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

Sivaporn Sivasinprasasn · Piangkwan Sa-nguanmoo ·
Wasana Pratchayasakul · Sirinart Kumfu ·
Siriporn C. Chattipakorn · Nipon Chattipakorn

Received: 2 January 2015 / Accepted: 9 March 2015 / Published online: 20 March 2015
© American Aging Association 2015

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) ($n=6/\text{group}$) 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.

S. Sivasinprasasn · P. Sa-nguanmoo · W. Pratchayasakul ·
S. Kumfu · S. C. Chattipakorn · N. Chattipakorn
Cardiac Electrophysiology Research and Training Center,
Faculty of Medicine, Chiang Mai University, Chiang
Mai 50200, Thailand

S. Sivasinprasasn
School of Health Science, Mae Fah Luang University, Chiang
Rai 57100, Thailand

S. Sivasinprasasn · P. Sa-nguanmoo · W. Pratchayasakul ·
S. Kumfu · N. Chattipakorn
Cardiac Electrophysiology Unit, Department of Physiology,
Faculty of Medicine, Chiang Mai University, Chiang
Mai 50200, Thailand

S. C. Chattipakorn
Department of Oral Biology and Diagnostic Science, Faculty
of Dentistry, Chiang Mai University, Chiang Mai 50200,
Thailand
e-mail: nchattip@gmail.com

S. Sivasinprasasn · P. Sa-nguanmoo · W. Pratchayasakul ·
S. Kumfu · S. C. Chattipakorn · N. Chattipakorn (✉)
Center of Excellence in Cardiac Electrophysiology Research,
Chiang Mai University, Chiang Mai 50200, Thailand
e-mail: nchattip@gmail.com

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

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, atherosclerosis, 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 zoltil (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: $FS\% = [(LVIDd - LVIDs) / LVIDd] \times 100$ (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 dichlorohydrofluorescein 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 H₃PO₄ and 0.6 % thiobabutaric 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 mM 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 polyvinylidene 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-mM 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 deprivation (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 cardio-metabolic protection that therefore result in cardio-metabolic 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 sympatho-vagal 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.

Acknowledgments This work was supported by a NSTDA Research Chair Grant from the National Science and Technology Development Agency (NC), the Thailand Research Fund RTA5580006 (NC), BRG5780016 (SC), TRG5680018 (WP), TRG5780002 (SK), Faculty of Medicine Endowment Fund (WP), and the Chiang Mai University Center of Excellence Award (NC).

Conflict of interest None

References

- Apaijai N, Pintana H, Chattipakorn SC, Chattipakorn N (2013) Effects of vildagliptin versus sitagliptin, on cardiac function, heart rate variability and mitochondrial function in obese insulin-resistant rats. *Br J Pharmacol* 169:1048–1057
- Apaijai N, Chinda K, Palee S, Chattipakorn S, Chattipakorn N (2014) Combined vildagliptin and metformin exert better cardioprotection than monotherapy against ischemia-reperfusion injury in obese-insulin resistant rats. *PLoS One* 9:e102374
- Bonithon-Kopp C, Scarabin PY, Darme B, Malmejac A, Guize L (1990) Menopause-related changes in lipoproteins and some other cardiovascular risk factors. *Int J Epidemiol* 19:42–48
- Campos C, Casali KR, Baraldi D, Conzatti A, Araujo AS, Khaper N, Llesuy S, Rigatto K, Bello-Klein A (2014) Efficacy of a low dose of estrogen on antioxidant defenses and heart rate variability. *Oxid Med Cell Longev* 2014:218749
- Carr MC (2003) The emergence of the metabolic syndrome with menopause. *J Clin Endocrinol Metab* 88:2404–2411
- Chattipakorn N, Incharoen T, Kanlop N, Chattipakorn S (2007) Heart rate variability in myocardial infarction and heart failure. *Int J Cardiol* 120:289–296
- Chinda K, Sanit J, Chattipakorn S, Chattipakorn N (2014) Dipeptidyl peptidase-4 inhibitor reduces infarct size and preserves cardiac function via mitochondrial protection in ischaemia-reperfusion rat heart. *Diab Vasc Dis Res* 11:75–83
- Ginsberg HN (2000) Insulin resistance and cardiovascular disease. *J Clin Invest* 106:453–458
- Higashi Y, Noma K, Yoshizumi M, Kihara Y (2009) Endothelial function and oxidative stress in cardiovascular diseases. *Circ J* 73:411–418
- Karmazyn M, Purdham DM, Rajapurohitam V, Zeidan A (2008) Signalling mechanisms underlying the metabolic and other effects of adipokines on the heart. *Cardiovasc Res* 79:279–286
- Leite RD, Prestes J, Bernardes CF, Shiguemoto GE, Pereira GB, Duarte JO, Domingos MM, Baldissera V, de Andrade Perez SE (2009) Effects of ovariectomy and resistance training on lipid content in skeletal muscle, liver, and heart; fat depots; and lipid profile. *Appl Physiol Nutr Metab* 34:1079–1086

