

Testosterone replacement attenuates mitochondrial damage in a rat model of myocardial infarction

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Abstract

Testosterone can affect cardiovascular disease, but its effects on mitochondrial dynamics in the post-infarct myocardium remain unclear. To observe the effects of testosterone replacement, a rat model of castration-myocardial infarction (MI) was established by ligating the left anterior descending coronary artery 2 weeks after castration with or without testosterone treatment. Expression of mitochondrial fission and fusion proteins was detected by western blot and immunofluorescence 14 days after MI. Cardiac function, myocardial inflammatory infiltration and fibrosis, cardiomyocyte apoptosis, mitochondrial microstructure, and ATP levels were also assessed. Compared with MI rats, castrated rats showed aggravated mitochondrial and myocardial insults, including mitochondrial swelling and disordered arrangement; loss of cristae, reduced mitochondrial length; decreased ATP levels; cardiomyocyte apoptosis; and impaired cardiac function. Results of western blotting analyses indicated that castration downregulated peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1A) and mitofusin 2, but upregulated dynamin-related protein 1. The results were also supported by results obtained using immunofluorescence. However, these detrimental effects were reversed by testosterone supplementation, which also elevated the upstream AMP-activated protein kinase (AMPK) activation of PGC1A. Thus, testosterone can protect mitochondria in the post-infarct myocardium, partly via the AMPK–PGC1A pathway, thereby decreasing mitochondrial dysfunction and cardiomyocyte apoptosis. The effects of testosterone were confirmed by the results of ELISA analyses.

Key Words

- ▶ testosterone
- ▶ mitochondrial dynamics
- ▶ AMPK
- ▶ PGC1A
- ▶ ATP
- ▶ myocardial infarction

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Introduction

Testosterone, a steroid hormone, affects the cardiovascular system. Results of epidemiological studies have indicated that testosterone deficiency is highly prevalent in men with cardiovascular disease (CVD) and is negatively associated with cardiovascular risk factors (Kelly & Jones 2014).

Reports of some studies described an increase in overall and cardiovascular-related mortality in men with low testosterone levels (Khaw *et al.* 2007, Laughlin *et al.* 2008, Ponikowska *et al.* 2010). Moreover, according to the results of some trials, low testosterone levels should be recognized

as an independent risk factor for CVD (Jones & Saad 2009, Jones 2010). These findings implicate testosterone as a potential therapeutic agent for CVD.

Testosterone replacement therapy has been found to significantly reduce mortality in men with low testosterone (Shores *et al.* 2012, Muraleedharan *et al.* 2013). Previous meta-analyses have not revealed testosterone replacement to have any adverse cardiovascular events, including mortality (Haddad *et al.* 2007, Fernandez-Balsells *et al.* 2010, Carson & Rosano 2012). Results of a recent meta-analysis (Xu *et al.* 2013) that included the Testosterone in Older Men (TOM) trial (Basaria *et al.* 2010) indicated that exogenous testosterone increased the risk of cardiovascular-related events. However, the results of a more recent meta-analysis indicated that testosterone supplementation wasn't related to any increase in cardiovascular risk, supporting the hypothesis that there isn't a causal link between testosterone supplementation and adverse cardiovascular events (Corona *et al.* 2014). Although much work has been reported recently in this field, the potential of testosterone replacement therapy to improve outcomes is still an uncertain matter. Given the widespread application of testosterone, and the confusion regarding the role of testosterone therapy in the treatment of CVD, continuing to study the effects of testosterone on CVD is important.

Mitochondria dysfunction has been identified in both the human and rodent post-infarct myocardium (Hori & Nishida 2009, Sena *et al.* 2009, Heather *et al.* 2010). Mitochondria are dynamic organelles that are characterized by mitochondrial fusion and fission, and the process of fusion and fission to generate an elongated interconnected mitochondrial network or a fragmented discrete phenotype is called mitochondrial dynamics. Mitochondrial fusion is driven by mitofusin 1, 2 (MFN1, 2) and optic atrophy 1, which are located in the mitochondrial outer and inner membrane respectively. The fission proteins include dynamin-related protein 1 (DRP1) and mitochondrial fission protein 1, which are located in the cytosol and mitochondrial outer membrane respectively. Recently, the field of mitochondrial dynamics has attracted increasing attention (Chen *et al.* 2009, Ong & Hausenloy 2010), and an imbalance between mitochondrial fusion and fission can lead to impaired mitochondrial structural and function.

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1A) can modulate the balance in mitochondrial networks by regulating the expression of dynamic proteins (Garnier *et al.* 2005). AMP-activated protein kinase (AMPK) can be activated in many situations, such as ischemia, pressure overload, and heart

failure, and its activation can upregulate the expression of PGC1A (Rowe *et al.* 2010, Zaha & Young 2012). Both decreased PGC1A and adverse mitochondrial network dynamics (reduced fusion and increased fission) have been found in post-infarct myocardium (Jiang *et al.* 2014).

Testosterone replacement therapy has been shown to modulate the cardiac B-adrenergic system, reduce wall stress and left ventricular end-diastolic pressure, promote angiogenesis, and improve cardiac function following myocardial infarction (MI; Nahrendorf *et al.* 2003, Sun *et al.* 2011, Chen *et al.* 2012). However, experiments investigating how testosterone affects mitochondrial network proteins and functions post-MI remain scarce. Therefore, this experiment was conducted to assess the effects of testosterone replacement therapy on mitochondrial damage in the post-infarct ischemic myocardium.

