Testosterone Deprivation Aggravates Left-Ventricular Dysfunction in Male Obese Insulin-Resistant Rats via Impairing Cardiac Mitochondrial Function and Dynamics Proteins

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Keywords

Testosterone deprivation · Obesity-related insulin resistance · Cardiac function · Mitochondrial function · Mitochondrial dynamics proteins

Abstract

Background: We have previously reported that testosterone deprivation at a very young age accelerated, but did not aggravate, left-ventricular (LV) dysfunction in obese insulin-resistant rats. However, the effects of testosterone deprivation during adulthood on LV function in obese insulin-resistant rats remains unclear. We hypothesized that testosterone deprivation aggravates LV dysfunction and cardiac autonomic imbalance via the impairment of cardiac mitochondrial function and dynamics proteins, a reduction in insulin receptor function, and an increase in apoptosis in obese insulin-resistant rats. **Methods:** Male rats were fed on either a normal diet (ND) or a high-fat diet (HFD) for 12 weeks. They were then subdivided into 2 groups: sham operation (NDS, HFS) and orchiectomy (NDO, HFO). Metabolic

parameters, blood pressure, heart rate variability (HRV), and LV function were determined at baseline and before and after orchiectomy. Mitochondrial function and dynamics proteins, insulin signaling, and apoptosis were determined 12 weeks postoperatively. **Results:** HFS rats exhibited obese insulin resistance, depressed HRV, and LV dysfunction. In HFO rats, systolic blood pressure was increased with more excessive depression of HRV and increased LV dysfunction, compared with HFS rats. These adverse cardiac effects were consistent with markedly increased mitochondrial dysfunction, reduced mitochondrial complex I and III proteins, reduced mitochondrial fusion proteins, and increased apoptosis, compared with HFS rats. However, testosterone deprivation did not lead to any alteration in the insulin-resistant condition in HFO rats, compared with HFS rats. **Conclusion:** We concluded that testosterone deprivation during adulthood aggravated the impairment of mitochondrial function, mitochondrial respiratory complex, mitochondrial dynamics proteins, and apoptosis, leading to LV dysfunction in obese insulin-resistant rats.

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Introduction

Both obesity-related insulin resistance and testosterone deprivation are known to increase the risk of cardiovascular disease [1], and in both human [2] and animal studies have been shown to be associated with left-ventricular (LV) dysfunction and cardiac autonomic imbalance [3–5]. Previous reports suggested that these adverse cardiac effects are due to mitochondrial dysfunction [4, 6]. Mitochondria are highly mobile organelles, and their function is controlled by mitochondrial dynamics [7, 8]. Changes in the fission/fusion balance in mitochondria can lead to mitochondrial dysfunction and apoptosis [9, 10]. Mitochondrial fission is regulated by dynamin-related protein 1 (Drp1). Mitochondrial fusion is regulated by mitofusin2 (Mfn2) and optic atrophy1 (OPA1), these proteins being responsible for outer and inner mitochondrial membrane fusion, respectively [7]. It has been shown that Drp1 protein levels were increased whereas Mfn2 protein levels were decreased in testosterone-deprived rats with myocardial infarction, compared with the levels in rats with myocardial infarction alone [8].

Although testosterone levels usually decline with age, obesity is also associated with a reduction of testosterone level [11]. Our previous study using obese insulin-resistant rats demonstrated that testosterone deprivation at a very young age followed by long-term consumption of a high-fat diet (HFD) could accelerate but not aggravate LV dysfunction [4]. A recent study showed that obese insulin-resistant rats subjected to testosterone deprivation in middle age had an exacerbated insulin-resistant condition as indicated by increased HOMA-IR [12]. In addition, LV mass was also reduced and serum CK-MB levels were increased in testosterone-deprived obese insulin-resistant rats subjected to myocardial ischemia reperfusion injury [13]. Currently, the process involved in the alteration of cardiometabolic function in testosterone-deprived obese insulin-resistant rats remains unknown. Furthermore, the underlying mechanism responsible for the changes in LV function in testosterone-deprived obese insulin-resistant rats is still unclear. We hypothesized that testosterone deprivation aggravates LV dysfunction and cardiac autonomic imbalance via the impairment of cardiac mitochondrial function and mitochondrial dynamics, reduced myocardial insulin receptor function, and increased apoptosis in obese insulin-resistant rats.

