Testosterone enhances estradiol's cardioprotection in ovariectomized rats

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Abstract

After menopause, the development of cardiovascular disease (CVD) is due not only to estrogen decline but also to androgen decline. This study examined the effects of either estradiol (E₂) or testosterone replacement alone or E₂-testosterone combination on isolated myocytes in ovariectomized (Ovx) rats subjected to ischemia/reperfusion (I/R). Furthermore, we determined whether the effects are associated with β_2 -adrenoceptor (β_2 -AR). Five groups of adult female Sprague-Dawley rats were used: Sham operation (Sham) rats, bilateral Ovx rats, Ovx rats with E₂ 40 μ g/kg per day (Ovx + E), Ovx rats with testosterone 150 μ g/kg per day (Ovx + T), and Ovx rats with E₂ 40 μ g/kg per day + testosterone 150 μ g/kg per day (Ovx + E/T). We determined the lactate dehydrogenase (LDH) release, percentage of rod-shaped cells and apoptosis of ventricular myocytes from rats of all groups subjected to I/R. Then, we determined the

above indices and contractile function with or without a selective β_2 -AR antagonist ICI 118 551. We also determined the expression of β_2 -AR. Our data show that either E₂ or testosterone replacement alone or E₂ and testosterone in combination decreased the LDH release, increased the percentage of rod-shaped cells, reduced apoptotic cells (%), and combination treatment appeared to be more effective than either E₂ or testosterone replacement alone. ICI 118 551 abolished the effects of the three. Combination supplementation also enhanced the expression of β_2 -AR. We concluded that in Ovx rats, testosterone enhances E₂'s cardioprotection, while E₂ and testosterone in combination was more effective and the protective effects may be associated with β_2 -AR. The study highlights the potential therapeutic application for CVD in postmenopausal women.

Journal of Endocrinology (2012) **212**, 61–69

Introduction

Epidemiological data showed that premenopausal women have a lower incidence of cardiovascular disease (CVD), compared with age-matched men. However, after menopause, the incidence of CVD rises sharply and remains the number one cause of death among women. Montalcini *et al.* (2007) found that the development of CVD after menopause is due not only to estrogen decline but also to androgen decline.

There is compelling evidence to suggest the role of estrogen in protecting the cardiomyocyte after ischemia/reperfusion (I/R) injury. However, the cardioprotective role of estrogen was challenged by the findings of the Women's Health Initiative study (Rossouw *et al.* 2002), as well as a study published in the *BMJ* (Vickers *et al.* 2007). In these studies, estrogen replacement therapy increased cardiovascular events in postmenopausal women. As a result, the potential use of estrogen replacement was much reduced, and interest began

to focus on the safety and efficacy of hormone therapy among postmenopausal women.

Recently, there is increasing interest in the use of testosterone as part of postmenopausal hormone therapy. Sievers *et al.* (2010) reported that low baseline testosterone in relatively older women is associated with increased all-cause mortality and cardiovascular events, which is largely independent of traditional risk factors. Low-dose testosterone supplementation improved functional capacity, insulin sensitivity and muscle strength in elderly female patients with chronic heart failure, and no androgenic side effects were detected (Iellamo *et al.* 2010). In several studies, addition of testosterone to estrogen therapy has been reported to improve sexual function and well-being in naturally and surgically menopausal women (Shifren *et al.* 2006). These findings prompted us to postulate that addition of physiological dose of testosterone to estrogen therapy may confer cardioprotection by direct action on the myocardium.

Our previous study showed that estradiol (E₂) plays a cardioprotective role in female rat hearts subjected to I/R, and the effects are associated with increased expression of β_2 -adrenoceptor (β_2 -AR) (Wu *et al.* 2008). In addition, studies have demonstrated that testosterone at physiological levels increases the number of β_2 -ARs (Xu *et al.* 1991), enhances the expression of α_{1A} -, β_1 -, and β_2 -AR in rat hearts (Tsang *et al.* 2008, 2009). So, we speculated that β_2 -AR may be a common downstream target of estrogen and testosterone that is involved in cardioprotection.

We hypothesized that E₂ and testosterone (E/T) combination confers cardioprotection against I/R injury and the action is associated with β_2 -AR. In this study, we evaluated the effect of E/T combination on lactate dehydrogenase (LDH) release, percentage of rod-shaped cells, apoptosis, and contractile function. To further elucidate whether the effect was associated with the activation of β_2 -AR, we inhibited β_2 -AR using a selective β_2 -AR antagonist ICI 118 551, then, reassessed the above effects and ascertained the level of expression of β_2 -AR using western blotting analysis.