Materials and methods

Animals

All of the experimental procedures were performed in accordance with the National Research Council's protocol for the care and use of laboratory animals. Male Wistar rats ($n=100$, weight 200–250 g) were purchased from the Laboratory Animal Center of Harbin Medical University (Harbin, China). The rats were housed in the temperature-controlled Laboratory Animal Center (22–24 °C) under circadian conditions with free access to standard chow and tap water.

Castration and testosterone replacement

Male rats randomly underwent castration (Cas) or sham castration (S-Cas) operations. The rats were anesthetized with i.p. chloral hydrate injections (3 ml/kg). While in the supine position, the bilateral testes were either removed or left intact. The rats were subsequently divided into four groups: i) S-Cas+placebo (S-Cas), ii) Cas+placebo (Cas), iii) Cas+testosterone (Cas+T), and iv) Cas+T+flutamide (Cas+T+F). The various interventions were administered according to these groupings on the same day as the surgery. Testosterone propionate (Tianjin Jinyao Amino Acid Co., Ltd., Tianjin, China) was injected subcutaneously at a physiological dose of 2 mg/kg per day. Flutamide (Sigma Chemical Co.) dissolved in propylene glycol was administered at a dose of 30 mg/kg per day. Peanut oil, used as a placebo, was injected similarly at the same dosage as testosterone.

MI model

Two weeks later, the rats were anesthetized with i.p. injections of chloral hydrate (3 ml/kg). Mechanical positive pressure ventilation with a frequency of 60–65/min was accomplished with a ventilator. The operation was conducted by ligating the left coronary artery with a 3/8 needle and 5.0 sutures. The model's success was confirmed by typical ST-elevation and QRS cluster broadening on the electrocardiogram (ECG). At 14 days after ligation, the rats were killed (six to eight from each group). For control groups, we selected rats that received no operation and underwent the same procedure. The above experimental process was based on a protocol described previously (Chen *et al.* 2012).

Echocardiography and, blood and myocardium collection

Echocardiography was performed under anesthesia at 14 days after ligation. The rats were placed in the proper posture after the thoracic walls were shaved clean. Two-dimensional and M-mode echocardiography was used to assess the morphology and function of the heart using an 8-MHz transducer connected to the Siemens Sequoia 512 echocardiographic system. The images used for measurements were those obtained from both the parasternal long- and short-axis views. The left ventricular end-diastolic and systolic diameters (LVDD and LVSD) were recorded. Fractional shortening (FS) and ejection fraction (EF) were calculated using the following formulas: $FS = (LVDD - LVSD) / LVDD \times 100\%$ and $EF = (LVDD^3 - LVSD^3) / LVDD^3 \times 100\%$. All parameters were based on the mean values of three cardiac cycles.

Blood samples were collected by cardiac puncture. The rats underwent thoracotomy immediately after killing. The connection between the heart and great vessels was cut; the heart was irrigated clean with cold saline; and the atria, great vessels, and valves were removed. Part of the myocardium was fixed in 2.5% glutaraldehyde and 4% paraformaldehyde for 24 h, while the remaining was stored in a refrigerator at -80°C after rapid freezing by liquid nitrogen.

Hematoxylin and eosin staining

The left ventricular myocardial tissue was embedded in paraffin and cut into 5 μm sections. Hematoxylin and eosin (H&E) staining was performed following the usual five-step protocol: deparaffinization, rehydration, staining, dehydration, and sealing in resin. Four sections were randomly selected from three rats in each group for the observation of myocardial inflammatory infiltration and fibrosis.

TUNEL assay

The standard assay has been described previously (Chen *et al.* 2012). Staining was performed according to the instructions in the *In Situ* Cell Death Detection Kit, POD (Roche). Positive cells were counted in at least five randomly selected fields under $400\times$ using a microscope.

Transmission electron microscopy

The left ventricular myocardium was cut into 1 mm³ cubes, treated with 2.5% glutaraldehyde for 24 h at 4 °C and immersed in 1% osmium tetroxide. It was then dehydrated through graded ethanol and embedded in paraffin. The specimens were cut into 90 nm ultrathin sections and then double-stained with uranyl acetate and lead citrate. Six magnification fields were randomly selected from three rats in each group for examination. The microstructures of mitochondria were photographed using a transmission electron microscope (JEOL, JEM 1210) at 80 or 60 KV onto electron microscope film (Kodak, ESTAR thick base). Mitochondrial cristae were identified manually from high-resolution, high-magnification images, and the area of mitochondria was calculated using an optical pen in Image J to calculate cristae number. The mitochondrial length was also measured.

Western blotting analysis

A standard western blotting assay was used to analyze protein expression. Myocardial tissue was ground with protein lysate every 5 min, and after six times, it was centrifuged for 10 min at 12 000 g. The supernatant was extracted, diluted with 5 \times buffer, and heated for 10 min at 95 °C. The samples were separated onto 8–10% SDS-polyacrylamide gels and then transferred to a PVDF membrane. The membranes were blocked with 5% nonfat milk buffer for 1 h at room temperature and subsequently incubated with anti-pAMPK (1:1000; Cell Signaling, Boston, MA, USA), anti-AMPKA (1:1000; Cell Signaling), anti-PGC1A, anti-MFN2, anti-DRP1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-GAPDH (1:10 000; ZhongShan, Beijing, China) antibodies overnight at 4 °C. The membranes were then incubated with the corresponding secondary antibodies for 1 h at room temperature. The blots were imaged with ECL reagent, and the intensity was quantified with Image J Software (National Institutes of Health, Bethesda, MD, USA). All experiments were done three times.