Materials and Methods

Ethical Approval

All experiments in this study were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee (permit No. 15/2559), in compliance with NIH guidelines and in accordance with the ARRIVE guidelines for reporting animal research.

Animals and Experimental Design

Thirty-two male Wistar rats (6 weeks old; 150–180 g) were obtained from Nomura Siam International Co., Ltd. (Bangkok, Thailand). The rats were housed in a room with controlled temperature and humidity, and were allowed access to water and food ad libitum. After 1 week of acclimatization, the rats were randomly divided into 2 dietary groups: a normal diet group (ND, a diet containing 19.77% energy from fat) and an HFD group (a diet containing 52.98% energy from fat). The rats were given their assigned diet for 12 weeks [14]. Then, the rats in each dietary group were randomly subdivided into 2 subgroups: a sham operation group ($n = 16$; NDS: $n = 8$ and HFS: $n = 8$) and a bilateral orchiectomy (ORX) group ($n = 16$; NDO: $n = 8$ and HFO: $n = 8$). Metabolic parameters, blood pressure, cardiac autonomic balance, and LV function were determined at baseline, after 12 weeks of HFD feeding, and 1, 2, 4, 8, and 12 weeks postoperatively. LV function was determined invasively at 12 weeks postoperatively. Insulin (10 IU/kg; Eli Lilly and Company, IA, USA) was injected afterwards to stimulate myocardial insulin signaling [15]. Thirty minutes after the insulin injection, the rats were euthanized using Zoletil 100 (150 mg/kg; Virbac, Bangkok, Thailand) and Xylazine (9 mg/kg; LBS Labs, Bangkok, Thailand) via intramuscular injection, followed by decapitation, and the heart was rapidly excised to enable the determination of cardiac mitochondrial function, mitochondrial dynamics, myocardial insulin signaling, and apoptotic protein expression. The experimental protocol is shown in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000487188).

Bilateral ORX

Rats were anesthetized and maintained using 2% isoflurane with 98% oxygen. The ORX was performed under sterile conditions, the incision being made in the scrotal area [4]. The blood vessels and vas deferens were permanently ligated with 5-0 silk sutures, and the testes were then removed. The skin incision was closed using a nonabsorbable suture. The rats were carefully monitored to prevent them from chewing their sutures and to record any other complications. An analgesic drug (4 mg/kg, 4% tolifenamic acid; Vetoquinol, France), and antibiotics (2 mg/kg, 2% marbofloxacin; Vetoquinol) were administered via subcutaneous injection for 3 days after surgery [4].

Determination of Metabolic Parameters

Body weight and food intake were recorded weekly, and visceral fat weight was recorded after euthanasia. Blood was collected from the tail vein and centrifuged at 6,000 rpm for 10 min. The plasma was collected to allow the determination of plasma testosterone, insulin, glucose, triglyceride, total cholesterol, and HDL levels [16].

Plasma testosterone levels were determined using the ELISA technique at the Central Laboratory Service of Maharaj Nakorn

Chiang Mai Hospital (Faculty of Medicine, Chiang Mai University) [4]. Plasma insulin levels were determined using a commercial ELISA kit (Merck Millipore, MA, USA). Plasma glucose, total cholesterol, and triglyceride levels were determined using commercial colorimetric kits (Erba Mannheim, Mannheim, Germany). The degree of insulin resistance was assessed by the HOMA index, which was calculated from fasting plasma insulin and fasting plasma glucose concentration. Plasma HDL levels were determined using a commercial colorimetric kit (BioVision Inc., CA, USA). Plasma LDL levels were calculated using the Friedewald formula [16].

Blood Pressure Determination

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were determined in conscious rats using a noninvasive volume-pressure recording tail cuff method (CODA-2; Kent Scientific, CT, USA) [16].

Heart Rate Variability Determination

A frequency domain of heart rate variability (HRV) including a high-frequency (HF) and low-frequency (LF) band were used to represent cardiac autonomic balance for a short-term recording [17]. A lead II electrocardiogram (ECG) was recorded in conscious rats. The ECG signals were recorded through a signal transducer (PowerLab 4/25T; AD Instruments, Sydney, NSW, Australia), and operated through the Chart 5.0 program (AD Instruments). Power spectra of RR intervals were obtained using the fast Fourier transform algorithm. Both the LF (0.2–0.6 Hz) and HF (0.6–3 Hz) bands were detected where LF represents sympathetic tone and HF represents parasympathetic tone. Therefore, an increased LF/HF ratio indicated cardiac sympathovagal imbalance [16].

