats.

Keywords Obesity · Insulin resistance · Estrogen deprivation · Mitochondria · Cardiometabolic function

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Introduction

Cardiovascular disease (CVD) has been the major cause of death for several decades and is expected to remain so

until 2030 (Mathers and Loncar 2006). It has been shown that women have a lower incidence of CVD than men at a similar age, but the incidence increases after the onset of menopause (Vitale et al. 2009). A bilateral ovariectomy (OVX) in women has also been shown to be associated with increased mortality from CVDs (Rivera et al. 2009), which may be due to the fact that estrogen is essentially involved in the regulation of cardiac function. It has been shown that a cardiac sympathovagal imbalance is commonly found in postmenopausal women, and estrogen replacement helps improve this cardiac autonomic dysfunction (Yang et al. 2013). Estrogen deficiency has been shown to reduce myocardium contractile response to calcium and beta-adrenergic receptor stimulation indicating contractile dysfunction of the heart (Ribeiro et al. 2013). Depletion of endogenous estrogen also causes endothelial dysfunction indicated by decreased endothelial relaxation and increased endothelial contraction of arterioles via reactive oxidative species augmentation and vascular nitric oxide reduction (Wang et al. 2014). These increase in cases of oxidative stress and endothelial dysfunction after estrogen deprivation could induce arterial stiffness, inflammation, arthrosclerosis, and plaque rupture leading to tissue ischemia and infarction (Higashi et al. 2009).

Obese-insulin resistance is considered an important risk factor for CVDs due to its negative impact on glucose and lipid metabolism, blood clotting factors, arterial wall health, left ventricular (LV) mass, and blood pressure regulation (Ginsberg 2000). Accumulated adipocytes in obesity caused an increase in adipocytokines and a decrease in adiponectin levels resulting in inflammation, thrombosis, and association with coronary artery disease and myocardial ischemia (Karmazyn et al. 2008). Ovariectomized rats also exhibited an altered plasma lipid profile and increased fat deposition in liver, muscle, and heart (Leite et al. 2009). Since menopause is associated with adverse effects on platelet function and systolic blood pressure (Bonithon-Kopp et al. 1990), combined conditions of estrogen deprivation and obese-insulin resistance would be expected to aggravate the deleterious effects of either one on the heart. Clinical evidence also shows that postmenopausal women have an increased prevalence of metabolic syndrome which is also another risk factor for CVD (Carr 2003).

Although the adverse impact of estrogen deprivation and metabolic syndromes is interrelated and both conditions are known to be associated with the development of cardiac dysfunction, the mechanisms involved as well as the onsets of metabolic and cardiac disorders following estrogen deprivation are still not well-understood. In addition to cardiac performance, the changes in cardiac mitochondrial function such as ROS production, mitochondrial membrane potential, and mitochondrial swelling at the different time points after estrogen deprivation, along with obese-insulin resistance, are also unknown. Therefore, in this study, the influences of female sex hormone deprivation and obese-insulin resistance on metabolic status, cardiac autonomic regulation, LV contractile function, and cardiac mitochondrial function, as well as the underlying mechanisms, have been investigated at several time points to clarify their effects on the progression of cardiometabolic dysfunction. We tested the hypothesis that the obese-insulin resistant condition accelerates and aggravates the development of metabolic disorders, cardiac autonomic imbalance, and cardiac mitochondrial dysfunction in estrogen-deprived female rats.

Methods

Animals

Female Wistar rats (weighing 200–220 g, n=72) were obtained from the National Animal Center (Salaya campus, Mahidol University, Bangkok, Thailand). The rats were housed in a temperature-controlled room (25 °C) with a 12/12-h dark/light cycle setting. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University, in compliance with NIH guidelines.

Experimental protocol

Rats were randomized into a sham (S)-operated group and a bilateral ovariectomized (OVX) group. One week after surgery, rats from both groups were divided into two further groups, one to be fed on a normal diet (ND, contain 19.77 % energy from fat) and the second on a high-fat diet (HF, contain 59.28 % energy from fat) (Pratchayasakul et al. 2011). This gave a total of four experimental groups (n=18/group) including (a) normal diet-fed sham-operated rats (NDS), (b) high-fat diet-fed sham-operated rats (HFS), (c) normal diet-fed

ovariectomized rats (NDO), and (d) high-fat diet-fed ovariectomized rats (HFO). In each of these four experimental groups, rats were fed with their prescribed diet and subdivided to be terminated at the three different times of 4, 8, and 12 weeks (n=6 for each time course). Body weight and food intake were recorded throughout the experiment. At the end of each assigned time course in each experimental group, blood samples were collected from tail vein for determinations of metabolic parameters and estrogen levels. Oral glucose tolerance testing (OGTT), heart rate variability (HRV) for cardiac autonomic balance, and echocardiography were carried out at each time course. At the end of the study protocol, rats were anesthetized with isoflurane via inhalation and terminated by decapitation. The hearts were rapidly removed and homogenized for cardiac mitochondrial function and biochemical studies.

Surgical procedure of ovariectomy

Rats were anesthetized with xylazine (0.15 ml/kg) and zolitil (50 mg/kg) via intraperitoneal injection and ventilated with room air (Pratchayasakul et al. 2014). The bilateral ovariectomy procedure was performed as described previously (Pratchayasakul et al. 2014). After hair shaving and skin cleaning, a midline dorsal skin incision between the inferior crest of the rib cage and superior base of the thigh was made to access the abdominal pelvic cavity. The uterine tube and ovary were identified. Both ovaries were completely removed, and uterine horns were returned into the pelvic cavity. Then, the abdominal wall was sutured, and the incision was closed. After the operation, rats were individually housed in a clear box with dry bedding for 1 week before being divided into the normal diet or high-fat diet-fed groups.

Metabolic function and estrogen level determinations

Fasting plasma insulin levels were detected using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Millipore, MI, USA). Fasting plasma glucose, cholesterol, and triglyceride (TG) concentrations were determined by colorimetric assay using a commercially available kit (Biotech, Bangkok, Thailand). Degrees of insulin resistance were assessed using homeostasis model assessment (HOMA) which uses the fasting plasma insulin level and fasting plasma glucose concentration its calculation (Pratchayasakul et al. 2014). Plasma estrogen concentration was measured by using a competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, MI, USA).

HRV test

HRV is a noninvasive assessment of cardiac autonomic function (Chattipakorn et al. 2007). Electrocardiograms (ECG), lead II, were recorded from each rat using a signal transducer (PowerLab 4/25 T, ADInstrument) and operated through the Chart 5.0 program for 20 min. ECG data were analyzed using a frequency-domain method using the MATLAB program. A high-frequency (HF) component (ranging between 0.15–0.40 Hz) is considered to be a marker of parasympathetic tone while low-frequency (LF) component (ranging between 0.04–0.15 Hz) is considered to be a marker of parasympathetic and sympathetic tone (Chattipakorn et al. 2007). Cardiac sympathetic/parasympathetic balance was reported as a LF/HF ratio. Increased LF/HF ratio indicates imbalanced cardiac autonomic regulation (i.e. depressed HRV) (Apaijai et al. 2013; Chattipakorn et al. 2007).

Echocardiography

Echocardiography was used to noninvasively assess the LV function. Animals were lightly anesthetized with 2 % isoflurane with oxygen (2 l/min) via inhalation. The echocardiography probe (S12, Hewlett Packard) which was connected to an echocardiograph (SONOS4500, Philips) was placed on the chest and moved enabling collection of the data along the long and short axes of the heart. Signals from M-mode echocardiography at the level of the papillary muscles were recorded to determine left ventricular internal diameter at the end of diastole (LVIDd) and left ventricular internal diameter at the end of the systole (LVIDs) (Semaming et al. 2014). Fractional shortening (FS) was calculated to estimate contractile function using the formula: FS% = [(LVIDd − LVIDs) / LVIDd] × 100 (Overbeek et al. 2006).

Cardiac mitochondrial function study

For cardiac mitochondrial function study, the mitochondrial ROS production, mitochondrial membrane potential changes, and mitochondrial swelling were determined using the method as described previously

(Apaijai et al. 2013; Palee et al. 2011). In brief, in each rat, the heart was removed at the end of the study and chopped into small pieces on an ice-cold plate. Then, cardiac tissues were homogenized and centrifuged to isolate cardiac mitochondria. Cardiac mitochondrial ROS production was measured by staining cardiac mitochondria with dichlorohydrofluoresce in diacetate (DCFDA) dye for 25 min, after which a fluorescent microplate reader (Model and brand) was used to detect the ROS level using the excitation wavelength of 485 nm and emission wavelength at 530 nm (Palee et al. 2011; Apaijai et al. 2013). For mitochondrial membrane potential change, the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used. The green fluorescent (JC-1 monomer) was excited at the wavelength of 485 nm and the emission detected at 590 nm while the red fluorescent (JC-1 aggregates) was excited at the wavelength of 485 nm and the emission detected at 530 nm. A decreased red/green fluorescence intensity ratio indicates depolarization of the mitochondrial membrane (Chinda et al. 2014). Isolated mitochondria were also measured for the change in the absorbance using a spectrophotometer at 540 nm for 30 min to identify any cardiac mitochondrial swelling. Decreased absorbance in a mitochondrial suspension indicates mitochondrial swelling (Chinda et al. 2014). Cardiac mitochondrial morphology was also studied using a transmission electron microscope (TEM; JEM-1200 EX II, JEOL Ltd., Japan).

Determination of oxidative stress

Malondialdehyde (MDA) concentrations in cardiac tissues and serum were measured by a high performance liquid chromatography (HPLC) system (Thermo Scientific, Bangkok, Thailand) as described previously (Apaijai et al. 2014). Serum and protein from cardiac tissues were mixed with 10 % trichloroacetic acid (TCA) containing BHT then heated at 90 °C for 30 min and cooled down to room temperature. The mixture was centrifuged, and the supernatant was mixed with 0.44 M H_3PO_4 and 0.6 % thiobabituric acid (TBA) solution to generate thiobarbituric acid reactive substances (TBARS). The solution was filtered through a syringe filter (polysulfone type membrane, pore size 0.45 μm, Whatman International, Maidstone, UK) and analyzed with the HPLC system. Data were analyzed with BDS software (BarSpec Ltd., Rehovot, Israel), and plasma TBARS concentration was determined directly from a standard curve generated from a standard reagent for MDA at different concentrations (0–10 μM) and reported as MDA equivalent concentration (Apaijai et al. 2013).

Cardiac expression of Bax and Bcl-2

Western blot analysis was used for protein expression of Bax and Bcl-2 as described previously (Chinda et al. 2014). Briefly, rat myocardial tissues were homogenized in a lysis buffer (containing 1 % Nonidet P-40, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate (SDS) in 1× PBS) in order to extract protein. Then, the homogenate was centrifuged at 13,000 rpm for 10 min. Total protein (50–80 mg) was mixed with the loading buffer (consists of 5 % mercaptoethanol, 0.05 % bromophenol blue, 75 nM Tris-HCl, 2 % SDS, and 10 % glycerol with pH 6.8) in 1-mg/ml proportion, and the mixture was boiled for 5 min and loaded into 10 % gradient SDS-polyacrylamide gel. After that, proteins were transferred to a polyvinyldene difluoride (PVDF) membrane with the presence of a glycine/methanol transfer buffer (containing 20-mM Tris base, 0.15-M glycine, and 20 % methanol) in a transfer system (Bio-Rad). PVDF membranes were incubated in 5 % skim milk in 1× TBS-T buffer (containing 20-mM Tris-HCl (pH 7.6), 137-nM NaCl, and 0.05 % Tween-20) for 1 h at room temperature then exposed to anti-Bax, anti-Bcl-2 (Cell Signaling Technology, Danvers, MA, USA) and anti-actin (Sigma-Aldrich, St. Louis, MO, USA) for 12 h. Bound antibody was detected by horseradish peroxidase conjugated with anti-rabbit or anti-mouse IgG. Enhanced chemiluminescence (ECL) detection reagents were administered to visualize peroxidase reaction products.

Statistical analysis

The data for each experiment were presented as mean± SE. For all comparisons, the significance of the difference between the mean was calculated by SPSS program (SPSS version 16, SPSS Inc.) using one-way ANOVA followed by post hoc, LSD test. P value less than 0.05 ($P<0.05$) was considered statistically significant.

Results

High-fat diet consumption accelerated and aggravated adverse changes of metabolic profiles and oxidative stress in estrogen-deprived rats

In week 4, both estradiol levels and uterus weight showed significant decrease in both NDO and HFO groups indicating endogenous estrogen-deprived conditions from the removal of ovaries. Ingestion of a high-fat diet for 4 weeks markedly increased body weight in HFO rats when compared with NDS, NDO, and HFS rats (Table 1). Visceral fat deposition significantly increased in both HFS and HFO rats; however, the amount of daily food intake was not significantly different between the groups during the 4 weeks (Table 1). No significant differences between groups were found for the metabolic parameters and serum and cardiac MDA at this time-course.