Immunofluorescence

The hearts were removed after thoracotomy, irrigated clean with cold saline, and then made into frozen sections immediately (8 μ m). The frozen tissue sections were fixed in acetone at 4 °C for 15 min and blocked with horse serum for 1 h. Anti-MFN2 (1:25; Santa Cruz Biotechnology) and DRP1 (1:25; Santa Cruz Biotechnology) primary antibodies incubated overnight at 4 °C. Following washing with PBS solution, the sections were incubated with secondary antibodies (1:200; Life Technologies) for 1 h. Finally, the slides were mounted with 4',6-diamidino-2-phenylindole (DAPI) and visualized under an epifluorescence microscope (Olympus). Three sections were randomly selected from three rats in each group for examination.

HPLC

The myocardial cell suspensions were placed in 1.5 ml tubes with 0.4 mol/l HClO₄ at 4 °C and set aside for 10 min before centrifugation for 10 min at 3000 g. The pH of the supernatant was neutralized to 6.5 by adding 0.5 mol/l KOH, and the standing and centrifugation steps were repeated. The levels of ATP in the final supernatant were quantified by HPLC.

ELISA

The blood samples were collected in red vacuum blood collection tubes and allowed to coagulate for 2 h at room temperature before centrifugation for 20 min at approximately 1000 g to obtain serum. ELISA Kits (Uscn Life Science, Inc., Wuhan, China) were used to detect serum testosterone and estradiol (E₂) levels. All steps complied with the manual. The detection range was 1.235–10 ng/ml for testosterone and 12.35–1000 pg/ml for E₂.

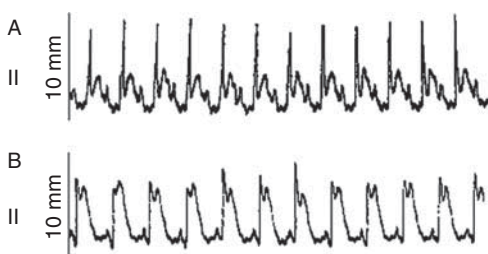


Figure 1
Representative ECG. (A) Normal ECG was recorded before operation. (B) Typical ST-elevation and widening of the QRS cluster was recorded after the left coronary artery had been ligated.

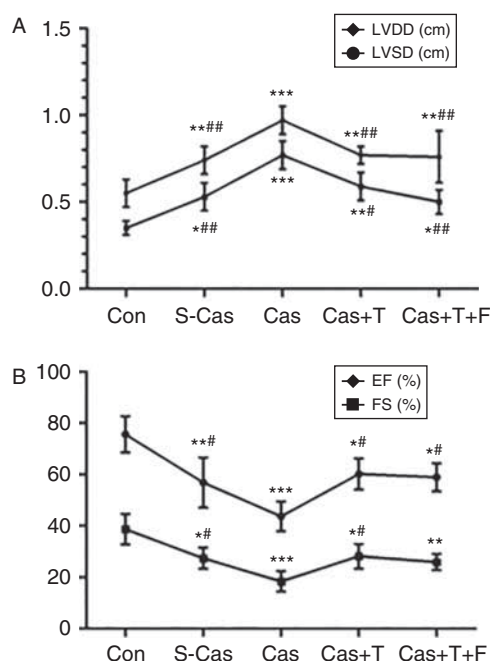


Figure 2
Echocardiography results 14 days after ligation or sham operation. (A) LVDD and LVSD results for all groups. (B) EF and FS measurements for all groups. Values are expressed as means \pm s.d., $n=3$. Con, control; S-Cas, sham-castration; Cas+T, castration + testosterone; Cas+T+F, castration + T + flutamide. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus Con and # $P<0.05$, ## $P<0.01$ versus Cas.

Statistical analysis

Results were expressed as mean \pm s.d. Statistical significance was evaluated by one-way ANOVA. A P value of 0.05 was considered to be statistically significant. Statistical analysis was carried out using SPSS 20.0 Software.

Results

Electrocardiography

ECG was performed to evaluate the MI model. Figure 1A shows a normal ECG. As shown in Fig. 1B, the MI model was confirmed by typical ST-elevation and the widening of the QRS cluster.

Effects of testosterone on cardiac function

Echocardiography was conducted to evaluate cardiac function 14 days after the ligation or sham operation (Fig. 2). Figure 2A shows LVDD and LVSD measurements for all groups. EF and FS are shown in Fig. 2B. Compared with the control group, MI significantly decreased the EF

($P < 0.01$) and FS ($P < 0.05$) and increased the LVDD ($P < 0.01$) and LVSD ($P < 0.05$). Compared with sham castrated rats, castrated rats had significantly dilated left ventricles (Cas versus S-Cas, $P < 0.01$) and greater systolic dysfunction (Cas versus S-Cas, $P < 0.05$). Testosterone replacement ameliorated these deficits (Cas+T versus Cas: LVDD, $P < 0.01$; LVSD, EF, FS, $P < 0.05$). Flutamide had no effect on myocardial performance.

Myocardial inflammatory infiltration and fibrosis

H&E staining was performed 14 days after surgery to examine myocardial inflammatory infiltration and fibrosis (Fig. 3). The rats with MI had obvious inflammatory infiltration and fibrosis. Inflammation and fibrosis was more pronounced in the Cas group than the S-Cas group, and testosterone replacement alleviated these effects of castration.