Materials and Methods

Experimental animals and materials

These experiments were approved by the Animal Ethics Committee of the Medical College of Xuzhou. Seventy-day-old female Sprague–Dawley rats (Clean grade, Xuzhou Medical College, China) weighing 180–200 g were randomly divided into two groups. One group was Sham operated and received daily injections of vehicle and served as normal control (sham group). The other group underwent bilateral ovariectomy and was divided into four subgroups. Two weeks after being ovariectomized (Ovx), four subgroups of the Oxv rats received daily injections of 1) vehicle (Ovx group), 2) E₂ (40 μ g/kg, s.c., Sigma) (Ovx + E group), 3) testosterone (150 μ g/kg, s.c., Sigma) (Ovx + T group), and 4) E₂ (40 μ g/kg, s.c., Sigma) + testosterone (150 μ g/kg, s.c., Sigma) (Ovx + E/T group) for 4 weeks according to previous studies (Tsang *et al.* 2008, Wu *et al.* 2008). All surgical procedures were performed under anesthesia with sodium pentobarbitone (60 mg/kg body weight, i.p.), chloral hydrate (300 mg/kg body weight, i.p.), and ketamine (20 mg/kg

body weight, i.p.). The adequacy of anesthesia was monitored by observing limb withdrawal and palpebral reflexes.

Experimental protocol

Ventricular myocytes were isolated from the hearts of all rats, and cultured in Dulbecco’s minimal essential medium at a density of 2×10^4 cells/well on 12-well culture dishes. The culture dishes were not coated in any way. The cells in medium were incubated at cell culture chamber with 37 °C, 100% humidity and 5% CO₂. The myocytes of Sham group did not experience the treatment of I/R. The myocytes of other groups were cultured for 6 h, and then subjected to 3 h ischemia followed by 4 h reperfusion. During the entire simulated ischemic period, the cell medium was replaced with an ‘ischemic buffer’ that contained (mM) 118 NaCl, 24 NaHCO₃, 1.0 NaH₂PO₄, 2.5 CaCl₂·2H₂O, 20 sodium lactate, 16 KCl, and 10 2-deoxyglucose (pH adjusted to 6.2) as reported previously (Das & Sarkar 2006), and the cells were incubated at 37 °C in tri-gas incubator equilibrated with 94% N₂ + 1% O₂ + 5% CO₂ for 3 h. Reperfusion was accomplished by replacing the ischemic buffer with normal cell medium and myocytes were incubated at cell culture chamber with 37 °C 100% humidity and 5% CO₂ for 4 h. In addition, the cells in Oxv, Oxv + E, Oxv + T, and Oxv + E/T groups were incubated 1 h with or without a selective β_2 -AR antagonist ICI 118 551 (Sigma) and then were subjected to I/R. The LDH release, percentage of rod-shaped cells, apoptosis, and contractile function were assessed at the end of reperfusion.

Isolation of ventricular myocytes

The rats were heparinized, anesthetized with sodium pentobarbitone (60 mg/kg body weight, i.p.) and ketamine (20 mg/kg body weight, i.p.), and then humanely killed by cervical dislocation. Ventricular myocytes were isolated, using the collagenase perfusion method described previously (Sun *et al.* 2006, Wu *et al.* 2008, Hao *et al.* 2009). There was no statistically significant difference in the viability of freshly prepared myocytes from all the groups, which ensures that percentage survival is equivalent among individual preparations/plates. After isolation, they were allowed to stabilize for at least 30 min before experiments.

Table 1 General features of experimental animals (n=10 per group). Data shown are mean $\pm$ s.e.m.

	Sham	Ovx	Ovx + E	Ovx + T	Ovx + E/T
Body weight (g)	248 $\pm$ 7	350 $\pm$ 10 [#]	271 $\pm$ 7*	336 $\pm$ 7 [#] \$	249 $\pm$ 4* ⁺
Heart weight (g)	0.92 $\pm$ 0.02	1.04 $\pm$ 0.04	1.01 $\pm$ 0.03	1.04 $\pm$ 0.03	0.93 $\pm$ 0.03
Heart weight/body weight ratio (mg/g)	3.70 $\pm$ 0.05	3.00 $\pm$ 0.05 [#]	3.60 $\pm$ 0.06*	3.10 $\pm$ 0.04 [#] \$	3.70 $\pm$ 0.05* ⁺
Serum E ₂ (pg/ml)	66 $\pm$ 9	13 $\pm$ 2 [#]	62 $\pm$ 8*	13 $\pm$ 3 [#] \$\$	63 $\pm$ 10* ⁺⁺
Serum testosterone (ng/ml)	1.65 $\pm$ 0.07	1.12 $\pm$ 0.04 [#]	0.79 $\pm$ 0.04 [#] ***	1.56 $\pm$ 0.10 [#] **\$	1.48 $\pm$ 0.11 [#] ***\$

[#]P<0.001 and ^{##}P<0.01 vs Sham, *P<0.001, **P<0.01, and ***P<0.05 vs Oxv, \$P<0.001 and \$\$P<0.01 vs Oxv + E, + P<0.001 and ++ P<0.01 vs Oxv + T.