At week 8, peripheral insulin resistance had developed in NDO, HFS, and HFO rats as demonstrated by a significant increase of insulin level and HOMA index in these groups (Table 2). However, only HFO rats showed a markedly increased level of plasma glucose and total cholesterol compared with NDS and NDO rats. Moreover, HFO rats also had higher levels of visceral fat, body weight, and total cholesterol than HFS rats, indicating the early development with worse impairment of the metabolic parameters in the HFO group at this time-course (Table 2).

At week 12, similar to results seen in week 8, the HFO rats still demonstrated the worst metabolic impairment as indicated by an increase of the body weight, visceral fat, plasma glucose, and plasma cholesterol when compared with the other groups at this time-course (Table 3).

Consistent with the metabolic parameters, the serum MDA level was also increased only in HFO rats beginning at week 8 (Table 2). However, it was not until week 12 that the plasma MDA was elevated in NDO and HFS rats (Table 3). The serum MDA in HFO rats was found to be the highest compared to NDO and HFS groups at this time-course (Table 3). Regarding cardiac MDA level, there was no significant difference between all groups during week 4 and week 8, but they were elevated in NDO, HFS, and HFO groups only at week 12 (Table 3).

Obese-insulin resistance due to high-fat diet consumption accelerated LV contractile dysfunction and cardiac sympathovagal imbalance in estrogen-deprived rats

For cardiac function, although both fractional shortening (%FS) and ejection fraction (%EF) were not

Table 1 Metabolic parameters investigated at 4 weeks after ovariectomy

Parameters	Normal diet		High-fat diet	
	Sham	OVX	Sham	OVX
Body weight (g)	266.66±9.18	285.83±6.88	284.16±6.37	327.00±4.35*†‡
Visceral fat (g)	11.83±1.21	10.12±0.90	19.58±1.32*†	20.51±1.65*†
Uterus weight (g)	0.33±0.02	0.10±0.00*‡	0.36±0.05†	0.10±0.01*‡
Glucose (mg%)	119.30±6.43	120.39±8.75	121.18±7.01	122.84±8.80
Cholesterol (mg%)	96.62±6.04	103.03±7.35	103.89±9.90	105.82±13.35
Triglyceride (mg%)	37.76±3.62	32.54±3.90	38.23±3.80	32.10±3.44
Insulin (ng/ml)	0.82±0.07	0.82±0.05	0.87±0.12	0.97±0.09
HOMA index	5.71±0.25	6.53±0.55	6.54±1.70	7.10±1.36
Estradiol level (pg/ml)	139.26±34.87	43.32±10.64*‡	132.29±26.12†	49.85±19.25*‡
Serum MDA (μmol/ml)	3.45±0.61	3.62±0.40	3.53±0.34	3.60±0.32
Cardiac MDA (μmol/ml)	2.27±0.20	1.92±0.77	2.19±0.07	2.31±0.37
Food intake (g/day)	16.65±0.98	16.10±1.13	17.57±0.43	17.60±0.30

Values are mean±SE for six rats per group

OVX ovariectomized, *HOMA* homeostasis model assessment, *MDA* malondialdehyde

*$P<0.05$ vs. NDS, †$P<0.05$ vs. NDO, ‡$P<0.05$ vs. HFS

Table 2 Metabolic parameters investigated at 8 weeks after ovariectomy

Parameters	Normal diet		High-fat diet	
	Sham	OVX	Sham	OVX
Body weight (g)	275.0±5.52	306.42±3.57*	313.7±8.22*	378.57±8.5*†‡
Visceral fat (g)	11.34±1.24	15.36±1.24*	24.11±1.48*†	30.34±1.49*†‡
Uterus weight (g)	0.37±0.02	0.09±0.00*‡	0.39±0.01†	0.09±0.00*‡
Glucose (mg%)	125.74±7.29	126.09±4.82	130.02±5.90	149.70±8.47*†
Cholesterol (mg%)	101.63±4.46	106.42±2.69	105.50±4.95	138.96±3.30*†‡
Triglyceride (mg%)	41.99±5.73	36.39±0.22	35.61±2.50	34.49±2.35
Insulin (ng/ml)	1.01±0.12	1.59±0.18*	1.52±0.18*	1.55±0.12*
HOMA index	6.48±0.94	11.75±1.88*	12.05±1.42*	13.30±1.43*
Oestradiol level (pg/ml)	117.66±10.11	50.76±2.21*‡	117.44±16.78†	59.61±2.62*‡
Serum MDA (μmol/ml)	3.47±0.16	4.00±0.22	4.04±0.19	4.67±0.27*†‡
Cardiac MDA (μmol/ml)	1.36±0.84	1.37±0.55	1.90±0.28	1.97±0.74
Food intake (g/day)	15.71±1.14	15.20±1.14	16.19±0.74	16.55±0.65

Values are mean±SE for six rats per group

OVX ovariectomized, *HOMA* homeostasis model assessment, *MDA* malondialdehyde

*$P<0.05$ vs. NDS, †$P<0.05$ vs. NDO, ‡$P<0.05$ vs. HFS

significantly different between all groups in week 4, HFO rats were the first group to exhibit significant reduction in both %FS (Fig. 1a) and %EF (Fig. 1b) beginning at week 8. LV dysfunction in the NDO and HFS rats was not observed until week 12 (Fig. 1a, b).

For cardiac autonomic function, there was no significant alteration in cardiac autonomic balance among all groups in week 4 (Fig. 1c). However, in week 8, only HFO rats developed depressed HRV as indicated by a significant increase in the LF/HF

Table 3 Metabolic parameters investigated at 12 weeks after ovariectomy

Parameters	Normal diet		High-fat diet	
	Sham	OVX	Sham	OVX
Body weight (g)	284.28±3.68	336.87±4.81*	342.22±4.93*	419.50±4.56*†‡
Visceral fat (g)	12.20±0.51	16.26±1.13*‡	31.14±1.62*†	35.20±0.94*†‡
Uterus weight (g)	0.39±0.02	0.10±0.00*‡	0.45±0.03†	0.10±0.00*‡
Glucose (mg%)	124.27±5.79	130.67±7.84	135.03±5.60	160.84±5.93 *†‡
Cholesterol (mg%)	88.72±5.84	106.74±10.06	125.63±13.5*†	156.75±11.8*†‡
Triglyceride (mg%)	31.60±3.25	32.47±2.10	31.70±3.01	31.58±3.07
Insulin (ng/ml)	0.87±0.14	1.57±0.27*	1.57±0.25*	1.58±0.11*
HOMA index	5.21±0.40	11.74±2.01*	11.92±2.27*	12.90±1.91*
Estradiol level (pg/ml)	127.65±20.95	62.37±7.23*‡	129.37±8.16†	74.37±7.11*‡
Serum MDA (μmol/ml)	3.52±0.16	4.31±0.14*	3.99±0.12*	4.80±0.16*†‡
Cardiac MDA (μmol/ml)	5.76±0.96	13.41±1.50*	12.09±2.35*	11.69±2.01*
Food intake (gram/day)	13.21±0.97	14.29±0.77	15.91±0.50*	16.30±0.58*

Values are mean±SE for six rats per group

OVX ovariectomized, *HOMA* homeostasis model assessment, *MDA* malondialdehyde

*$P<0.05$ vs. NDS, †$P<0.05$ vs. NDO, ‡$P<0.05$ vs. HFS

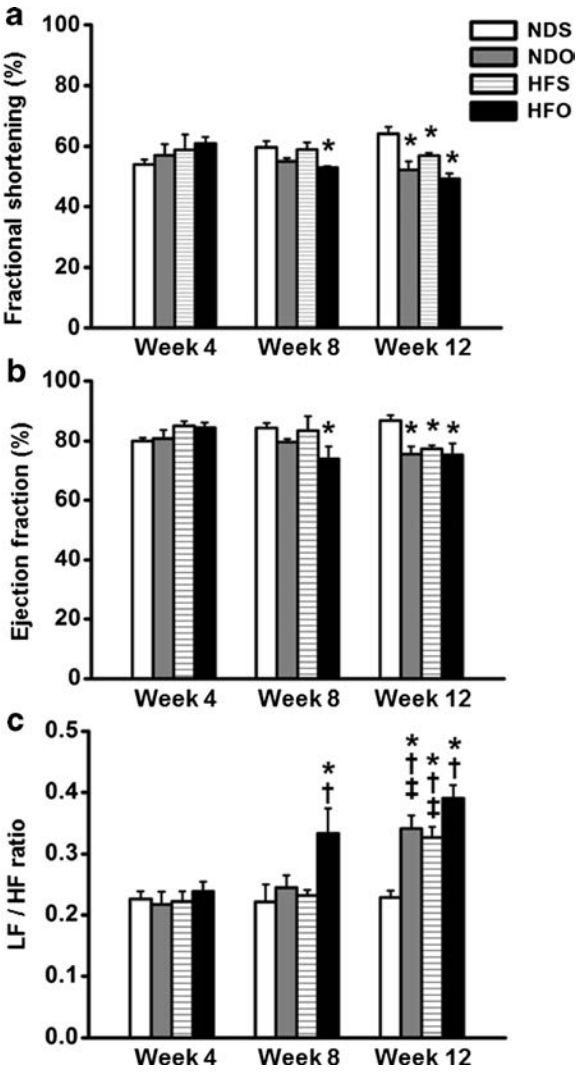

Fig. 1 Effects of obese-insulin resistance on left ventricular function and HRV in estrogen-deprived rats. High-fat fed ovariectomized rats (HFO) early exhibited impaired fractional shortening (FS) (**a**) and ejection fraction (%EF) (**b**) at 8 weeks while normal-diet fed ovariectomized rats (NDO) and high-fat fed sham-operated rats (HFS) were affected at 12 weeks (**c**). LF/HF ratio firstly increased in HFO at 8 weeks then in NDO and HFS at 12 weeks. Values are mean±SE for six rats per group. *$P<0.05$ vs. normal-diet fed sham-operated rats (NDS) in the same week, †$P<0.05$ vs. its 4-week data and ‡$P<0.05$ vs. its 8-week data

ratio (Fig. 1c), suggesting that cardiac autonomic imbalance was firstly initiated in this group at this time-course. In week 12, rats in NDO, HFS, and HFO groups had an increased LF/HF ratio when compared with the NDS group (Fig. 1c). Moreover, the LF/HF ratio in week 12 of HFO rats exhibited a greater increased LF/HF ratio when compared to that in week 4 and week 8 (Fig. 1c), indicating the progressive impairment of cardiac sympathovagal balance over time in HFO rats.

Obese-insulin resistance due to high-fat diet consumption decreased anti-apoptotic protein and accelerated cardiac mitochondrial impairment in estrogen-deprived rats

In the heart, the expressions of Bax and Bcl-2 showed no significant difference among all groups at week 4 (Fig. 2a, b). In week 8, the Bax level was also not different in all groups. It was not until week 12 that the level of Bax expression was increased in NDO, HFS, and HFO groups (Fig. 2a). In contrast, beginning at week 8, the Bcl-2 level was found to decrease only in the HFO rats, and it continued to decrease in week 12 (Fig. 2b). The Bcl-2 level in NDO and HFS was not altered at any time-course. Moreover, a reduced Bax/Bcl-2 ratio was found in NDO, HFS, and HFO groups in week 12 (Fig. 2c).

Cardiac mitochondrial ROS production, mitochondrial membrane potential change, and mitochondrial swelling showed no difference between all groups during week 4 (Fig. 3). In week 8, cardiac mitochondrial dysfunction was found only in HFO rats as indicated by significantly increased ROS levels, decreased $\Delta\Psi$ (indicating mitochondrial depolarization), and cardiac mitochondrial swelling (Fig. 3a–c). It was not until week 12 that NDO and HFS rats developed cardiac mitochondrial dysfunction (Fig. 3).

Discussion

The major findings from the present study clearly demonstrated that obese-insulin resistance induced by high-fat diet consumption not only aggravated the impairments of metabolic function but also accelerated the development of cardiac autonomic imbalance, LV dysfunction, oxidative stress, and cardiac mitochondrial dysfunction, when a rat was under estrogen-deprived conditions. A summary of these findings is showed in Table 4.