Effects of testosterone on cardiac cell apoptosis

TUNEL staining was used to assess apoptotic cell death in all five groups 14 days after MI. TUNEL-positive nuclei were observed in all groups (Fig. 4). Myocardial apoptosis was significantly exacerbated in the Cas group compared with the other groups ($P < 0.001$). Although the number of positive nuclei was also increased in MI and testosterone replacement rats, it was increased to a lesser extent than

that in the Cas group ($P < 0.001$). The results indicated that testosterone exerts a protective effect against cardiomyocyte apoptosis.

Effects of testosterone on myocardial mitochondria

Transmission electron microscopy was used to examine mitochondrial microstructures (Fig. 5). Changes in mitochondrial microstructures are shown in Fig. 5. In the 14-day groups, MI rats displayed swelling, disordered arrangement, loss of cristae number (S-Cas versus Con, $P < 0.01$), and mitochondria of reduced length and testosterone deprivation resulted in aggravated swelling and disordered arrangement, loss of cristae number and short in length (Cas versus S-Cas, $P < 0.05$).

Testosterone therapy alleviated the castration-induced changes in cristae number ($P < 0.01$) and mitochondrial length ($P < 0.01$). These results therefore indicate that castration exacerbates mitochondrial damage that can be ameliorated by testosterone treatment.

Testosterone mediates the activation effects of the AMPK–PGC1A pathway on mitochondrial fusion and fission

We performed western blotting to evaluate protein expression. As shown in Fig. 6A and B, 14 days after MI the mitochondrial fission protein DRP1 (S-Cas versus Con,

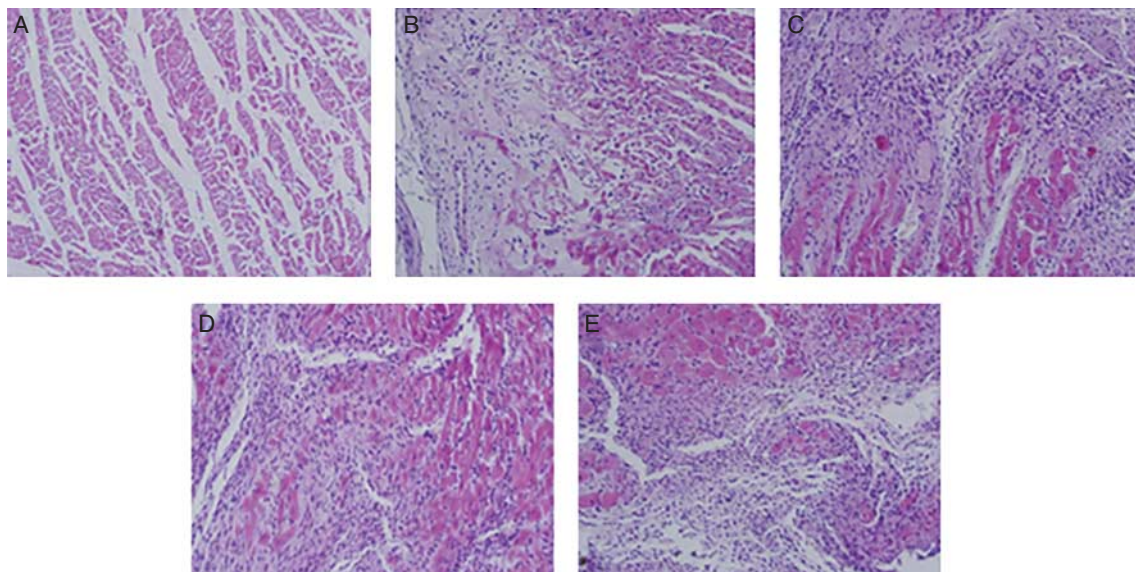
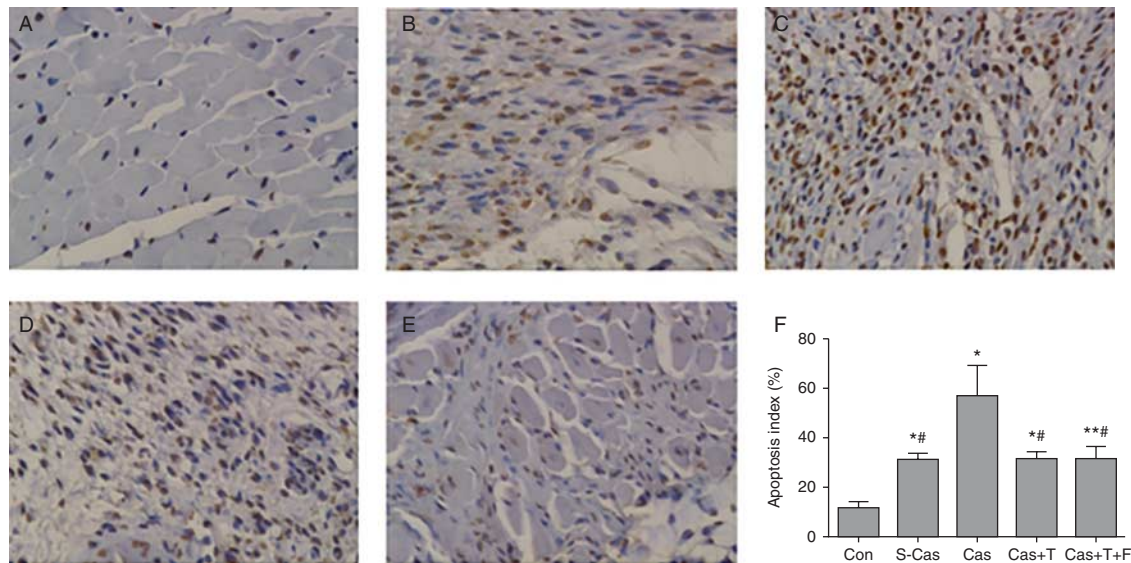


Figure 3

Myocardial histology staining of left ventricular 14 days after myocardial infarction. Representative H&E staining (200 \times) for each group. (A) Control group, (B) S-Cas group, (C) Cas group, (D) Cas+T group, and (E) Cas+T+F

group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0638>.