LV Function Determination

LV function was determined noninvasively using echocardiography at baseline, after 12 weeks of HFD feeding, at 1, 2, 4, 8, and 12 weeks postoperatively. The rats were anesthetized using 1% isoflurane. An S12 probe was placed on the chest at the parasternal short axis, and connected to the machine (GE Vivid-i; GE Healthcare, IL, USA). The M-mode echocardiogram was recorded at the papillary muscle, and the percentage of LV ejection fraction (%LVEF) was determined [16].

At 12 weeks postoperatively, an invasive LV function test was carried out using pressure-volume loop analysis. All rats were anesthetized using a combination of 50 mg/kg Zoletil (Virbac) and 3 mg/kg Xylazine (LBS Labs) via intramuscular injection. In each case the right carotid artery was identified, and a pressure-volume admittance catheter (1.9F VSL catheter; Transonic, NY, USA) was inserted through the right carotid artery and advanced into the LV. Then, the pressure-volume catheter was connected to the pressure-volume admittance system (ADV500; Transonic), and the signals were recorded via a Labscribe 2 program (iWorx, NH, USA). LV function including heart rate (HR), end-systolic and diastolic pressure (ESP, EDP), $\pm dp/dt$, end-systolic and diastolic volume (ESV, EDV), and stroke volume (SV) were determined [18].

Cardiac Mitochondrial Function

The heart tissue was minced and homogenized in an ice-cold buffer. Cardiac mitochondria were obtained using a differential centrifugation technique as previously described [19]. For cardiac mitochondrial ROS level determination, 0.4 mg/mL of cardiac mi-

tochondrial protein was incubated with 2 μ M DCFH-DA dye at 25 °C for 20 min. The fluorescence intensity of DCF was detected at λ_{ex} 485 nm and λ_{em} 530 nm using a fluorescence microplate reader (BioTek, VT, USA). Increased DCF fluorescence intensity indicates increased cardiac mitochondrial ROS levels. For cardiac mitochondrial membrane potential (MMP) changes, 0.4 mg/mL of cardiac mitochondrial protein was incubated with 5 μ M JC-1 dye. JC-1 is a ratiometric dye that exhibits potential-dependent accumulation in mitochondria. The red fluorescence intensity of JC-1 (J-aggregates) was detected at λ_{ex} 485 nm and λ_{em} 590 nm, and it indicates an intact MMP or healthy mitochondria. The green fluorescence intensity of JC-1 (J-monomers) was detected at λ_{ex} 485 nm and λ_{em} 530 nm, and it indicates low MMP or unhealthy mitochondria. Both red and green fluorescence intensity were measured by a fluorescence microplate reader (BioTek). Therefore, a decreased red/green fluorescence intensity ratio indicates mitochondrial membrane depolarization [18].

For cardiac mitochondrial function, cardiac mitochondrial respiration was assessed using a high-throughput automated 96-well extracellular flux analyzer (XFe96; Agilent Seahorse, CA, USA); 0.5 mg/mL of cardiac mitochondrial protein was used, and mitochondria were suspended in a medium buffer containing 100 mM KCl, 10 mM HEPES, 5 mM KH_2PO_4 , and pH 7.2 in the presence of 0.2% fatty acid-free BSA. The XFe96 plate was coated with polyethylenimine (1:15,000 dilution) and incubated overnight at 37 °C, and the polyethylenimine was removed on the day of the assay; 20 μ L of 0.5 mg/mL of isolated cardiac mitochondria was loaded into the XFe96 plate, and mitochondria were centrifuged at 3,000 g for 7 min at 4 °C. Then, the mitochondria were incubated for 30 min at 37 °C before starting the assay. First, pyruvate/malate (final concentration: 5 mM each) was added as NADH-linked substrate, followed by ADP (final concentration: 1 mM) administration to determine mitochondrial state 3 respiration. State 4 respiration was measured after adding oligomycin (final concentration: 1 μ M) [20, 21].