Chronic high-fat diet consumption is a common factor that contributes to the development of obesity and induces several subsequent clinical diseases such as insulin resistance, diabetes mellitus, and cardiovascular disease (Marinou et al. 2010). In HFO rats, increasing of body weight and visceral fat was accelerated as well as

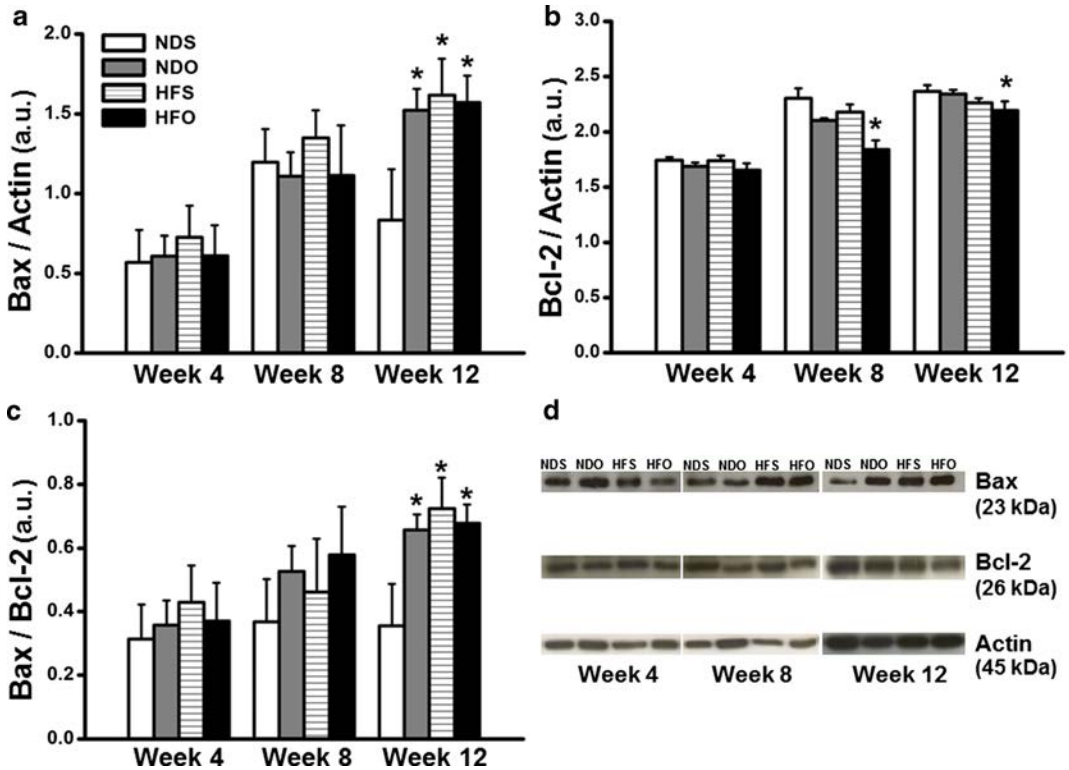

Fig. 2 Effects of obese-insulin resistance on cardiac cellular apoptosis in estrogen-deprived rats. Bax expression (**a**), Bcl-2 expression (**b**), Bax/Bcl-2 ratio (**c**), and representative bands of Bax, Bcl-2, and actin from blotting analysis (**d**). Bcl-2 level was reduced in HFO rats both at 8 and 12 weeks while levels of Bax and Bax/Bcl-2 ratio were increased in HFO, NDO, and HFS at 12 weeks. Values are mean±SE for six rats per group. *$P<0.05$ vs. NDS in the same time period

hyperglycemia and dyslipidemia which were aggravated compared with NDO rats, suggesting the negative effect of obese-insulin resistance on metabolic status in OVX rats. It has been shown that excessive fat accumulation increased oxidative stress, induced mitochondrial impairment, and has been implicated with insulin resistance (Apaijai et al. 2013). Higher levels of MDA and mitochondrial ROS found in HFO rats, compared with NDO and HFS rats, could be essentially contributed to not only the acceleration but also the aggravation of the metabolic disturbance observed in these rats.

The present study has also demonstrated the early development of impaired cardiac autonomic balance (i.e. depressed HRV) in HFO rats. It is well-known that impaired cardiac autonomic balance is associated with increased sympathetic activity and oxidative stress (Apaijai et al. 2013). It has been shown that increased oxidative stress, which was indicated by increased MDA and ROS levels, was an important factor that affected HRV since increased ROS level could cause sympathetic overactivity via inactivation of nitric oxide (Ye et al. 2006). Cardiac sympathovagal disturbance was also previously reported to occur in ovariectomized female rats and was restored by estrogen therapy to reduce oxidative stress, suggesting the considerable impact of oxidative stress on HRV (Campos et al. 2014). In the present study, it was found that the early development of oxidative stress in HFO rats could be responsible for the early development of impaired HRV in these rats, indicating that either obese-insulin resistance or estrogen deprivation could accelerate and aggravate the oxidative stress status when both conditions were combined, leading to early impairment of the HRV as seen in this study. Moreover, although either obese-insulin resistance or estrogen deprivation alone could impair cardiac autonomic balance, we demonstrated that there was earlier development of depressed HRV in the obese-insulin resistant with estrogen-deprived (HFO) rats. These findings indicate that obese-insulin resistance

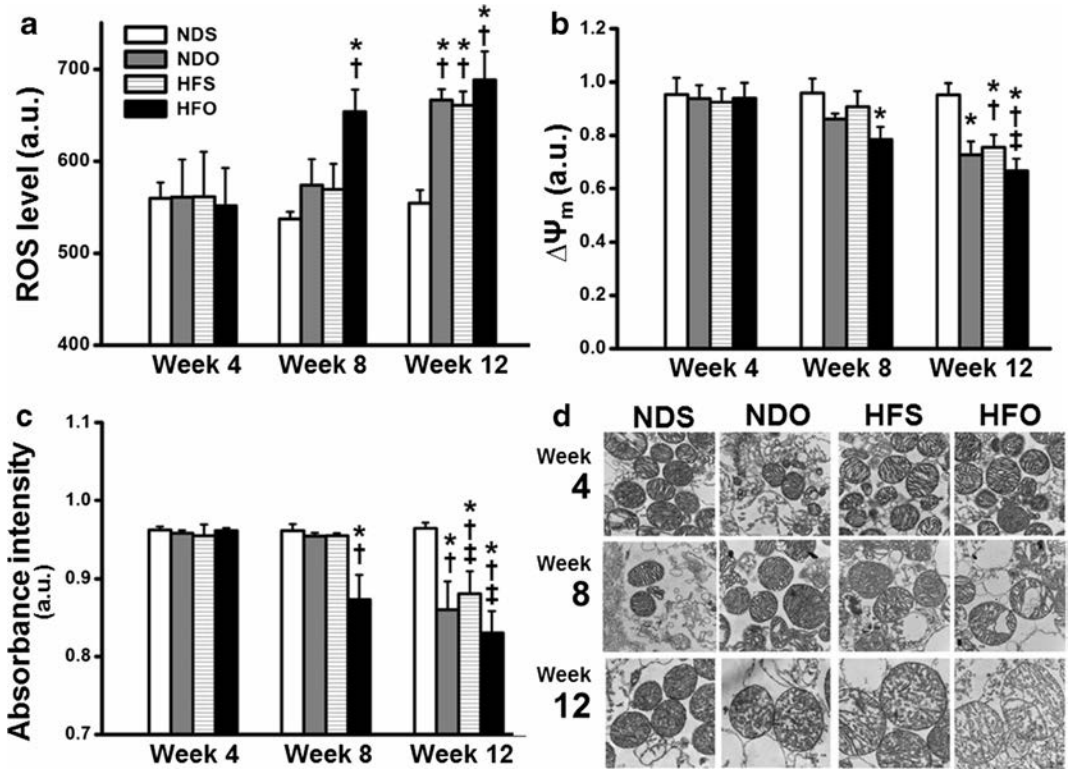

Fig. 3 Effects of obese-insulin resistance on cardiac mitochondrial function in estrogen-deprived rats. Cardiac mitochondrial ROS level (**a**), cardiac mitochondrial membrane potential change (**b**), cardiac mitochondrial swelling (**c**), and transmission electron micrographs illustrate cardiac mitochondria from rats at 4, 8, and 12 weeks in each group (**d**). Cardiac mitochondrial impairment was found earlier (week 8) in HFO rats and later (week 12) in NDO and HFS rats. Values are mean±SE for six rats per group. *$P<0.05$ vs. NDS in the same week, †$P<0.05$ vs. its 4-week data and ‡$P<0.05$ vs. its 8-week data

accelerated the development of HRV impairment in estrogen-deprived rats.

Cardiac function is the most important parameter that has been shown to be affected by either obese-insulin resistance or estrogen deprivation. It was suggested that obesity-associated regression of cardiac performance was mediated through myocardial metabolic change by increased myocardial fatty acid uptake and oxygen consumption (Peterson et al. 2004). Besides the metabolic pathway, impaired cardiac mitochondrial function including increased cardiac mitochondrial ROS production, mitochondrial membrane depolarization, and mitochondrial swelling has been shown to cause cellular apoptosis

Table 4 Summary of cardiometabolic impairment of the experiment groups

Impairment	8 week				12 week			
	NDS	NDO	HFS	HFO	NDS	NDO	HFS	HFO
Metabolic disturbance		✓	✓	✓✓		✓	✓	✓✓
LV contractile dysfunction				✓		✓	✓	✓
Cardiac autonomic imbalance				✓		✓	✓	✓
Oxidative stress				✓		✓	✓	✓✓
Cardiac mitochondrial impairment				✓		✓	✓	✓

(Chinda et al. 2014). In the present study, although either estrogen deprivatio (NDO) or obese-insulin resistance (HFS) alone could cause LV contractile dysfunction, this deleterious effect was accelerated in HFO rats when compared with NDO and HFS rats. These findings suggested that obese-insulin resistant conditions accelerated the development of this LV impairment. Moreover, the development of cardiac mitochondrial dysfunction was also observed earlier in HFO rats, suggesting that the impairment of cardiac mitochondrial function could be responsible for LV dysfunction. Moreover, reduced cardioprotection and higher apoptosis levels indicated by reduced Bcl-2 level together with increased Bax levels as demonstrated in this study support the important role of cardiac mitochondrial function as a vital mechanism underlying this cardiac contractile impairment.

In conclusion, our findings demonstrated that either the loss of endogenous estrogen by ovariectomy alone (Vogel et al. 2013) or obese-insulin resistance alone (Apaijai et al. 2013) could abrogate cardiometabolic protection that therefore result in cardiometabolic disorders. However, when obese insulin-resistant conditions were added to estrogen-deprived conditions, it not only aggravated the development of metabolic disturbance but also accelerated this deleterious effect as well as contributed to the development of LV dysfunction and cardiac sympathovagal imbalance. The underlying mechanisms could be due to the increased oxidative stress and the deterioration of cardiac mitochondrial function as demonstrated in the present study (Table 4). Therefore, our findings clearly demonstrated that obese-insulin resistance caused by high-fat diet consumption accelerated the development of cardiac and metabolic dysfunction through oxidative stress generation and mitochondrial dysfunction in estrogen-deprived female rats. The available treatment for estrogen deprivation with obese-insulin resistance, in addition to postmenopausal hormone therapy, may include antidiabetic or blood-glucose control agents in order to improve the impaired metabolic status. Our team had previously reported that an oral-antidiabetic drug, dipeptidyl peptidase-4 inhibitor, not only improves plasma insulin and cholesterol profiles but also helps decrease cardiac oxidative stress and mitochondrial dysfunction (Apaijai et al. 2013). Future studies are needed to investigate the roles of estrogen replacement therapy as well as the dipeptidyl peptidase-4 inhibitor in this study model.

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Conflict of interest None

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Obesity accelerates cognitive decline by aggravating mitochondrial dysfunction, insulin resistance and synaptic dysfunction under estrogen-deprived conditions

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ABSTRACT

Chronic consumption of a high-fat diet (HF) causes peripheral insulin resistance, brain insulin resistance, brain mitochondrial dysfunction and cognitive impairment. Estrogen deprivation has also been found to impair cognition. However, the combined effect of both conditions on the brain is unclear. We hypothesized that estrogen deprivation causes brain insulin resistance, brain mitochondrial dysfunction, hippocampal synaptic dysfunction and cognitive impairment, and that consumption of a HF accelerates these impairments in an estrogen-deprived condition. Seventy-two female rats were divided into sham (S) and ovariectomized (O) groups. Rats in each group were further divided into two subgroups to be fed with either a normal diet (ND) or HF for 4, 8 and 12 weeks. At the end of each period, the Morris water maze test was carried out, after which the blood and brain were collected for metabolic and brain function analysis. Obesity, peripheral insulin resistance, increased brain oxidative stress and hippocampal synaptic dysfunction were observed at the eighth week in the NDO, HFS and HFO rats. However, these impairments were worse in the HFO rats. Interestingly, brain insulin resistance, brain mitochondrial dysfunction and cognitive impairment developed earlier (week eight) in the HFO rats, whereas these conditions were observed later at week 12 in the NDO and HFS rats. Either estrogen deprivation or HF appears to cause peripheral insulin resistance, increased brain oxidative stress, hippocampal synaptic dysfunction, brain mitochondrial dysfunction and brain insulin resistance, which together can lead to cognitive impairment. A HF accelerates and aggravates these deleterious effects under estrogen-deprived conditions.