**Figure 4**

Cardiac apoptosis 14 days after ligation or sham surgery. Representative TUNEL staining (400 \times) of myocardium from (A) control group, (B) S-Cas group, (C) Cas group, (D) Cas+T group, and (E) Cas+T+F group. (F) Quantitative analysis of TUNEL-positive cells in each group to calculate

the apoptosis index. Values are expressed as means \pm s.d., $n=3$. S-Cas, sham-castration; T, testosterone; F, flutamide. $*P<0.001$, $**P<0.01$ versus Con and $^{\#}P<0.001$ versus Cas. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0638>.

$P<0.05$) was upregulated and the mitochondrial fusion protein MFN2 was downregulated (S-Cas versus Con, $P<0.01$). Castration further increased fission (Cas versus S-Cas, $P<0.05$) and decreased fusion (Cas versus S-Cas, $P<0.01$), and these effects were reversed by testosterone replacement. To further investigate possible mechanisms by which testosterone affects mitochondrial network dynamics, we assessed mitochondrial upstream AMPK–PGC1A pathways (Fig. 6C and D). PGC1A was downregulated under the ischemia conditions (S-Cas versus Con, $P<0.05$), and this downregulation was aggravated by testosterone deprivation (Cas versus S-Cas, $P<0.05$). Compared with castrated rats, testosterone replacement enhanced the expression of PGC1A ($P<0.05$). Increased activation of AMPK was observed in ischemic rats. However, this activation was downregulated by testosterone deficiency (Cas versus S-Cas, $P<0.01$) and the combination with testosterone resulted in greater activation of AMPK (Cas+T versus Cas, $P<0.05$). The results indicate that the phosphorylation of AMPK can upregulate PGC1A to further control the balance of mitochondrial dynamics.

Effects of testosterone on mitochondrial fusion and fission

Immunofluorescence was conducted to represent mitochondrial fusion and fission. The expression of

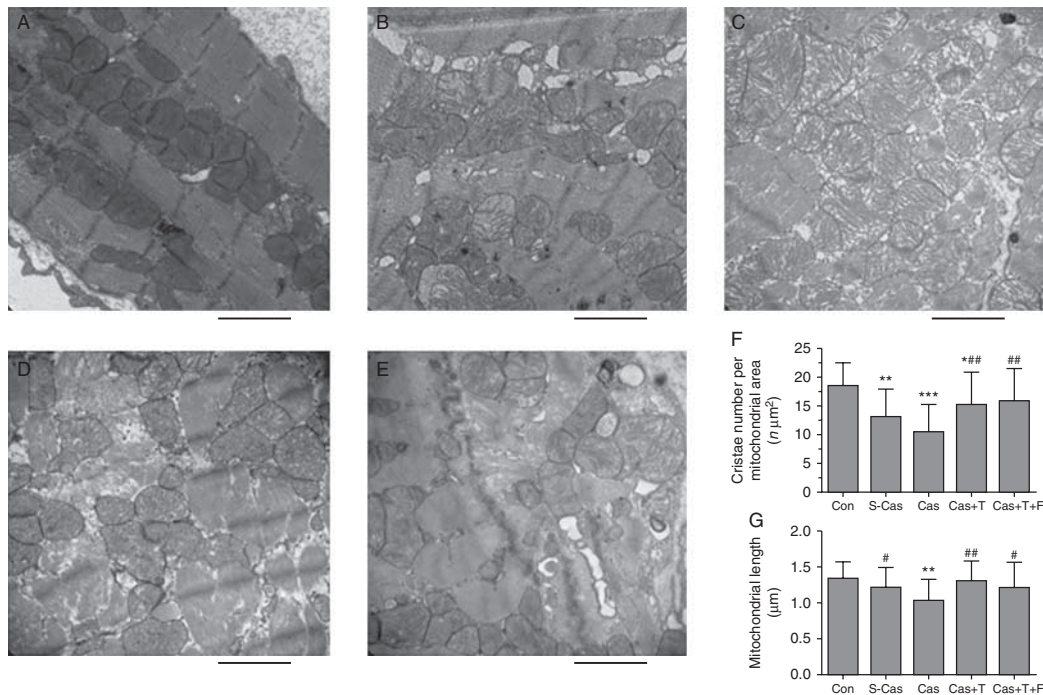
mitochondrial fusion protein MFN2 and fission indicator DRP1 is shown in Fig. 7. As shown in Fig. 7B, MI reduced the expression of MFN2 and increased the expression of DRP1. Testosterone deprivation further increased fission and decreased fusion (Fig. 7C), and these effects were reversed by testosterone therapy (Fig. 7D). The testosterone–flutamide combination induced no significant changes compared with the Cas+T group (Fig. 7E). These results therefore indicate that castration exacerbates mitochondrial dynamic imbalance that can be ameliorated by testosterone treatment.