Plasma and Cardiac Malondialdehyde Determination

In this study, cardiac tissue homogenates were used to determine cardiac malondialdehyde (MDA) levels as an indicator for cardiac oxidative stress. Plasma was used to determine plasma MDA levels as an indicator for circulating oxidative stress. These samples were mixed with 0.6% thiobarbituric acid (TBA) and 0.44 M H_3PO_4 , and then heated at 90 °C for 30 min to become pink-colored products called thiobarbituric acid reactive substances (TBARS). Plasma and cardiac TBARS concentration were determined directly from the standard curves using the HPLC system, and reported as MDA equivalent concentration. Plasma MDA concentration was expressed in μ M, and cardiac MDA concentration was expressed in μ M/mg protein [4, 19].

Western Blot Analysis

Sixty micrograms of the total protein were mixed with a loading buffer as previously described [16]. The protein was loaded onto 10 or 12.5% SDS-acrylamide gels, separated by electrophoresis, and then transferred onto 45- μ m nitrocellulose membrane (GE healthcare, Buckinghamshire, UK) in a wet-tank blotting system (Bio-Rad, CA, USA). The membranes were blocked in 5% skim milk or 5% BSA in TBST.

For mitochondrial dynamic and respiratory chain complex determination, membranes were incubated with primary antibodies

Fig. 1. Effects of testosterone deprivation on peripheral metabolic parameters ($n = 8/\text{group}$). **a** Plasma testosterone levels. **b** Daily food intake. **c** Body weight. **d** Visceral fat weight. * $p < 0.05$ versus NDS; † $p < 0.05$ versus NDO; ‡ $p < 0.05$ versus HFS. NDS, normal diet-fed rats and sham operation; NDO, normal diet-fed rats with orchiectomy; HFS, high-fat diet-fed rats with sham operation; HFO, high-fat diet-fed rats with orchiectomy.

including PGC1 α (1:200 dilution; Santa Cruz), CPT1 (1:1,000 dilution; Santa Cruz), OXPHOS (1:1,000 dilution; Abcam), p-Drp1^{ser616} (1:1,000 dilution), Drp1 (1:1,000 dilution), Mfn2 (1:1,000 dilution), and OPA1 (1:1,000 dilution; all Cell Signaling).

For apoptosis determination, membranes were incubated with primary antibodies including Bax (1:200 dilution; Santa Cruz), Bcl2 (1:1,000 dilution; Abcam), cleaved caspase 3 (1:1,000 dilution; Cell Signaling) and caspase 3 (1:1,000 dilution; Cell Signaling).

For myocardial insulin signaling determination, membranes were incubated with primary antibodies including p-IR^{tyr1162/1163} (1:200 dilution; Santa Cruz), IR (1:200 dilution; Santa Cruz), p-Akt^{ser473} (1:1,000 dilution; Cell Signaling) and Akt (1:1,000 dilution; Cell Signaling).

After incubation with primary antibodies at 4 °C overnight, membranes were transferred to secondary antibodies. They were then exposed to an enhanced chemiluminescent substrate (Clarity Western ECL Substrate; Bio-Rad). The Western blot images and densitometric analysis were carried out using the ChemiDoc Imaging system with Image Lab software (Bio-Rad) [16, 18].

Statistical Analysis

Data are presented as mean $\pm$ SE. All statistical analyses were performed using GraphPad 6.0. For time course analysis, data were

analyzed using a two-way ANOVA followed by a Tukey post hoc test. For single time point analysis, data were analyzed using a one-way ANOVA followed by a Tukey post hoc test. $p < 0.05$ was considered statistically significant.

Results

Testosterone Deprivation Did Not Aggravate Metabolic Impairment in Obese Insulin-Resistant Rats

At baseline there was no difference in body weight, food intake, plasma glucose, insulin, HOMA index, lipid profiles, and plasma testosterone levels between ND- and HFD-fed rats (Fig. 1b, c; online suppl. Fig. 2, 3). Plasma testosterone levels were increased in both the ND and HFD groups at week 12 (Fig. 2a).