Introduction

Both estrogen and obesity have been shown to be involved in the functioning of insulin receptors and also in cognition. It has been shown in a previous study that either estrogen deprivation or obesity increased the oxidative stress level, and led to the development of insulin resistance and cognitive impairment (Evsen et al., 2013; Henderson, 2008; Matsuzawa-Nagata et al., 2008; Pintana et al., 2012). Our previous studies demonstrated that obesity induced by consumption of a high-fat diet (HF) increased brain oxidative stress, as indicated by an increased brain corticosterone level, brain malondialdehyde level and brain mitochondrial dysfunction which subsequently led to impaired brain insulin sensitivity, as indicated by the impairment of insulin-induced long-term depression (LTD) and impaired brain insulin signaling (Pintana et al., 2012; Pipatpiboon et al., 2012, 2013; Pratchayasakul et al., 2011b). Moreover, obesity has been shown to cause cognitive impairment as demonstrated by rats in the Morris water maze test (Pintana et al., 2012; Pipatpiboon et al., 2013) as well as impaired hippocampal synaptic function (Karimi et al., 2013; Stranahan et al., 2008).

Previous studies demonstrated that estrogen deprivation following a bilateral ovariectomy (OVX) caused impaired hippocampal synaptic function as indicated by decreased long-term potentiation (LTP) amplitude, dendritic spine density and synaptic proteins as well as cognitive impairment (Daniel et al., 1997; Luine et al., 1998). These impairments following OVX were alleviated by estrogen administration (Qu et al., 2013; Roseman et al., 2012; Sherwin, 2005). Although several studies demonstrated that either estrogen deprivation or obesity could impair

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hippocampal synaptic function and cognitive function, it is still unclear how estrogen deprivation could affect brain insulin sensitivity and brain mitochondrial function. Moreover, whether obesity aggravates the impairment of and/or accelerates the development of peripheral and brain insulin sensitivity, oxidative stress, brain mitochondrial function, hippocampal synaptic function and cognitive behaviors under conditions of estrogen-deprivation have never been investigated. In the present study, the hypotheses tested were that: 1) estrogen deprivation alone causes the impairment of brain insulin sensitivity, brain mitochondrial function, hippocampal synaptic function and cognitive behaviors, and 2) obesity due to long-term HF consumption accelerates and aggravates those impairments in estrogen-deprived rats.

Materials and methods

Animal models and experimental protocols

All experimental protocols were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Female Wistar rats (200–220 g, n = 72) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were housed individually in a temperature-controlled environment under a light–dark cycle of 12:12 h. One week after arrival, the rats were divided into sham-operated (S) and bilateral ovariectomized (O) groups. One week after surgery, rats were randomly assigned to either a normal diet (ND; 28.24% E protein, 51.99% E carbohydrates, 19.77% E fat) group, which received a standard laboratory pellet diet (CP 082; C.P. Company, Bangkok, Thailand) or a high-fat diet (HF; 26.45% E protein, 14.27% E carbohydrates, 59.28% E fat) group, which were fed a diet containing 59.28% total energy from fat as described in our previous study (Pratchayasakul et al., 2011b). All animals were given ad libitum access to food and water. At the end of weeks 4, 8 and 12, the locomotor activity test began 1 day prior to the first day of the spatial learning and memory behavior test. After that, the animals were allowed to rest for 1 day before an oral glucose tolerance test was performed. Then, animals were deeply anesthetized with isoflurane and killed by decapitation. Blood samples were collected for the determination of metabolic parameters. The brain was rapidly removed for electrophysiological study and biochemical analyses.

Ovariectomy procedure

Female rats were anesthetized using an intraperitoneal injection with xylazine (LBS Laboratories, Bangkok, Thailand; 0.15 ml/kg) and Zoletil (VirbacLabolatories, Carros, France; 50 mg/kg). The bilateral ovariectomy was performed through a midline dorsal section, through which the ovaries were removed and then the incision closed.

Chemical analysis for metabolic parameters

Fasting plasma glucose, HDL, LDL, cholesterol and triglyceride levels were determined using commercially available kits (ERBA diagnostic, Mannheim, Germany). The fasting plasma insulin levels were investigated using ELISA kits (LINCO Research, Missouri, USA). Serum estrogen levels were determined using an EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA). All chemical analyses used to identify metabolic parameters were performed in duplicate in the same assay. The coefficient of variation for the intra- and inter-assays were 6.12% and 10.12% for glucose; 2.35% and 7.16% for HDL; 2.12% and 5.37% for LDL; 2.44% and 5.52% for cholesterol; 7.97% and 13.24% for triglycerides; 4.67% and 10.43% for insulin; and 6.10% and 9.88% for estrogen, respectively.

Table 1
The metabolic parameters in sham and OVX rats receiving either ND or HFD feeding for 4, 8 and 12 weeks.

Parameter	4 weeks				8 weeks				12 weeks			
	NDS	NDO	HFS	HFO	NDS	NDO	HFS	HFO	NDS	NDO	HFS	HFO
Body weight (g)	255.0 ± 4.3	285.8 ± 6.9	292.1 ± 9.6*	356.9 ± 16.8*,†,‡	275.5 + 6.8	310.0 ± 3.8*	307.3 ± 7.5*	387.1 ± 11.0*,†,‡	282.5 ± 3.9	334.3 ± 6.8*	328.1 ± 7.8*	418.1 ± 5.9*,†,‡
Visceral fat (g)	11.0 ± 1.3	9.3 ± 0.8	20.7 ± 1.6*,†	22.1 ± 2.2*,†	10.6 ± 1.2	14.2 ± 1.0*	25.1 ± 2.3*,†	29.2 ± 2.0*,†	11.3 ± 0.6	16.3 ± 1.7*	27.7 ± 2.3*,†	33.4 ± 1.2*,†,‡
Uterus (g)	0.4 ± 0.0	0.1 ± 0.0*,‡	0.3 ± 0.1	0.1 ± 0.0*,‡	0.3 ± 0.0	0.1 ± 0.0*,‡	0.4 ± 0.0	0.1 ± 0.0*,‡	0.4 ± 0.0	0.1 ± 0.0*,‡	0.4 ± 0.0	0.1 ± 0.0*,‡
Food intake (g/day)	16.7 ± 1.0	16.1 ± 1.1	17.6 ± 4.4	18.5 ± 1.0	16.6 ± 0.9	15.9 ± 1.1	15.7 ± 0.7	16.1 ± 0.6	15.3 ± 0.7	14.2 ± 1.1	15.2 ± 0.4	15.4 ± 0.5
Glucose (mg %)	128.4 ± 6.7	125.6 ± 8.1	128.1 ± 10.6	128.3 ± 11.4	122.8 ± 7.3	133.6 ± 7.3	133.9 ± 6.8	148.8 ± 7.3*	122.7 ± 5.3	128.4 ± 10.8	127.6 ± 4.1	151.6 ± 6.8*,†,‡
Cholesterol (mg/dl)	93.2 ± 6.4	104.0 ± 8.6	103.9 ± 9.9	101.1 ± 12.7	107.3 ± 5.0	111.6 ± 4.5	132.1 ± 17.0*	137.2 ± 2.5*,†	92.9 ± 5.1	112.1 ± 12.1	137.6 ± 11.5*,†	146.4 ± 12.1*,†
Triglyceride (mg/dl)	34.0 ± 3.6	32.2 ± 3.5	33.7 ± 3.6	31.6 ± 3.7	47.7 ± 2.6	45.7 ± 4.6	47.8 ± 3.3	46.3 ± 4.8	40.8 ± 3.2	43.8 ± 3.6	43.0 ± 3.3	40.5 ± 6.2
HDL (mg/dl)	5.6 ± 0.32	6.0 ± 0.1	5.6 ± 0.5	5.7 ± 0.4	6.4 ± 0.2	6.6 ± 0.2	6.2 ± 0.2	6.38 ± 0.8	5.7 ± 0.3	5.7 ± 0.2	5.7 ± 0.2	5.8 ± 0.3
LDL (mg/dl)	76.2 ± 3.8	82.7 ± 6.6	75.9 ± 8.5	70.4 ± 8.9	76.3 ± 5.7	87.6 ± 3.6	92.4 ± 5.0*	122.3 ± 2.8*,†,‡	68.7 ± 5.5	69.0 ± 1.9	113.1 ± 17.3*,†	131.1 ± 16.0*,†
Insulin (ng/ml)	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	1.4 ± 0.2*	1.5 ± 0.2*	1.6 ± 0.2*	0.9 ± 0.1	1.5 ± 0.3*	1.4 ± 1.2*	1.5 ± 0.2*
HOMA index	6.3 ± 1.6	6.1 ± 0.1	7.1 ± 1.4	7.6 ± 1.3	5.6 ± 1.1	11.14 ± 1.70*	12.4 ± 1.9*	12.6 ± 1.5*	7.3 ± 1.8	13.5 ± 2.4*	13.8 ± 2.7*	13.9 ± 2.0*
Serum estrogen (pg/ml)	167.8 ± 39.3	58.7 ± 17.5*,‡	151.9 ± 21.2†	63.2 ± 20.6*,‡	114.7 ± 12.1	53.29 ± 1.99*,‡	117.2 ± 16.9	58.4 ± 3.7*,‡	129.4 ± 17.8	63.9 ± 6.5*,‡	127.2 ± 7.4†	71.3 ± 6.9*,‡
Serum MDA (μM)	3.4 ± 0.6	3.4 ± 0.4	3.5 ± 0.3	3.45 ± 0.33	3.9 ± 0.1	4.19 ± 0.15*	4.1 ± 0.1*	4.8 ± 0.3*,†,‡	3.39 ± 0.16	4.1 ± 0.2*	4.0 ± 0.1*	4.9 ± 0.2*,†,‡
Brain MDA (μM)	4.7 ± 0.1	4.6 ± 0.1	4.9 ± 0.1	4.61 ± 0.07	5.7 ± 1.3	8.10 ± 0.13*	7.9 ± 0.1*	9.8 ± 0.4*,†,‡	5.38 ± 1.19	8.0 ± 0.3*	7.9 ± 0.4*	10.1 ± 0.8*,†,‡

Data was presented as mean ± SEM. *, p < 0.05 compared with NDS, †, p < 0.05 compared with NDO, ‡, p < 0.05 compared with HFS; n = 5–6/group; NDS: sham ND-fed rats, NDO: ovariectomized ND-fed rats, HFS: sham HFD-fed rats, HFO: ovariectomized HFD-fed rat.

Determination of insulin resistance

Insulin resistance was evaluated using the Homeostasis Model Assessment (HOMA) index (Matthews et al., 1985) and the total area under the curve of the oral glucose tolerance test (OGTT) as described in our previous study (Pratchayasakul et al., 2011b). HOMA was calculated from fasting plasma insulin and fasting plasma glucose concentrations. A higher HOMA index indicated a higher degree of insulin resistance. The HOMA index was determined using the following equation: [(fasting plasma insulin μU/ml) × (fasting plasma glucose mmol/l)] / 22.5.

In addition, the OGTT was investigated at the end of weeks 4, 8 and 12 of the dietary periods. Briefly, the animals were fasted for 12 h before they underwent an OGTT. An OGTT consisted of 2 g/kg body weight glucose feeding by gavage. Blood was collected from a small cut at the tip of the tail immediately before and at 15, 30, 60 and 120 min after glucose feeding. The plasma was utilized for the investigation, and glucose analysis was performed with a commercially available kit (Biotech, Bangkok, Thailand).

Determination of malondialdehyde (MDA) levels

The MDA level was determined by a high performance liquid chromatography method following the protocol used in the previous study (Candan and Tuzmen, 2008).

Extracellular recordings of hippocampal slices for insulin-induced LTD and electrical-induced LTP

Brain slice preparation for extracellular recordings was performed following the guidelines in the previous study (Chattipakorn and McMahon, 2002). Field excitatory postsynaptic potentials (fEPSPs) were recorded according to our previous protocol (Pratchayasakul et al., 2011a,2011b). In the LTD protocol, hippocampal slices were perfused with aCSF for 10 min. as a baseline. After that, the insulin-induced LTD condition was created by perfusion with aCSF plus 500 nM insulin (Humulin R, Eli Lilly, Giessen, Germany) for an additional 10 min, and then the slices were perfused with aCSF for a further 50 min. In the LTP protocol, LTP was induced by delivering high-frequency stimulation (HFS; 4 trains at 100 Hz; 0.5 s duration; 20 s interval). Experiments were performed at least 40 min after HFS. The amount of potentiation was calculated at 40 min after HFS.