ATP assay

The ATP content in the myocardium was assayed by HPLC 14 days after MI (Fig. 8). The results indicate that myocardial ischemia decreased ATP synthesis (S-Cas versus Con, $P<0.05$) and that testosterone insufficiency exacerbated this detrimental effect (Cas versus S-Cas, $P<0.05$). A physiological dose of testosterone successfully restored the concentration of ATP, preventing further deterioration (Cas+T versus Cas, $P<0.05$).

Measurement of serum testosterone and E_2 levels

Serum testosterone and E_2 levels were measured by ELISA and are shown in Fig. 9A and B respectively. As expected,

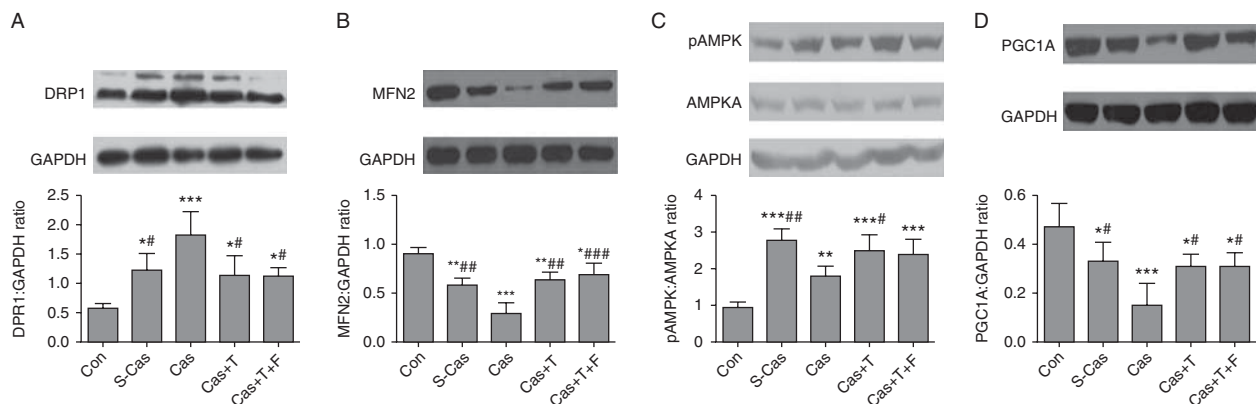
**Figure 5**

Mitochondrial microstructural changes in left ventricular tissue from each group. Representative images (20 000 \times) are shown. Scale = 2 μ m. (A) Control group, (B) S-Cas group, (C) Cas group, (D) Cas+T group, and (E) Cas+T+F group. (F and G) Effects of castration and testosterone on

mitochondrial cristae number and mitochondrial length. Data represent means \pm s.d., $n=20$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus Con and # $P<0.05$, ## $P<0.01$ versus Cas.

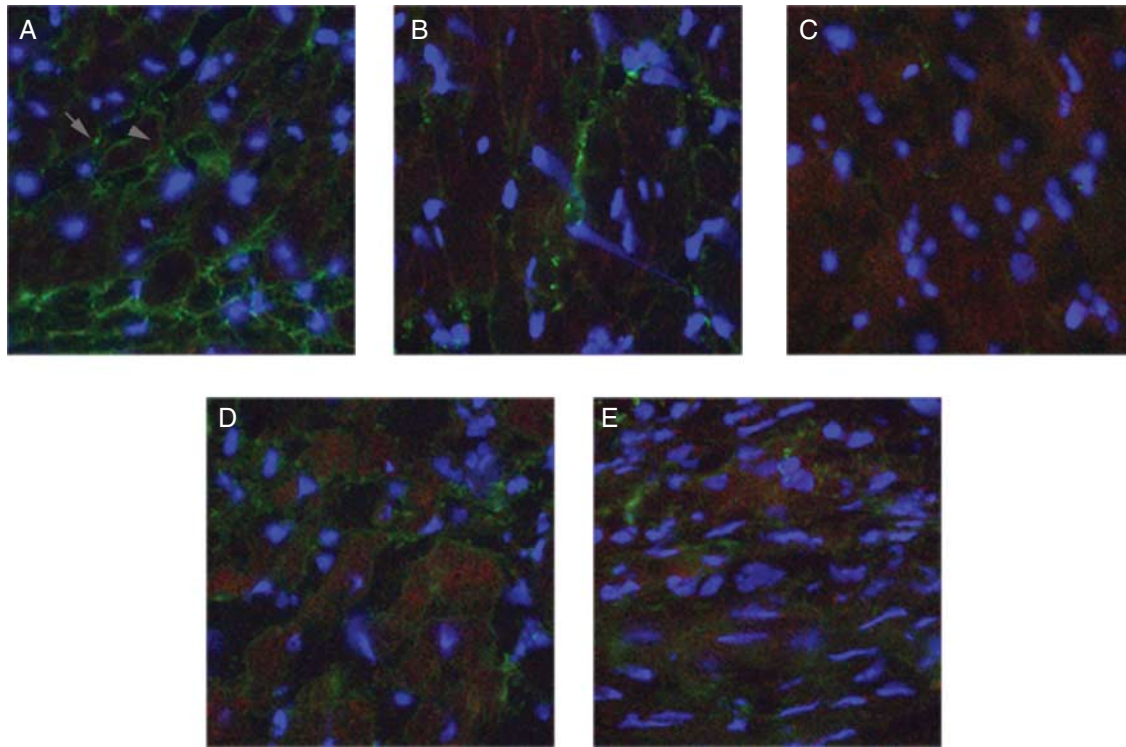
testosterone supplementation restored serum concentrations close to physiological levels. As shown in Fig. 9A, castration resulted in a significant decrease in testosterone levels at 2 and 4 weeks (Cas versus S-Cas, $P<0.05$), and

concentrations of this gonadal steroid were completely restored by testosterone therapy (Cas versus Cas+T, $P<0.05$). The testosterone–flutamide treatment produced no significant changes compared with the Cas+T group.