After ORX, plasma testosterone levels were decreased in both NDO and HFO rats, compared with NDS and HFS groups (Fig. 1a). Body weight and food intake were

Fig. 2. Effects of testosterone deprivation on blood pressure, cardiac autonomic balance, and LV function in normal and obese insulin-resistant rats ($n = 8/\text{group}$). **a** SBP. **b** DBP. **c** LF/HF ratio. **d** %LVEF. * $p < 0.05$ versus NDS; † $p < 0.05$ versus NDO; ‡ $p < 0.05$ versus HFS. NDS, normal diet-fed rats and sham operation; NDO, normal diet-fed rats with orchietomy; HFS, high-fat diet-fed rats with sham operation; HFO, high-fat diet-fed rats with orchietomy; SBP, systolic blood pressure; DBP, diastolic blood pressure; LF/HF ratio: low-frequency/high-frequency ratio of heart rate variability; %LVEF, percentage of left-ventricular ejection fraction.

reduced in both NDO and HFO groups, compared with NDS and HFS groups (Fig. 1b, c). Visceral fat weight was determined at 12 weeks post-ORX, and our data showed that it was reduced in both NDO and HFO groups, compared with NDS and HFS groups (Fig. 1d).

All rats fed on HFD for 12 weeks developed obesity-related insulin resistance. At 1, 2, 4, 8, and 12 weeks after ORX, all metabolic parameters including plasma glucose, insulin levels, HOMA-IR, and lipid profiles showed no difference from baseline in NDO and HFO groups, compared with NDS and HFS groups (see online suppl. Fig. 2, 3), suggesting that testosterone deprivation did not alter peripheral insulin resistance in both ND- and HFD-fed rats.

Testosterone Deprivation Aggravated LV Dysfunction in Obese Insulin-Resistant Rats

At baseline, SBP, DBP, LF/HF ratio, and %LVEF were no different between groups. After 12 weeks of HFD feeding, LF/HF ratio was increased and %LVEF was decreased in both HFS and HFO groups, compared with NDS and NDO groups (Fig. 2a–d). However, there was no difference in SBP and DBP between ND- and HFD-fed rats (Fig. 2a–d).

After ORX, in the NDO group, SBP was increased at 12 weeks post-ORX, LF/HF ratio was increased at 8 and 12 weeks post-ORX, and %LVEF was reduced at 8 and 12 weeks post-ORX, compared with the NDS group (Fig. 2a–d). In the HFO group, SBP was markedly increased at 4, 8, and 12 weeks post-ORX, LF/HF ratio was

Table 1. Effects of testosterone deprivation on invasive left-ventricular function parameters in ND- and HFD-fed rats

Parameters	NDS	NDO	HFS	HFO
HR, bpm	331±48	344±35	450±27*, †	448±32*, †
ESP, mm Hg	130±5	115±3*	112±7*, †	115±10*, †
EDP, mm Hg	6±2	5±2	14±3*, †	21±5*, †, ‡
+dP/dt, mm Hg/s	8,832±368	8,732±412	6,131±212*, †	6,014±107*, †
-dP/dt, mm Hg/s	-5,307±213	-5,136±347	3,341±475*, †	2,987±351*, †, ‡
ESV, µL	20±2	18±4	24±7*, †	25±6*, †
EDV, µL	322±35	335±41	411±31*, †	432±32*, †, ‡
SV, µL/g	0.5±0.04	0.6±0.02	0.32±0.05*, †	0.35±0.06*, †
%LVEF	96.8±1.4	92.1±0.4*	87±2.35*, †	78±2.25*, †, ‡

ND, normal diet; HFD, high-fat diet; NDS, normal diet-fed rats and sham operation; NDO, normal diet-fed rats with orchietomy; HFS, high-fat diet-fed rats with sham operation; HFO, high-fat diet-fed rats with orchietomy; HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; ESV, end-systolic volume; EDV, end-diastolic volume; SV, stroke volume; %LVEF, percentage of left-ventricular ejection fraction. *p < 0.05 versus NDS; †p < 0.05 versus NDO; ‡p < 0.05 versus HFS.

increased at 4, 8, and 12 weeks post-ORX, and %LVEF was reduced at 8 and 12 weeks post-ORX, compared with the HFS group (Fig. 2a–d).

In addition, LV function was determined invasively at 12 weeks post-ORX. In the NDO group, ESP and %LVEF were lower than in the NDS group, indicating LV dysfunction (Table 1). In the HFS group, HR, EDP, ESV, and EDV were increased, and ESP, $\pm$ dP/dt, SV, and %LVEF were decreased, compared with the NDS group, again indicating LV dysfunction (Table 1). In the HFO group, EDP and EDV were markedly increased, and -dP/dt and %LVEF were markedly decreased, compared with the HFS group (Table 1). These data suggested that testosterone deprivation aggravated LV dysfunction in HFD-fed rats.