- Marinou K, Tousoulis D, Antonopoulos AS, Stefanadi E, Stefanadis C (2010) Obesity and cardiovascular disease: from pathophysiology to risk stratification. *Int J Cardiol* 138:3–8
- Mathers CD, Loncar D (2006) Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* 3:e442
- Overbeek LI, Kapusta L, Peer PG, de Korte CL, Thijssen JM, Daniels O (2006) New reference values for echocardiographic dimensions of healthy Dutch children. *Eur J Echocardiogr* 7:113–121
- Palee S, Weerateerangkul P, Surinkeaw S, Chattipakorn S, Chattipakorn N (2011) Effect of rosiglitazone on cardiac electrophysiology, infarct size and mitochondrial function in ischaemia and reperfusion of swine and rat heart. *Exp Physiol* 96:778–789
- Peterson LR, Waggoner AD, Schechtman KB, Meyer T, Gropler RJ, Barzilai B, Davila-Roman VG (2004) Alterations in left ventricular structure and function in young healthy obese women: assessment by echocardiography and tissue Doppler imaging. *J Am Coll Cardiol* 43:1399–1404
- Pratchayasakul W, Chattipakorn N, Chattipakorn SC (2011) Effects of estrogen in preventing neuronal insulin resistance in hippocampus of obese rats are different between genders. *Life Sci* 89:702–707
- Pratchayasakul W, Chattipakorn N, Chattipakorn SC (2014) Estrogen restores brain insulin sensitivity in ovariectomized non-obese rats, but not in ovariectomized obese rats. *Metabolism* 63:851–859
- Ribeiro RF Jr, Potratz FF, Pavan BM, Forechi L, Lima FL, Fiorim J, Fernandes AA, Vassallo DV, Stefanon I (2013) Carvedilol prevents ovariectomy-induced myocardial contractile dysfunction in female rat. *PLoS One* 8:e53226
- Rivera CM, Grossardt BR, Rhodes DJ, Brown RD Jr, Roger VL, Melton LJ III, Rocca WA (2009) Increased cardiovascular mortality after early bilateral oophorectomy. *Menopause* 16: 15–23
- Semaming Y, Kumfu S, Pannangpetch P, Chattipakorn SC, Chattipakorn N (2014) Protocatechuic acid exerts a cardioprotective effect in type 1 diabetic rats. *J Endocrinol* 223:13–23
- Vitale C, Mendelsohn ME, Rosano GM (2009) Gender differences in the cardiovascular effect of sex hormones. *Nat Rev Cardiol* 6:532–542
- Vogel H, Mirhashemi F, Liehl B, Taugner F, Kluth O, Kluge R, Joost HG, Schurmann A (2013) Estrogen deficiency aggravates insulin resistance and induces beta-cell loss and diabetes in female New Zealand obese mice. *Horm Metab Res* 45: 430–435
- Wang D, Wang C, Wu X, Zheng W, Sandberg K, Ji H, Welch WJ, Wilcox CS (2014) Endothelial dysfunction and enhanced contractility in microvessels from ovariectomized rats: roles of oxidative stress and perivascular adipose tissue. *Hypertension* 63:1063–1069
- Yang SG, Mlcek M, Kittnar O (2013) Estrogen can modulate menopausal women's heart rate variability. *Physiol Res* 62(Suppl 1):S165–S171
- Ye S, Zhong H, Yanamadala S, Campese VM (2006) Oxidative stress mediates the stimulation of sympathetic nerve activity in the phenol renal injury model of hypertension. *Hypertension* 48:309–315