**Figure 6**

Testosterone mediates the activation effects of the AMPK–PGC-1A pathway on mitochondrial fusion and fission. Mitochondrial fission protein DRP1 (A) was significantly increased and mitochondrial fusion protein MFN2 (B) was downregulated 14 days after MI. Castration increased mitochondrial fission and decreased mitochondrial fusion compared with the S-Cas group, testosterone replacement reversed the detriment. AMPK–PGC1A signaling was activated by testosterone to mediate partially the mitochondrial fission

and fusion. (C) Testosterone further increased the phosphorylation of AMPK compared with castration rats. (D) Testosterone deprivation downregulated the expression of PGC1A, testosterone supplementation restored PGC1A levels. The data are expressed as the means \pm s.d., $n=3$. Con, control; S-Cas, sham-castration; Cas+T, castration+testosterone; Cas+T+F, castration+T+flutamide. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus Con and # $P<0.05$, ## $P<0.01$, ### $P<0.001$ versus Cas.

**Figure 7**

Detection of MFN2 and DRP1 in myocardium. Representative images (200 \times) are shown. Nuclei were stained with DAPI (blue). Mitochondrial fusion protein MFN2 which is located in the mitochondrial inner membrane was dyed green (arrow), mitochondrial fission protein DRP1 which is

located in the cytosol was dyed red (triangle). (A) Control group, (B) 5-Cas group, (C) Cas group, (D) Cas+T group, and (E) Cas+T+F group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0638>.

There was no significant difference in average serum E_2 concentrations between the groups (Fig. 9B).

Discussion

In this study, we first report the effects of testosterone replacement on mitochondrial network dynamics in post-MI hearts. MI resulted in pathologically remodeled mitochondrial dynamics, and castration aggravated the damage by disrupting mitochondrial dynamics with a reduction in cristae number and mitochondrial length, resulted in decreased ATP levels, and cardiomyocyte apoptosis, while testosterone replacement protected the network dynamics against further dysfunction. The testosterone-induced activation of AMPK–PGC1A may be a key mechanism accounting for balanced mitochondrial fusion and fission.

Recently, mitochondrial dynamics have attracted increased attention owing to their associations with multiple mitochondrial functions. Mitochondrial fusion and fission are essential to maintaining balanced mitochondrial dynamics, and even mild mitochondrial defects can result in disease. Proper mitochondrial fusion, which

requires MFN2, and proper fission events, which require DRP1, are essential for mitochondrial integrity, respiratory capacity, oxidative phosphorylation, apoptosis, response to cellular stress, and mitophagy (Garnier *et al.* 2005, Chan 2012). Oxidative phosphorylation is the major method of ATP generation. Once mitochondrial dynamics are damaged, ATP generation decreases. Our results indicated that testosterone deprivation aggravates mitochondrial deficits and decreases ATP levels. Testosterone replacement ameliorated the negative effects, elevated ATP levels, and ultimately improved myocardial function.

In this study, results of western blotting indicated that MI downregulated PGC1A and MFN2, but upregulated DRP1. Immunofluorescence results also indicated that MI reduced mitochondrial fusion and increased mitochondrial fission. It has been reported that excessive fission and fragmentation contribute to cell death (Twig *et al.* 2008, Disatnik *et al.* 2013). Our results support this through the observed further upregulation of DRP1 and increased myocardial apoptosis induced by castration. PGC1A is a critical regulator of mitochondrial fusion and fission (Garnier *et al.* 2005, Jiang *et al.* 2014). In our experiment,

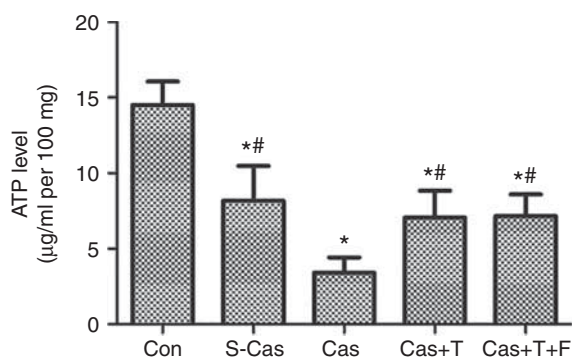


Figure 8

The effects of castration and testosterone on ATP levels in myocardium 14 days after MI. Data represent means \pm s.d., $n=6$. S-Cas, sham-castration; T, testosterone; F, flutamide. $*P<0.05$ versus Con and $^{\#}P<0.05$ versus Cas.

testosterone elevated the level of PGC1A but down-regulated DRP1. The increased expression of PGC1A can lead to the upregulation of both MFN2 and DRP1 (Garnier *et al.* 2005). We therefore suspect that there is another pathway involved in the regulation of mitochondrial fission. Jiang *et al.* (2014) showed that MI induced pathological mitochondrial fission through JNK/ERK-P53 signaling in addition to PGC1A.