Testosterone Deprivation Aggravated Cardiac Mitochondrial Dysfunction in Obese Insulin-Resistant Rats

Our data showed that cardiac mitochondrial ROS levels were higher in the NDO group than in the NDS group, whereas MMP changes and mitochondrial swelling were no different between these two groups (Fig. 3a–c). In the HFS group, mitochondrial ROS levels and mitochondrial swelling were increased, and MMP changes were decreased, compared with the NDS group (Fig. 3a–c). In the HFO group, mitochondrial ROS and mitochondrial swelling were markedly increased, and MMP changes were markedly decreased, compared with the HFS group

(Fig. 3a–c). Representative images of cardiac mitochondria morphology of rats in the NDS, NDO, HFS, and HFO groups are shown in Figure 3e. In addition, our data demonstrated that state 3 respiration was decreased in NDO rats, compared with NDS rats. In HFD groups, HFS and HFO rats had lower state 3 respiration than NDS rats, and HFO rats had the lowest state 3 respiration, compared with NDS, NDO, and HFS rats (Fig. 3d). State 4 respiration was decreased in both HFS and HFO rats, compared with NDS and NDO rats. However, state 4 respiration was not different between HFS and HFO rats. These data suggested that testosterone deprivation aggravated cardiac mitochondrial dysfunction by decreasing ADP-stimulated mitochondrial respiration in obese insulin-resistant rats.

Testosterone Deprivation Aggravated Circulating and Cardiac Oxidative Stress in Obese Insulin-Resistant Rats

When considering the results from the ND-fed rats, plasma and cardiac MDA were increased in NDO rats, compared with these parameters in the NDS rats (see online suppl. Fig. 5a, b). In HFD-fed rats, our data showed that plasma and cardiac MDA were higher in HFS rats than in both NDS and NDO rats, and also that these parameters were markedly increased in HFO rats, compared with HFS rats (Fig. 4a, b), suggesting that testosterone deprivation aggravated circulating and cardiac oxidative stress in both ND- and HFD-fed rats.

Fig. 3. Effects of testosterone deprivation on cardiac mitochondrial function in normal and obese insulin-resistant rats ($n = 8$ /group). **a** ROS levels. **b** MMP changes. **c** Swelling. **d** Cardiac mitochondrial oxygen consumption rate. **e** Representative images of cardiac mitochondria. * $p < 0.05$ versus NDS; † $p < 0.05$ versus

NDO; ‡ $p < 0.05$ versus HFS. NDS, normal diet-fed rats and sham operation; NDO, normal diet-fed rats with orchietomy; HFS, high-fat diet-fed rats with sham operation; HFO, high-fat diet-fed rats with orchietomy; ROS, reactive oxygen species; MMP, mitochondrial membrane potential.

Testosterone Deprivation Aggravated the Reduction of Mitochondrial Respiratory Complex and Mitochondrial Fusion Proteins, and Aggravated Cardiac Apoptosis, but Did Not Alter Myocardial Insulin Receptor Function in Obese Insulin-Resistant Rats

In ND-fed rats, PGC1 α , CPT1, mitochondrial respiratory complex I–V, p-Drp1^{ser616}/Drp1, Mfn2, and OPA1 protein levels were no different between NDS and NDO groups (Fig. 4a–d; online suppl. Fig. 5a, b). In the HFS group, CPT1, mitochondrial respiratory complex I, III, and V, Mfn2, and OPA1 protein levels were decreased, compared with the NDS and NDO groups (Fig. 4b–d; online suppl. Fig. 5b). In the HFO group, PGC1 α , mitochondrial respiratory complex I and III, Mfn2, and OPA1 pro-

tein levels were markedly decreased, compared with the HFS group (Fig. 4b–c; online suppl. Fig. 5a). However, there was no difference in CPT1 and complex V protein levels between the HFS and HFO groups (Fig. 4c; online suppl. Fig. 5b). Moreover, p-Drp1^{ser616}/Drp1 protein levels were not different among groups (Fig. 4a).