Immunoblotting

The subsequent brain homogenates for immunoblotting were prepared as described in our previous study (Pratchayasakul et al., 2011b). Briefly, brain homogenates for immunoblotting were prepared by incubating whole brain slices into aCSF or aCSF plus 500 nM insulin (Humulin R, Eli Lilly, Giessen, Germany) for 5 min. After that, whole brain slices in each conditioned group were homogenized, and used for further immunoblotting. IR protein, Akt/PKB protein, IR tyrosine phosphorylation and Akt/PKB at serine 473 kinases phosphorylation were analyzed by electrophoresis and immunoblotting was carried out with rabbit antibodies for the IR (Santa Cruz Biotechnology, CA, USA; sc-711; 1:1000), Akt/PKB (Cell Signaling Technology, MA, USA; #9272; 1:1000), IR at tyrosine (Santa Cruz Biotechnology, CA, USA; sc-25103-R; 1:1000) and Akt/PKB at serine 473 (Cell Signaling Technology, MA, USA; #9271; 1:1000), respectively. All blots were incubated with a horseradish peroxidase conjugated anti-rabbit secondary antibody (1:2000, Cell Signaling Technology, MA, USA). The protein bands were developed with Amersham hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

Brain mitochondrial function

After decapitation, the brain tissue was transferred into 10 ml of ice-cold MSE-nagarse solution and homogenized at 600 rpm/min

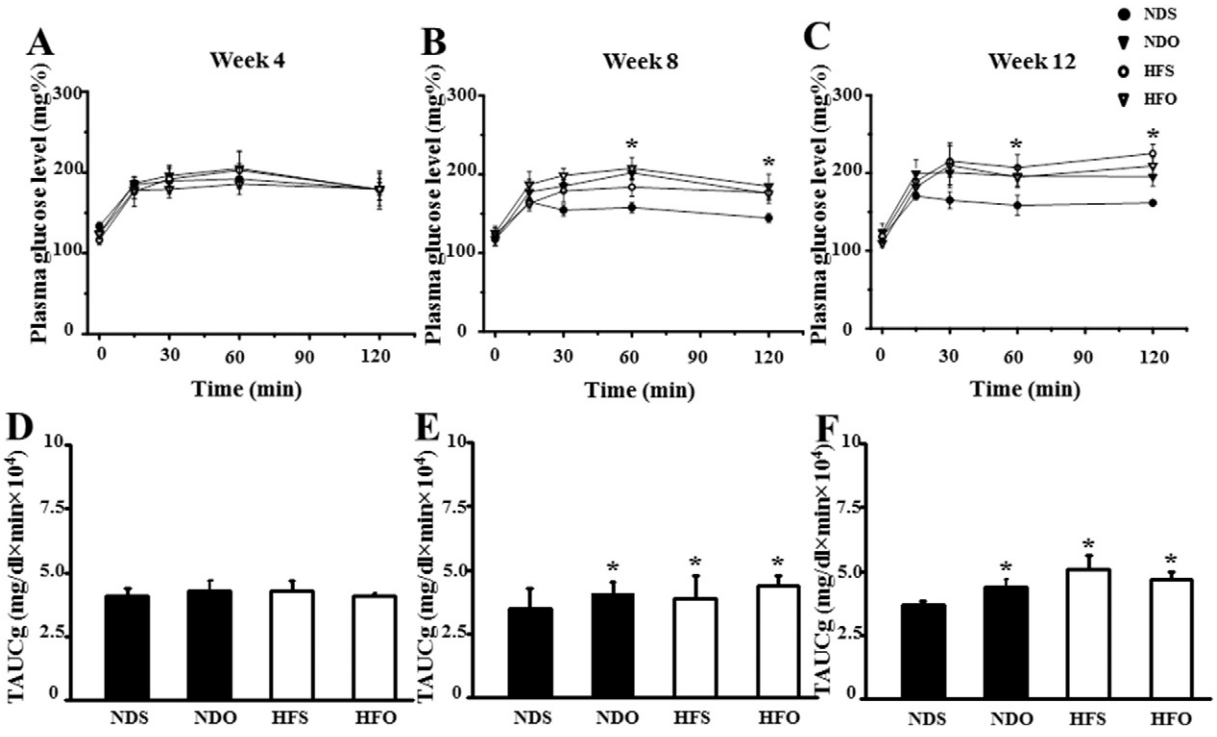

Fig. 1. Glucose response during oral glucose tolerance test from NDS, NDO, HFS and HFO at 4 weeks (A), 8 weeks (B) and 12 weeks (C); and total area under the curve of plasma glucose during an oral glucose tolerance test (TAUCg) from NDS, NDO, HFS and HFO at 4 weeks (D), 8 weeks (E) and 12 weeks (F); *, $p < 0.05$ compared with NDS; n = 5–6/group.

using a homogenizer. The subsequent brain mitochondrial isolation was prepared as described in our previous study (Pipatpiboon et al., 2012). Brain mitochondrial function was determined using a brain mitochondrial reactive oxygen species (ROS) assay, a brain mitochondrial membrane potential (ΔΨm) assay and a brain mitochondrial swelling assay. The details of brain mitochondrial function were previously described (Pipatpiboon et al., 2012).

Golgi impregnation and analysis

Since increased dendritic spine density has been reported to be related to LTP expression (Bramham, 2008; Hill and Zito, 2013), we therefore aimed to investigate the dendritic spine density in our experiment. After decapitation, brains were removed and rinsed with double distilled water, and they were then processed for Golgi staining using a commercially available kit (FD Neurotechnologies kit, PK 401, Ellicott City, USA). The details of dendritic spine density analysis were previously described (Sripetchwandee et al., 2014). Briefly, two segments from a pyramidal cell in the CA1 area of the hippocampus were randomly measured. This was the same area for fEPSP recording in our extracellular recordings of hippocampal slices for insulin-induced LTD and electrical-induced LTP. Both segments were located on the tertiary apical dendrites and were viewed through an inverted microscope (IX-81, Olympus, Tokyo, Japan). Three neuronal cells from each brain slice were selected for quantitative analysis (3 slices/animals, n 5–6 animals/group). The number of spines was counted using a hand counter, and the dendritic lengths were measured using Xcellence imaging software (Olympus, Tokyo, Japan).

Morris water maze test (MWM)

In this study, the protocol of MWM was modified from a previous study (Vorhees and Williams, 2006). This experiment determined learning and memory ability with two different types of MWM, acquisition test (hidden platform) and probe trial test (removal of the platform from the water pool) as described in our previous study (Pintana et al., 2012). In the acquisition test, rats were trained to navigate a route to the platform in 4 trials/day for 5 days, with an inter-trial interval of 15 s. For each trial, rats were given 120 s to find the platform, and the time taken to reach the platform was recorded. In the probe trial test, the platform was removed, and the time spent in the target quadrant was recorded. After testing was completed, times taken to reach the platform, times spent in the target quadrant, and swim speeds were calculated using Smart 3.0 software (Panlab, Harvard Apparatus, Barcelona, Spain). In addition, the open-field test was developed to determine the locomotor activity, and was modified from the method used by Arakawa (Arakawa, 2005). The open field consisted of a black platform with a 75 cm × 75 cm floor and 40-cm walls. The box floor was drawn with white lines (6 mm) to form 25 equal squares. The animals were placed into the box and observed for 2 min. The numbers of lines which the animals crossed were counted as a representation of locomotor activity.

Statistical methods

Data was presented as mean ± SEM. All statistical analyses were performed using the program SPSS (version 17; SPSS, Chicago, Ill., USA). The significance of the difference in all parameters was calculated using a two-way ANOVA (diet X surgery) followed by post-hoc Fisher's protected least significant difference (LSD) test. A two-way ANOVA with repeated measurements was used to compare the training trials of the MWM test. For the main effects, the effect sizes were calculated using Eta-squared, η^2. For all comparisons, a value of $p < 0.05$ was considered to be statistically significant.

Results

A High-fat diet aggravated the impairment of peripheral insulin sensitivity under estrogen-deprived conditions

Initial animal body weight, plasma glucose and plasma insulin levels showed no significant difference between all groups. The effects of the ovariectomy were confirmed by determining uterine weight and serum estrogen levels at the end of 4, 8 and 12-weeks of the dietary periods. The ovariectomy resulted in a decrease in uterine weight and serum estrogen levels in both the ND and HF rats (for uterine weight: $F(1,52) = 203.44$, $p < 0.001$, $\eta^2 = 0.69$ at 4 weeks; $F(1,45) = 210.87$, $p < 0.001$, $\eta^2 = 0.29$ at 8 weeks; $F(1,49) = 205.56$, $p < 0.001$, $\eta^2 = 0.74$ at 12 weeks; for estrogen: $F(1,31) = 40.28$, $p < 0.001$, $\eta^2 = 0.47$ at 4 weeks; $F(1,24) = 34.68$, $p < 0.001$, $\eta^2 = 0.61$ at 8 weeks; $F(1,27) = 35.92$, $p < 0.001$, $\eta^2 = 0.56$ at 12 weeks; Table 1).

At the end of week four, sham-operated HF-fed rats (HFS) and ovariectomized HF-fed rats (HFO) had significantly increased body weight and visceral fat compared to sham ND-fed rats (NDS) and ovariectomized ND-fed rats (NDO) ($F(1,14) = 20.64$, $p < 0.001$, $\eta^2 = 0.65$ for body weight; $F(1,49) = 41.34$, $p < 0.001$, $\eta^2 = 0.67$ for visceral fat; Table 1). However, the levels of glucose, cholesterol, triglyceride, HDL, LDL, insulin, HOMA index and the total area under the glucose curve (TAUCg) from the OGTT showed no significant difference between groups (Table 1 and Figs. 1A, D).

By weeks 8 and 12, the NDO, HFS and HFO rats had developed obesity, indicated by increased body weight and visceral fat (for body

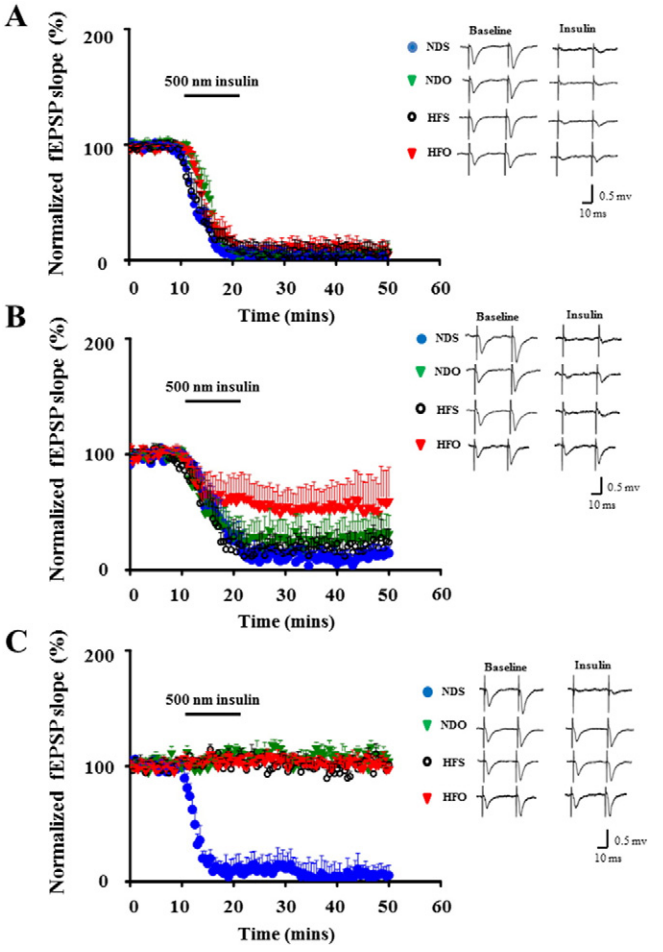

Fig. 2. The degree of insulin-mediated LTD observed from hippocampal slices of NDS, NDO, HFS and HFO at 4 weeks (A), 8 weeks (B) and 12 weeks (C); (n = 5–7 independent slices, n = 6 animals/group).

weight: $F(1,51) = 53.51$, $p < 0.001$, $\eta^2 = 0.65$ at 8 weeks; $F(1,51) = 123.72$, $p < 0.001$, $\eta^2 = 0.61$ at 12 weeks; for visceral fat: $F(1,43) = 48.04$, $p < 0.001$, $\eta^2 = 0.63$ at 8 weeks; $F(1,46) = 111.45$, $p < 0.001$, $\eta^2 = 0.65$ at 12 weeks; Table 1), and the impairment of peripheral insulin sensitivity, demonstrated by an increase in insulin level, HOMA index and TAUCg, when compared with the NDS rats (for insulin: $F(1,34) = 4.38$, $p = 0.044$, $\eta^2 = 0.19$ at 8 weeks; $F(1,93) = 3.95$, $p = 0.047$, $\eta^2 = 0.13$ at 12 weeks; for HOMA: $F(1,32) = 5.84$, $p = 0.022$, $\eta^2 = 0.26$ at 8 weeks; $F(1,35) = 4.15$, $p = 0.048$, $\eta^2 = 0.12$ at 12 weeks; for TAUCg: $F(1,16) = 5.11$, $p = 0.038$, $\eta^2 = 0.17$ at 8 weeks; $F(1,29) = 17.14$, $p = 0.001$, $\eta^2 = 0.54$ at 12 weeks; Table 1 and Figs. 1B, C, E, F). In addition, plasma LDL and cholesterol levels of the HFS and HFO rats showed a significant increase when compared to those of the NDS rats (for LDL: $F(1,15) = 25.81$, $p < 0.001$, $\eta^2 = 0.72$ at 8 weeks; $F(1,23) = 13.72$, $p = 0.001$, $\eta^2 = 0.41$ at 12 weeks; for cholesterol: $F(1,29) = 9.50$, $p = 0.004$, $\eta^2 = 0.37$ at 8 weeks; $F(1,35) = 8.47$, $p = 0.006$, $\eta^2 = 0.20$ at 12 weeks; Table 1). Interestingly, these impaired metabolic parameters were highest in the HFO rats ($p < 0.05$; Table 1). These findings at weeks 8 and 12 suggest that either estrogen deprivation alone or a high-fat diet alone impaired peripheral insulin sensitivity, and that high-fat diet aggravated the severity of that impairment under conditions of estrogen-deprivation.