In ARKO mice, the activation of ERK1/2 and 5 induced by Ang-II was decreased compared with WT mice, proving that testosterone can indirectly control ERKs (Ikeda *et al.* 2005). Testosterone might protect mitochondria against excessive fission through the ERKs pathway. Results described in one report also indicated that testosterone can upregulate mitochondrial transcription factor A, reduce oxidative stress, and finally exert a protective effect on mitochondria via AKT activation (Ikeda *et al.* 2010). This body of evidence indicates that testosterone protects mitochondria.

AMPK in heart can be activated by a wide array of stimulations, with ischemia being one of the most important factors (Zaha & Young 2012). There is evidence indicating that ischemia-induced AMPK activation exerts a protective effect on the heart (Russell *et al.* 2004, Calvert *et al.* 2008). Testosterone induced further AMPK activation and upregulated PGC1A compared with testosterone deprivation, indicating that testosterone replacement protected mitochondrial and cardiac function. In addition, AMPK is an upstream regulatory factor of PGC1A (Rowe *et al.* 2010), and therefore testosterone may regulate PGC1A through AMPK phosphorylation. Although testosterone elevated the phosphorylation

of AMPK, it was lower than that in MI rats. The once daily s.c. injection may be a factor.

Flutamide is an antagonist of the androgen receptor and can prevent the effects of androgen (Chen *et al.* 2012). According to the findings of this study, the testosterone-flutamide combination induced no significant changes in post-infarct myocardium compared with the Cas+T group. The fact that flutamide was unable to block the effects of testosterone may be associated with the mechanism by which testosterone affected the cells through AR-independent mechanism. Androgen might exert a non-genomic action through binding to G-protein coupled membrane receptors (Heinlein & Chang 2002). G-protein-coupled membrane receptors control the activation of PKA and PKC and influence intracellular calcium (Ca^{2+}) concentrations (Sladek & Song 2012). In cultured endothelial cells, the activation of PKC could influence liver kinase B1 (LKB1) to stimulate AMPK (Xie *et al.* 2006). Testosterone can modulate Ca^{2+} channels of the cell membrane directly (Kelly & Jones 2013). The modulation of Ca^{2+} channels will also change intracellular Ca^{2+} concentrations. The activation of calcium calmodulin

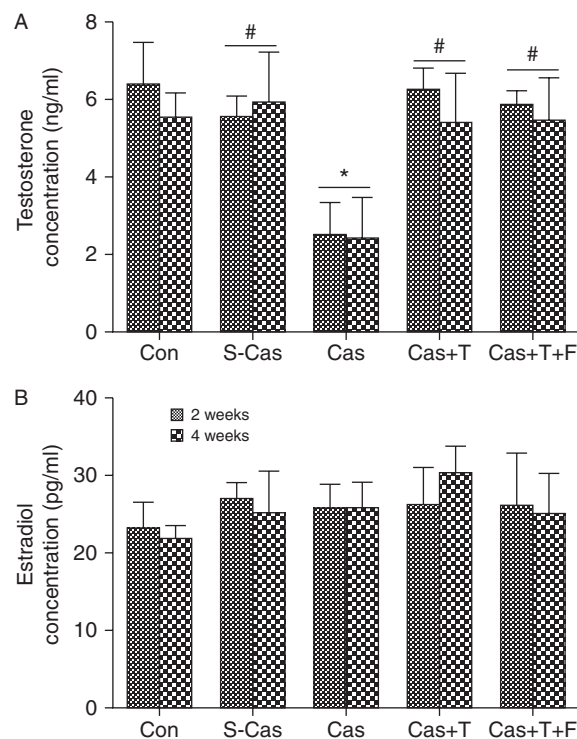


Figure 9

Serum testosterone levels (A) and estradiol levels (B) at 2 and 4 weeks after castration. Values are means \pm s.d., $n=6$ (ng/ml). Cas, castration; S-Cas, sham-castration; T, testosterone; F, flutamide. $*P<0.05$ versus Con and $^{\#}P<0.05$ versus Cas.

activated protein kinase kinase B (CaMKKB) can be mediated by changes in intracellular Ca²⁺ concentration induced via both G-protein-coupled membrane receptors and Ca²⁺ channels to further regulate the phosphorylation of AMPK (Zaha & Young 2012). Therefore, we suspect that testosterone might stimulate AMPK through binding to G-protein-coupled membrane receptors or influencing Ca²⁺ channels.

To exclude the influence of E₂, we measured serum E₂ concentrations, but this was not enough. As we know, testosterone can be converted into E₂ by aromatase in the local tissue without increasing serum E₂ levels (Simpson 2003, Bell *et al.* 2013). In order to completely exclude the effect of E₂, in the next step we will use testosterone plus an aromatase inhibitor and analyze the expression of progesterone receptor, which was considered to be a highly estrogen-dependent gene (Comeglio *et al.* 2014), within myocardial tissue.

In summary, testosterone deficiency aggravated mitochondrial damage, in part via the AMPK–PGC1A pathway, which downregulated MFN2 and upregulated DRP1 thereby increasing mitochondrial swelling, disordered arrangement, and loss of cristae, reducing mitochondrial length, and leading to a decrease in ATP levels and cardiomyocyte apoptosis in ischemic myocardium. Testosterone replacement therapy significantly reversed these changes. This demonstrates that testosterone, similarly to insulin, is indispensable to the human body.

Declaration of interest

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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