As regards cardiac apoptosis in ND-fed rats, there was no difference in Bax, Bcl2, and cleaved caspase 3/caspase 3 protein levels between NDS and NDO groups (see online suppl. Fig. 5c, d). However, HFS and HFO groups had decreased Bcl2 protein levels, whereas Bax and cleaved caspase 3/caspase 3 protein levels were not different in the NDS, NDO, and HFS groups (see online suppl. Fig. 5c, d). In the HFO group, cleaved caspase 3/caspase 3 protein levels were increased when compared with the

Fig. 4. Effects of testosterone deprivation on mitochondrial dynamics and respiratory complex in normal and obese insulin-resistant rats ($n = 8/\text{group}$). **a** p-Drp1^{S616}/Drp1. **b** Mfn2 protein levels. **c** OPA1 protein levels. **d** Mitochondrial respiratory complex I–V protein levels. * $p < 0.05$ versus NDS; † $p < 0.05$ versus NDO;

‡ $p < 0.05$ versus HFS. NDS, normal diet-fed rats with sham operation; NDO, normal diet-fed rats with orchietomy; HFS, high-fat diet-fed rats with sham operation; HFO, high-fat diet-fed rats with orchietomy; Drp1, dynamic-related protein1; Mfn2, mitofusin2; OPA1, optic atrophy1.

HFS group (see online suppl. Fig. 5e). However, there was no difference in the Bax and Bcl2 proteins levels between HFS and HFO groups (see online suppl. Fig. 5c, d).

Myocardial insulin signaling, in ND-fed rats, p-IR^{tyr1162/1163}/IR and p-Akt^{ser473}/Akt were no different between NDS and NDO groups (see online suppl. Fig. 6). In HFD-fed rats, p-IR^{tyr1162/1163}/IR and p-Akt^{ser473}/Akt were decreased in both HFS and HFO groups, compared with NDS and NDO groups (see online suppl. Fig. 6). However, p-IR^{tyr1162/1163}/IR and p-Akt^{ser473}/Akt were not different between HFS and HFO groups (see online suppl. Fig. 6).

Discussion

Our study demonstrated the following: (i) in obese insulin-resistant rats testosterone deprivation reduced body weight, did not aggravate peripheral insulin response, but caused a decrease in myocardial insulin signaling; (ii) in obese insulin-resistant rats testosterone deprivation aggravated LV dysfunction and as there was, as a result, increased cardiac sympathovagal imbalance, increased systolic blood pressure, deterioration in cardiac mitochondrial dysfunction, reduced mitochondrial respiratory complex I and III protein levels, reduced mito-

chondrial fusion protein levels, and increased myocardial apoptosis; and (iii) in normal rats, testosterone deprivation caused cardiac sympathovagal imbalance and LV dysfunction via increased oxidative stress.

In this study, testosterone deprivation led to a reduction in body weight due to reduced food intake in both normal and obese insulin-resistant rats. Previous studies have demonstrated that circulating adiponectin levels were increased in both ND- and HFD-fed rodents with testosterone deprivation [22, 23]. Adiponectin is known to promote weight loss by increasing energy expenditure [24] and reducing appetite [25]. Although body weight was reduced following testosterone deprivation, the peripheral insulin-resistant condition was not affected in both normal and obese insulin-resistant rats. In addition, testosterone deprivation did not aggravate the impairment of myocardial insulin receptor function in these obese insulin-resistant rats. Our findings are inconsistent with a previous study by Donner et al. [12] which showed that testosterone deprivation, after the onset of obesity-related insulin resistance, aggravated the insulin-resistant condition in male rats. The discrepancy could be due to the difference in the diet used in that study to create the obese insulin-resistant model, and the duration of testosterone deprivation. In that study, they used a high-fat and high-sucrose (HFD/HS) diet to induce obesity-related insulin resistance, and metabolic parameters were determined at 20 weeks post-ORX [12] whilst we used HFD to induce obesity-related insulin resistance, and metabolic parameters were determined at 12 weeks post-ORX.

We previously demonstrated that testosterone deprivation in very young rats accelerated but did not aggravate LV dysfunction in obese insulin-resistant rats [4]. In this study, we found that testosterone deprivation during adulthood aggravated LV dysfunction and cardiac sympathovagal imbalance in obese insulin-resistant rats. Therefore, our data suggested that an alteration in LV function in obese insulin-resistant rats with testosterone deprivation depends upon the age at which testosterone deprivation occurs.