A high-fat diet accelerated the impairment of brain insulin sensitivity under conditions of estrogen-deprivation

At week four, the degree of insulin-induced LTD detected from brain slices of the NDS, NDO, HFS and HFO rats showed no statistically significant difference between the groups (Fig. 2A). At week eight, the degree of insulin-induced LTD was significantly reduced in HFO rats compared to that of the NDS, NDO and HFS rats ($F(1,19) = 6.42$, $p = 0.020$, $\eta^2 = 0.34$, Fig. 2B). At week 12, insulin-induced LTD was diminished in the NDO, HFS and HFO rats ($F(1,19) = 63.48$, $p < 0.001$, $\eta^2 = 0.91$, Fig. 2C).

In addition to insulin-induced LTD, brain insulin signaling was determined. At the end of weeks 4, 8 and 12, the expression of IR and Akt/PKB proteins showed no statistically significant difference between the groups (Figs. 3A–C, 4A–C). At week four, the levels of tyrosine phosphorylation of IR and Akt/PKB phosphorylation at the serine 473 site were also not significantly different (Figs. 3D, 4D). However, at week 8 the levels of tyrosine phosphorylation of IR (pIR) and Akt/PKB phosphorylation at the serine 473 site (Akt-ser) were significantly decreased in the HFO rats, when compared with those of the NDS, NDO and HFS rats ($F(1,13) = 4.87$, $p = 0.041$, $\eta^2 = 0.58$ for pIR; $F(1,8) = 5.71$, $p = 0.044$, $\eta^2 = 0.11$ for Akt-ser; Figs. 3E, 4E). At week 12, the levels of tyrosine phosphorylation of IR and Akt/PKB phosphorylation at the serine 473 site of the NDO, HFS and HFO rats were significantly reduced compared with those of the NDS rats ($F(1,39) = 4.11$, $p = 0.048$, $\eta^2 = 0.48$ for pIR; $F(1,23) = 8.42$, $p = 0.010$, $\eta^2 = 0.60$ for Akt-ser; Figs. 3F, 4F). These findings suggest that either estrogen deprivation alone or a high-fat diet alone impaired brain insulin sensitivity, and that a high-fat diet accelerated that impairment under estrogen-deprived conditions.

A high-fat diet accelerated the impairment of brain mitochondrial function under estrogen-deprived condition

Brain mitochondria play an important role in the regulation of Ca^{2+}, synaptic plasticity and neuronal survival (Mattson et al., 2008). Previous studies demonstrated that mitochondrial dysfunction affected learning and memory in several models including streptozotocin (STZ)-induced diabetic rats (Ye et al., 2011), old-age female rats (Platano et al., 2008) and HFD-fed rats (Pintana et al., 2012). Therefore, this study investigated the effects of a high-fat diet on brain mitochondrial function under conditions of estrogen deprivation. We found that brain mitochondrial function was not significantly different between groups at week four (Figs. 5A, D, G). At week eight, brain mitochondrial dysfunction occurred in the HFO rats as indicated by increased brain mitochondrial ROS production, brain mitochondrial membrane depolarization and brain mitochondrial swelling, when compared with the NDS rats ($F(1,9) = 5.24$, $p = 0.041$, $\eta^2 = 0.55$ for mitochondrial ROS production; $F(1,13) = 4.79$, $p = 0.046$, $\eta^2 = 0.22$ for mitochondrial membrane depolarization; $F(1,32) = 4.92$, $p = 0.041$, $\eta^2 = 0.29$ for mitochondrial swelling; Figs. 5B, E, H). At week 12, brain mitochondrial dysfunction was observed in the NDO, HFS and HFO rats ($F(1,16) = 5.24$, $p =$

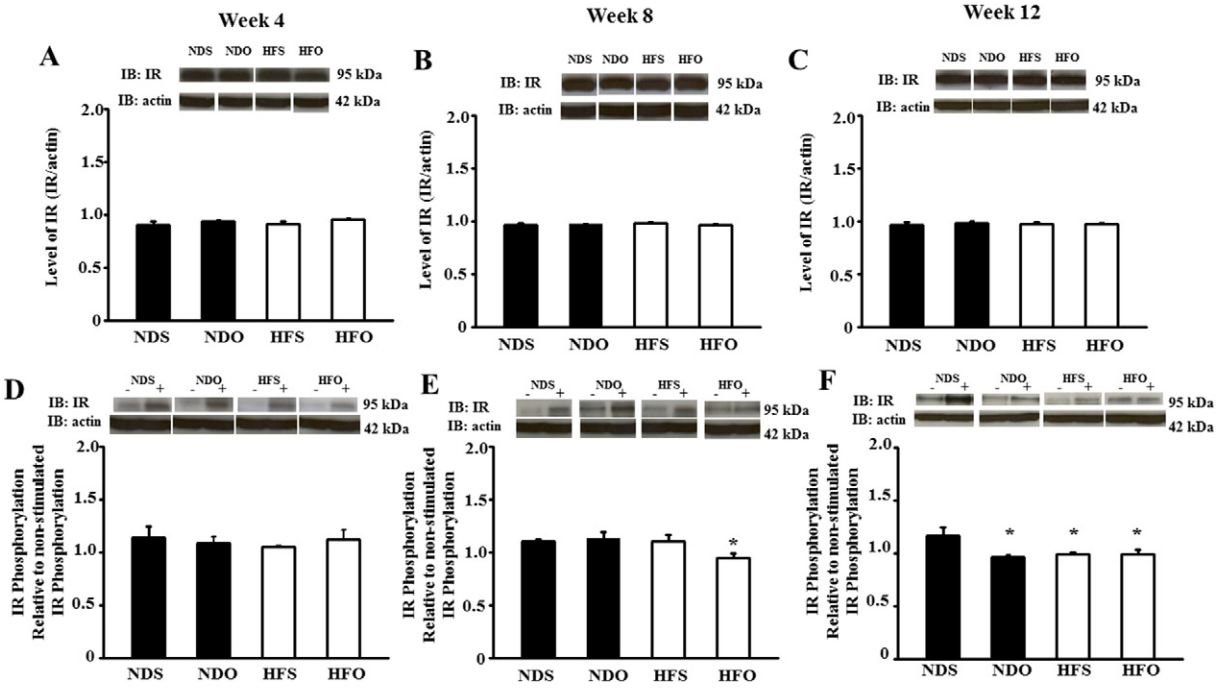

Fig. 3. Representative blots of IR protein in brain slices harvested from the NDS, NDO, HFS and HFO rats at 4 weeks (A), 8 weeks (B) and 12 weeks (C). Representative blots of IR tyrosine phosphorylation in brain slices harvested from NDS, NDO, HFS and HFO rats at 4 weeks (D), 8 weeks (E) and 12 weeks (F); *, $p < 0.05$ compared with NDS; n = 5–6/group; −, no insulin stimulation; +,insulin stimulation.

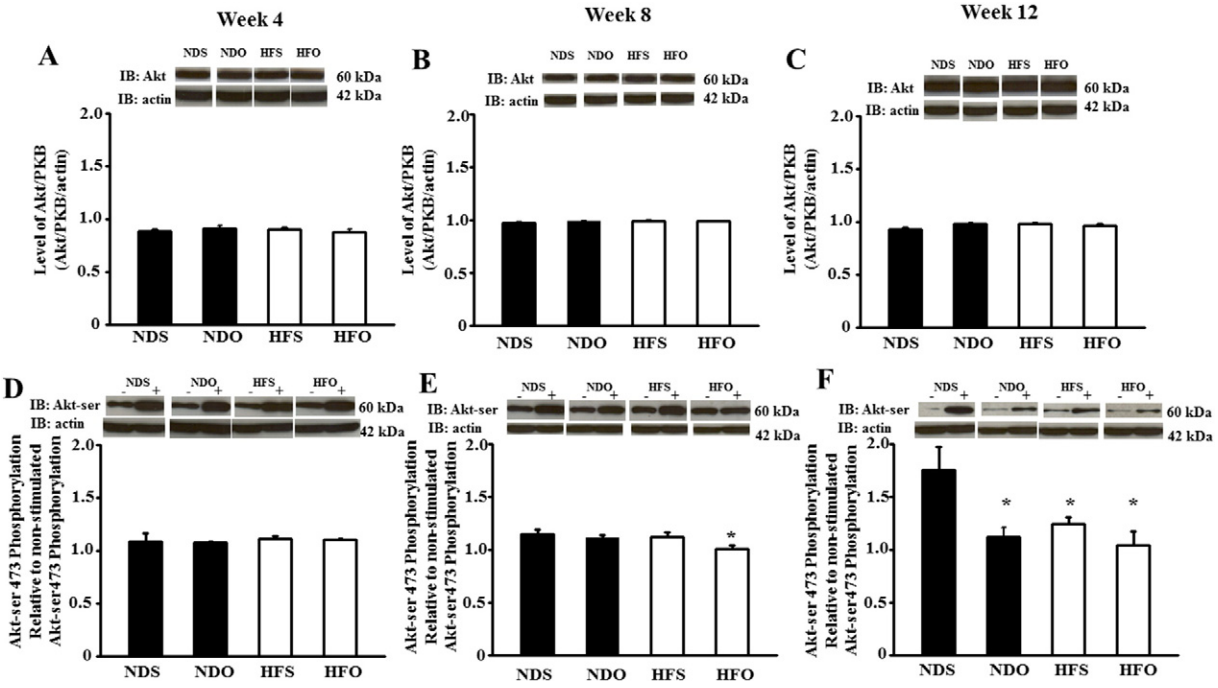

Fig. 4. Representative blots of protein level of Akt/PKB in brain slices harvested from the NDS, NDO, HFS and HFO rats at 4 weeks (A), 8 weeks (B) and 12 weeks (C). Representative blots of Akt/PKB phosphorylation at the serine 473 site in brain slices harvested from NDS, NDO, HFS and HFO rats at 4 weeks (D), 8 weeks (E) and 12 weeks (F); *, $p < 0.05$ compared with NDS; n = 5–6/group; −,no insulin stimulation; +, insulin stimulation.

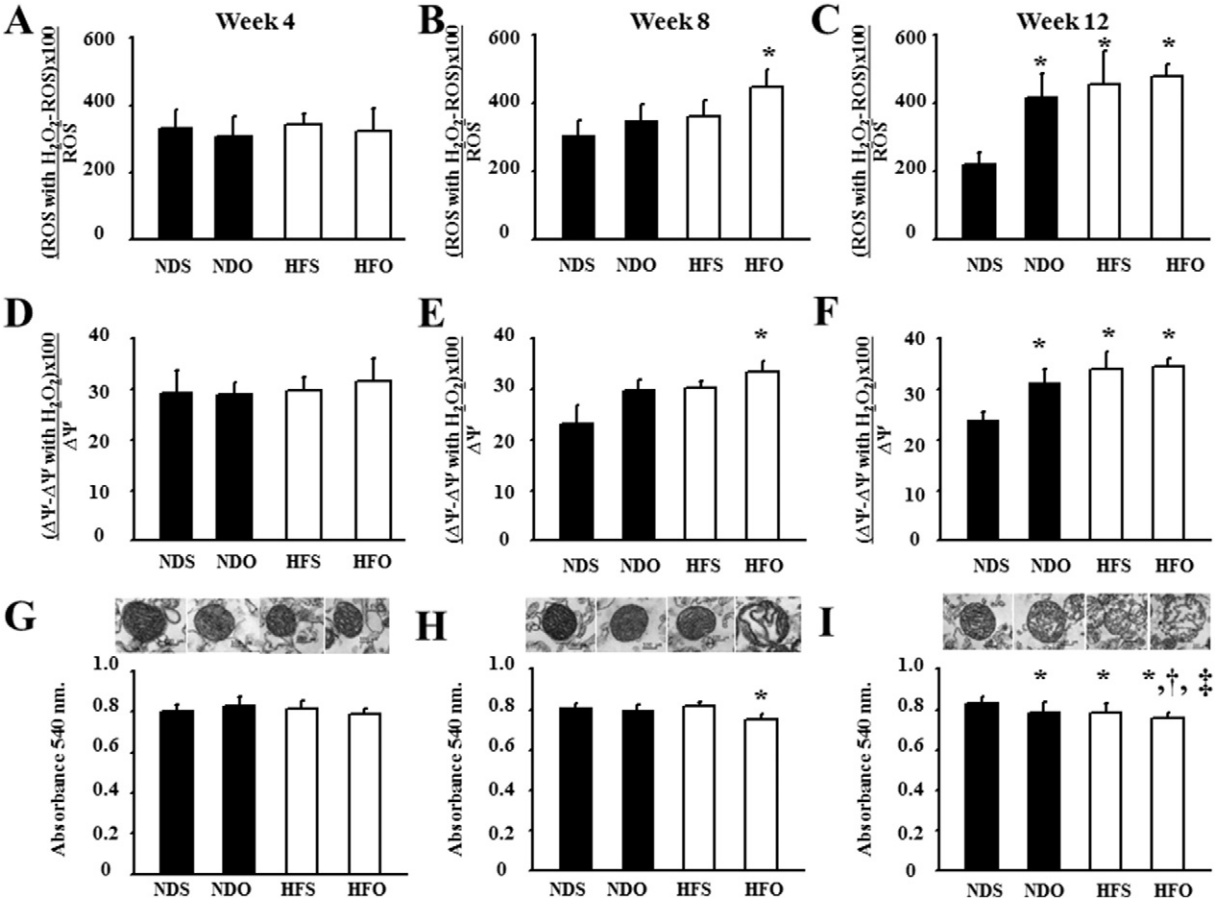

Fig. 5. Brain mitochondrial ROS production from the NDS, NDO, HFS and HFO rats at 4 weeks (A), 8 weeks (B) and 12 weeks (C); Brain mitochondrial membrane potential change ($\Delta\psi_m$) from the NDS, NDO, HFS and HFO rats at 4 weeks (D), 8 weeks (E) and 12 weeks (F); Brain mitochondrial swelling and transmission electron microscopy (original magnification ×25,000) shows the ultrastructure of brain mitochondria from the NDS, NDO, HFS and HFO rats at 4 weeks (G), 8 weeks (H) and 12 weeks (I); *, $p < 0.05$ compared with NDS, †, $p < 0.05$ compared with NDO, ‡, $p < 0.05$ compared with HFS; n = 5–6/group.

0.036, $\eta^2 = 0.36$ for mitochondrial ROS production; $F(1,27) = 7.51$, $p = 0.021$, $\eta^2 = 0.21$ for mitochondrial membrane depolarization; $F(1,26) = 13.94$, $p = 0.001$, $\eta^2 = 0.42$ for mitochondrial swelling; Figs. 5C, F, I). In addition, the degree of brain mitochondrial swelling was greatest in the HFO rats ($p < 0.05$; Fig. 5I). Also this disruption in brain mitochondrial morphology, indicated by unfolding and swelling, was observed earlier (at week eight) in the HFO rats, and later at week 12 in the NDO and HFS rats (Figs. 5H, I). These findings suggest that either estrogen deprivation alone or a high-fat diet alone caused the impairment of brain mitochondrial function, and that a high-fat diet accelerated that impairment under estrogen-deprived conditions.

A high-fat diet aggravated an increase in serum and brain oxidative stress under conditions of estrogen-deprivation

In addition to brain mitochondrial dysfunction, serum and brain oxidative stress levels were investigated. At week 4, serum and brain MDA levels were not significantly different between groups. However, at weeks 8 and 12, serum and brain MDA levels in the NDO, HFS and HFO rats were significantly increased, when compared with NDS rats (for serum MDA: $F(1,26) = 10.54$, $p = 0.003$, $\eta^2 = 0.63$ at 8 weeks; $F(1,28) = 24.54$, $p < 0.001$, $\eta^2 = 0.48$ at 12 weeks; for brain MDA: $F(1,14) = 7.84$, $p = 0.021$, $\eta^2 = 0.50$ at 8 weeks; $F(1,14) = 10.78$, $p = 0.005$, $\eta^2 = 0.60$ at 12 weeks; Table 1). Interestingly, the serum and brain MDA levels in the HFO rats were significantly higher than that of the NDO and HFS rats ($p < 0.05$; Table 1). These results suggest that either estrogen deprivation alone or a high-fat diet alone leads to increased serum and brain oxidative stress, and that a high-fat diet aggravated the severity of oxidative stress under estrogen-deprived conditions.

A high-fat diet aggravated the impairment of hippocampal synaptic plasticity under conditions of estrogen-deprivation

Several studies have demonstrated that the impairment of hippocampal synaptic plasticity was influenced by oxidative stress (Massaad and Klann, 2011; Ye, 2013). Therefore, hippocampal synaptic plasticity, including electrical-induced LTP as well as the dendritic spine density, was determined. At week four, the degree of electrical-mediated LTP, which was calculated using hippocampal slices of the NDS, NDO, HFS and HFO rats, was not significantly different (Fig. 6A). In addition, the number of dendritic spines in the CA1 hippocampus was not significantly different between groups (Fig. 7A).

At weeks 8 and 12, electrical-induced LTP of the NDO, HFS and HFO rats was significantly decreased, when compared with that of NDS rats ($F(1,11) = 22.70$, $p = 0.001$, $\eta^2 = 0.52$ at 8 weeks; $F(1,17) = 14.90$, $p = 0.001$, $\eta^2 = 0.44$ at 12 weeks; Figs. 6B, C). In addition, the electrical-induced LTP of the HFO rats was significantly attenuated, when compared with that of the NDO and HFS rats ($p < 0.05$; Figs. 6B, C). The density of dendritic spines in the CA1 hippocampus of the NDO, HFS and HFO rats was significantly decreased, when compared with that of the NDS rats ($F(1,12) = 15.60$, $p = 0.002$, $\eta^2 = 0.78$ at 8 weeks; $F(1,18) = 27.09$, $p < 0.001$, $\eta^2 = 0.85$ at 12 weeks; Figs. 7B, C). Interestingly, the number of dendritic spines in the HFO rats was the lowest among all groups ($p < 0.05$; Figs. 7B, C). These findings suggest that either estrogen deprivation alone or a high-fat diet alone impairs the hippocampal synaptic plasticity, and that a high-fat diet aggravates that impairment under conditions of estrogen-deprivation.

A high-fat diet accelerated the impairment of cognitive function under conditions of estrogen-deprivation

Before the MWM test at each time period, the locomotor activity of all groups was determined using the open-field test. The number of lines that the rats crossed during the test was not significantly different between all groups. This finding indicates that the locomotor activity of all groups before the cognitive test was not significantly different.

The time to reach the platform in the acquisition test from the first to the fifth day of training in all experimental groups significantly decreased ($F(4,27) = 23.87$, $p < 0.001$, $\eta^2 = 0.36$ at 4 weeks, $F(4,20) = 17.88$, $p < 0.001$, $\eta^2 = 0.27$ at 8 weeks, $F(4,22) = 19.08$, $p < 0.001$, $\eta^2 = 0.49$ at 12 weeks; Figs. 8A,B,C). However, the learning and memory tests were not significantly different between groups at week 4 (Figs. 8A, D). At week 8, the mean time to reach the platform during the acquisition test of the HFO rats was significantly increased, when compared with that of the NDS, NDO and HFS rats ($F(1,22) = 6.08$, $p = 0.022$, $\eta^2 = 0.29$; Fig. 8B). In addition, the mean time spent in the target quadrant during the probe test of the HFO rats was significantly decreased, when compared with that of the NDS, NDO and HFS rats ($F(1,78) = 3.97$, $p = 0.046$, $\eta^2 = 0.29$; Fig. 8E).

At week 12, decreased learning and memory behaviors of the NDO, HFS and HFO rats were observed (Figs. 8C, F), as indicated by the increased mean time to reach the platform during the acquisition test ($F(1,24) = 7.56$, $p = 0.036$, $\eta^2 = 0.23$; Fig. 8C), and the decreased mean time spent in the target quadrant during the probe test ($F(1,24) = 4.73$, $p = 0.040$, $\eta^2 = 0.26$; Fig. 8F). In addition, the swim speed of each rat was recorded, and there were no significant differences in the swim speed between all groups (24.71 ± 1.89, 23.71 ± 1.61, 23.04 ± 2.20 and 25.83 ± 1.20 cm/s, in NDS, NDO, HFS and HFO, respectively). These findings confirmed that the impairment of cognitive function was due to spatial memory deficit, instead of the differences in weight and fat content of the animals. All of these findings indicate that either estrogen deprivation alone or a high-fat diet alone impairs the learning and memory behaviors, and that a high-fat diet

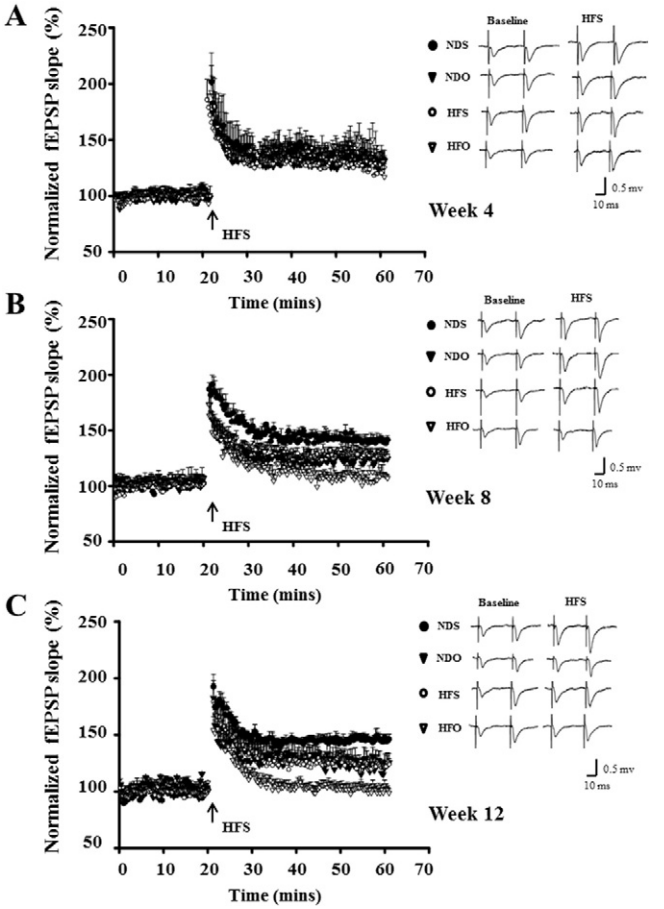

Fig. 6. The degree of electrical-mediated LTP observed from hippocampal slices of NDS, NDO, HFS and HFO at 4 weeks (A), 8 weeks (B) and 12 weeks (C); (n = 5–7 independent slices, n = 6 animals/group).