In this study, the potential mechanism responsible for the deleterious effects on LV function was determined. In normal rats subjected to testosterone deprivation, our data showed that systemic and cardiac oxidative stress levels were increased, together with an increase in cardiac mitochondrial ROS level when compared with normal rats without testosterone deprivation. However, cardiac MMP and mitochondrial swelling were within normal limits. Excessive levels of systemic and mitochondrial oxidative stress were found to stimulate central and periph-

eral sympathetic activity [26–28]. This abnormal activity was followed by cardiac autonomic imbalance, contributing to LV dysfunction [29] (see online suppl. Fig. 7).

Cardiac mitochondrial membrane depolarization and cardiac mitochondrial swelling were observed in obese insulin-resistant rats, but not in normal rats with testosterone deprivation, suggesting more severe cardiac mitochondrial dysfunction and mitochondrial damage. It is interesting that testosterone deprivation led to increased mitochondrial ROS levels, and aggravated cardiac mitochondrial membrane depolarization and swelling in obese insulin-resistant rats. These data suggested that testosterone deprivation aggravated cardiac mitochondrial damage in obese insulin-resistant rats.

After mitochondrial damage, the adaptation of mitochondrial dynamics is usually activated by increasing mitochondrial fission and fusion proteins [30, 31]. Mitochondrial fission helps to remove damaged mitochondria via the mitophagy mechanism [32]. In addition, the process of mitochondrial fusion enhances mitochondrial oxidative phosphorylation by upregulating activity of the mitochondrial respiratory complex [32]. However, in obese insulin-resistant rats with testosterone deprivation, mitochondrial fusion protein was reduced, as indicated by reduced Mfn2 and OPA1 proteins, whereas mitochondrial fission protein was not altered, when compared with their sham operation group. A reduction in Mfn2 protein results in reduced outer mitochondrial membrane fusion [33]. Previous studies demonstrated that silencing the Mfn2 increased sensitivity to apoptotic stimuli, and accelerated cytochrome c release [33]. Thus, our data suggested that testosterone deprivation reduced Mfn2 protein levels, which may lead to reduced outer mitochondrial membrane fusion, and promoted cardiac apoptosis as shown in these testosterone-deprived obese insulin-resistant rats (Fig. 4).

OPA1 is a marker for inner mitochondrial membrane fusion [34], which is responsible for maintaining mitochondrial cristae shape [34], and cristae are the critical site for the oxidative phosphorylation process [35]. Therefore, a reduction in OPA1 directly affects mitochondrial respiration via a reduction in the oxidative phosphorylation process [36]. In the obese insulin-resistant rats with testosterone deprivation, OPA1 protein levels were reduced along with a reduction in mitochondrial respiratory complexes I and III. Thus, our results suggest that testosterone deprivation reduced OPA1 protein, which possibly leads to reduced inner mitochondrial membrane fusion. Furthermore, we also found that testosterone deprivation reduced PGC1 α protein expression

in obese insulin-resistant rats. As PGC1 α mediates mitochondrial biogenesis and the oxidative phosphorylation process [35–38], these findings suggested that testosterone deprivation could reduce mitochondrial biogenesis and oxidative phosphorylation in obese insulin-resistant rats.

Although testosterone deprivation did not affect mitochondrial OXPHOS protein levels in normal rats, it decreased ADP-stimulated cardiac mitochondrial oxygen consumption. These data suggested that in normal rats, cardiac mitochondrial dysfunction occurred following testosterone deprivation, and preceded the reduction of OXPHOS protein levels. In obese insulin-resistant rats, testosterone deprivation aggravated cardiac mitochondrial dysfunction since it markedly decreased ADP-stimulated cardiac mitochondrial oxygen consumption and reduced complex I and III protein levels. However, testosterone deprivation did not affect state 4 respiration. Our data also showed that testosterone deprivation did not alter complex V protein levels in both normal rats and obese insulin-resistant rats.

In conclusion, our findings demonstrated that testosterone deprivation aggravated LV dysfunction in obese insulin-resistant rats by exacerbating cardiac mitochondrial dysfunction and decreasing mitochondrial fusion proteins, reducing mitochondrial oxidative phosphorylation proteins, and increasing apoptosis. However, these adverse cardiac effects are independent of the status of both peripheral and myocardial insulin signaling.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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