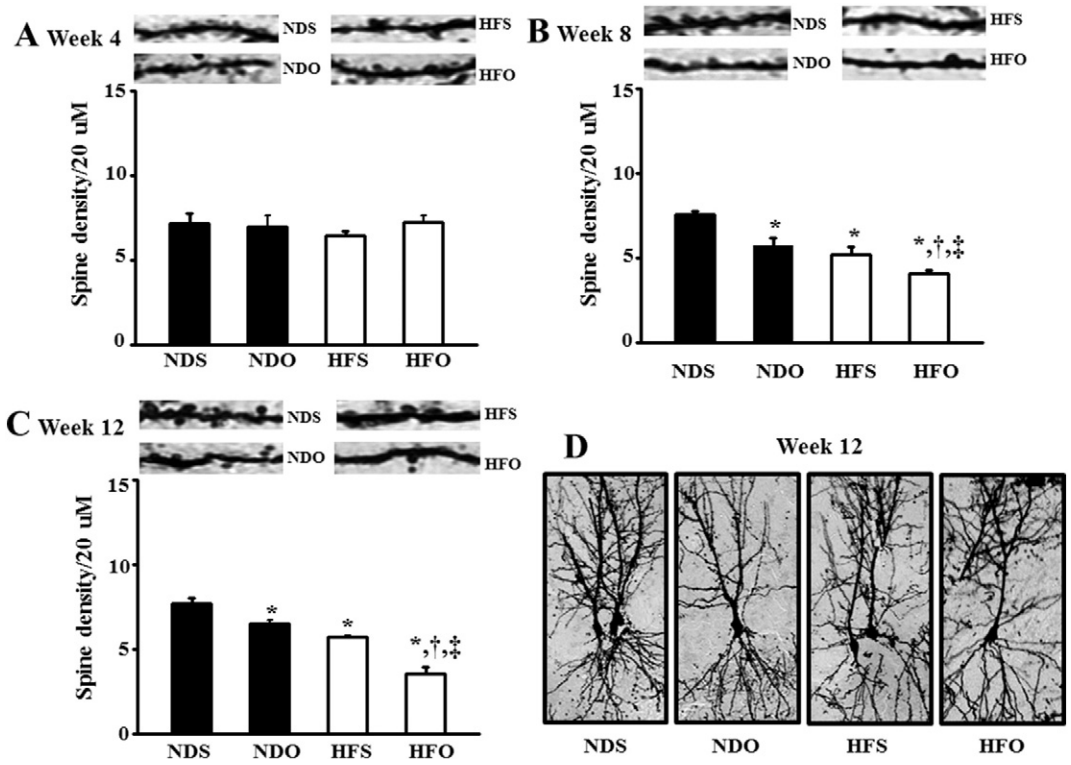

Fig. 7. The number of dendritic spines on tertiary dendrites in apical dendrite from the NDS, NDO, HFS and HFO rats at 4 weeks (A), 8 weeks (B) and 12 weeks (C); a picture of a representative neuron from 12 weeks of NDO, HFS and HFO rats (D) *, $p < 0.05$ compared with NDS, †, $p < 0.05$ compared with NDO, ‡, $p < 0.05$ compared with HFS; n = 5–6/group.

accelerates the cognitive impairment under conditions of estrogen-deprivation.

Discussion

The major findings of this study are as follows: 1) impaired peripheral insulin sensitivity was initially observed at week 8 in the NDO, HFS, and HFO rats; 2) the impairment of brain insulin sensitivity, indicated by the reduction of insulin-induced LTD, and brain insulin signaling, as well as brain mitochondrial dysfunction occurred earlier (at week 8) in the HFO rats, and was found later at week 12 in the NDO, HFS and HFO rats; 3) the occurrence of oxidative stress and hippocampal synaptic dysfunction was initially observed at week 8 in the NDO, HFS and HFO rats; 4) the impaired cognitive function developed earlier (at week

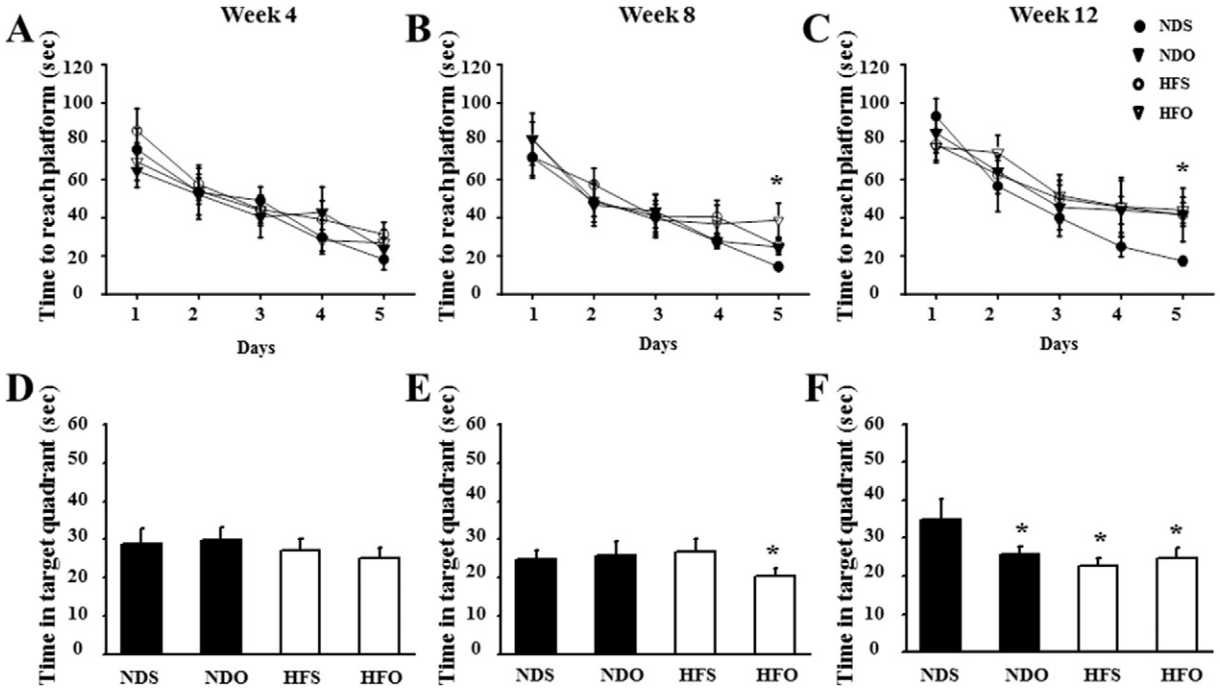

Fig. 8. Time to reach platform in acquisition test from the NDS, NDO, HFS and HFO rats at 4 weeks (A), 8 weeks (B) and 12 weeks (C); Time spent in target quadrant in probe test from the NDS, NDO, HFS and HFO rats at 4 weeks (D), 8 weeks (E) and 12 weeks (F); *, $p < 0.05$ compared with NDS; n = 5–6/group.

8) in the HFO group, and was found later at week 12 in the NDO and HFS groups; 5) either estrogen deprivation alone or a high-fat diet alone caused impaired peripheral insulin sensitivity, reduced brain insulin sensitivity, brain mitochondrial dysfunction, hippocampal synaptic dysfunction and cognitive impairment; 6) a high-fat diet significantly accelerated and aggravated the severity of these deleterious effects under conditions of estrogen-deprivation.

Previous studies demonstrated that either a high-fat diet alone or estrogen deprivation alone could impair peripheral insulin sensitivity (Funaki, 2009; Mauvais-Jarvis et al., 2013; Pratchayasakul et al., 2011b). Our previous studies (Pipatpiboon et al., 2012; Pratchayasakul et al., 2011b) and the present study confirmed that obesity induced by HF consumption impaired peripheral insulin sensitivity, as indicated by increased body weight, visceral fat, plasma glucose level, plasma insulin level, HOMA index, and the total area under the curve (TAUCg). In addition, it has been shown that estrogen deprivation alone also caused obesity, leading to the development of peripheral insulin resistance (Alonso et al., 2006; Babaei et al., 2010). Consistent with those studies, this study showed that 8 weeks after a bilateral ovariectomy, rats had both increased body weight and visceral fat, leading to reduced peripheral insulin sensitivity. Interestingly, the severity of impaired peripheral insulin sensitivity in rats on a high-fat diet and estrogen deprived rats was significantly greater than rats with a high-fat diet alone or with estrogen deprivation alone. These findings suggest that a high-fat diet aggravates the severity of peripheral insulin resistance in estrogen-deprived rats. On consideration of these findings, it is therefore possible that a high-fat diet on top of estrogen deprivation could lead to the development of Type II diabetes, as indicated in this study by the increased plasma glucose levels found in rats on both a high-fat diet and with estrogen-deprivation.

Interestingly, our results demonstrated that peripheral insulin resistance occurred earlier (at week 8), whereas the impairment of brain insulin sensitivity and brain mitochondrial function developed later (at week 12) in both the estrogen-deprived group and the high-fat diet group. However, the high-fat diet with estrogen-deprived rats developed the worst peripheral insulin resistance, and had brain insulin resistance and brain mitochondrial dysfunction occurring simultaneously with the occurrence of peripheral insulin resistance. Thus, we demonstrated for the first time that a high-fat diet in rats accelerated the development of brain insulin resistance under conditions of estrogen-deprivation. It has been shown that estrogen deprivation alone can cause an obese-insulin resistant condition (Babaei et al., 2010; Barros and Gustafsson, 2011). Although several studies demonstrated that impaired insulin sensitivity was caused by several mechanisms, increased oxidative stress has been shown to play a major role in this undesirable effect (Boden, 1997; Evans et al., 2002; Faraj et al., 2004; Smith and Minson, 2012). Moreover, brain insulin resistance is also known to be strongly associated with brain mitochondrial dysfunction (Boden, 1997; Pintana et al., 2012; Pipatpiboon et al., 2012; Pratchayasakul et al., 2011b). Furthermore, it has been shown that mitochondrial dysfunction affected the activation of several serine kinase pathways, leading to defective insulin signal transduction (Rhee, 2006; Saini, 2010) and brain insulin resistance (Pipatpiboon et al., 2012). In the present study, the findings that a high-fat diet in rats accelerated the development of brain insulin resistance under estrogen-deprived conditions could be due to the fact that rats under the combined high-fat diet with estrogen deprivation had the severest degree of oxidative stress, peripheral insulin resistance and brain mitochondrial dysfunction, thus leading to the early development of brain insulin resistance. Our findings emphasized the very important role of oxidative stress caused by either a high-fat diet or estrogen deprivation, which could lead to the reduction of peripheral and brain insulin sensitivity, as observed in the present study.

It has been shown that increased oxidative stress plays an important role in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease (Finkel and Holbrook, 2000) and dementia in aging (Krieglstein et al., 2000). These pathological conditions have been identified by increased neuronal apoptosis and impaired hippocampal synaptic plasticity, as indicated by decreased LTP amplitude and a reduction in dendritic spine density (Avila-Costa et al., 1999; Rivas-Arancibia et al., 2010). In this study, we have demonstrated that either estrogen deprivation alone or a high-fat diet alone impaired the hippocampal synaptic plasticity, as indicated by decreased LTP and decreases in the density of apical dendrite spines of the CA1 hippocampus. A high-fat diet aggravated those impairments in the estrogen-deprived rats. Although it has been shown that either a high-fat diet alone or estrogen deprivation alone could lead to increased oxidative stress levels in the brain leading to the impairment of hippocampal synaptic plasticity, here we demonstrated for the first time that a high-fat diet significantly aggravated that impairment in rats under an estrogen-deprived condition.

Previous studies demonstrated that rats fed with a HF with high glucose for eight months demonstrated impaired spatial learning ability, evidenced by a decreased dendritic spine density and reduced LTP at the Schaffer-CA1 synapse (Karimi et al., 2013; Stranahan et al., 2008). In addition, our previous study showed that a 12-week HF consumption in male rats caused cognitive impairment via increased brain MDA levels and mitochondrial ROS production, as well as impaired brain insulin sensitivity (Pintana et al., 2012). Also, ovariectomized rats showed obvious spatial learning and memory deficits in the MWM test with a significant loss of neurons and impaired synaptic function in the hippocampus (Qu et al., 2013; Roseman et al., 2012). In this study it has been demonstrated for the first time that a high-fat diet accelerated the development of cognitive impairment in rats under an estrogen-deprived condition. The possible explanation of the worsening cognitive impairment in rats on a high-fat diet under conditions of estrogen-deprivation could be due to the drastically increased peripheral insulin resistance and oxidative stress level in those rats, leading to brain mitochondrial dysfunction, brain insulin receptor dysfunction and reduced hippocampal synaptic plasticity.

In conclusion, this study demonstrated that either a high-fat diet alone or estrogen deprivation alone caused impaired peripheral insulin sensitivity, the reduction of brain insulin sensitivity, brain mitochondrial dysfunction, hippocampal synaptic dysfunction and cognitive impairment. Also, a high-fat diet significantly accelerated and aggravated those deleterious effects under conditions of estrogen-deprivation.

Limitation

One limitation of the present study was the lack of investigation into the effects of HF on brain function in non-obese rats. Although previous studies demonstrated the effects of obesity on cognitive decline (Gerges et al., 2003; Winocur et al., 2005), there is a report demonstrating that diet-induced cognitive impairment could precede weight gain and obesity (Davidson et al., 2013). This issue needs further investigation in the future.

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