Measurement of plasma E_2 and testosterone concentrations

Immediately after the heart was removed, 2.5 ml of blood was drawn from the thorax and put into a tube containing 0.6 ml of 5% sodium citrate. Sera were separated by centrifugation and stored at -80°C until assayed. The E_2 and testosterone concentrations were measured by a chemiluminescent enzyme immunoassay (Access, Beckman, Fullerton, CA, USA), according to the manufacturer's instructions. Sensitivity for the testosterone determination was 0.05 ng/ml and for E_2 was 10 pg/ml. Intra-assay variance for testosterone and E_2 was 6.3 and 5.5% respectively.

Measurement of LDH release in culture medium

The release of LDH in culture medium was determined after I/R as an indication of myocardial injury. The concentration of LDH was assayed spectrophotoelectrically as described previously (Lucchinetti *et al.* 2008). The sensitivity and intra-assay variance for LDH was 0.001 U/l and 2% respectively. All samples were assayed in triplicate.

Identification of rod-shaped cells

Rod-shaped cells are the viable cells and round-shaped cells are nonviable cells. Five micrographs were taken randomly per sample with an inverted microscope. All rod- and round-shaped myocytes in these fields were measured. In total, 600–800 cells were measured for one condition of one preparation. The percentage of rod cells in total cells was calculated with Image-Pro Express Software (Media Cybernetics, Glen Burnie, MD, USA). The sensitivity and intra-assay variance for percentage of rod-shaped cells was 0.01 and 2% respectively. The analysis of myocytes was done under blind conditions.

Measurement of cardiac myocyte contraction

Contractile function was monitored by a video edge detector system as described previously (Wu *et al.* 2008). Briefly, a few drops of medium containing ventricular myocytes were added to an open chamber on the stage of an inverted microscope (Olympus, Tokyo, Japan). Five minutes later, myocytes spontaneously attached to the bottom of the chamber, which was filled with Krebs–Henseleit buffer (KHB) and 100 nM isoprenaline to mimic the *in vivo* situation when there is sympathetic activity. KHB (2 ml/min, containing 2.0 mM Ca^{2+}) was adjusted to pH 7.4 by equilibration with 95% O_2 –5% CO_2 . The ventricular myocytes were paced with electrical field stimulation (0.5 Hz). The myocytes used were rod-shaped with clear sarcomeres. At least ten myocytes per experimental group per heart were studied. The outputs of the video edge detector were sent to a computer. Contractile function was assessed using the following indices: shortening amplitude, time-to-peak (TTP) contraction and time-to-100% relaxation (R_{100}). The sensitivity for the shortening amplitude, TTP, and R_{100} was 0.1%, 0.01 and 0.01 ms

respectively. Intra-assay variance for shortening amplitude, TTP, and R_{100} was less than ~5% for all.

Hoechst 33258 DNA staining

Nuclear staining with Hoechst 33258 was assessed to detect chromatin condensation or nuclear fragmentation, which is characteristic of apoptosis. Each of the group cells was cultured on sterile cover glasses placed in the six-well plates and fixed with 4% paraformaldehyde and stained with 1 $\mu\text{g}/\text{ml}$ Hoechst 33258 (Sigma) for 10 min at room temperature in the dark. The cells were then washed three times with sterilized, distilled H_2O . Cells were counted and 200 were isolated and scored for the incidence of apoptotic chromatin changes using a fluorescence microscope (Olympus). The sensitivity and intra-assay variance for apoptotic cells was 0.01 and 2% respectively.

Figure 1 E/T combination reduced injury and increased viability. (A) LDH release. (B) Percentage of rod-shaped cells. Ventricular myocytes were subjected to I/R. Data shown are mean $\pm$ S.E.M. $n=8$ hearts in each group. # $P<0.001$ and ## $P<0.01$ vs Sham, * $P<0.001$ vs Sham+I/R, $^{\$}P<0.001$ vs Ovxx+I/R, and $^{+}P<0.001$ vs Ovxx+E/T+I/R.

Western blot analysis

After I/R, ventricular myocytes were collected from each group and membrane protein from myocytes was extracted as described previously (Wu *et al.* 2008). In brief, ventricular myocytes were homogenized followed by centrifugation at 6660 *g* for 15 min at 4 °C. The pellet was dispersed in 400 µl of the original buffer. Protein concentration was determined with BCA Protein Assay kit (Beyotime Biotechnology, Haimen, China). The protein sample (60 µg) was separated by 10% SDS-PAGE, and then transferred onto nitrocellulose membranes, blocked and probed with polyclonal antibodies (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for the β_2 -ARs. An alkaline phosphatase-linked anti-rabbit IgG (1:1000 dilution) was used as secondary antibody. Protein bands were shown by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The membranes were scanned into the computer, and relative intensity of bands was analyzed by Image J 3.0 system (NIH, Bethesda, MD, USA).

Statistical analysis

In each experimental series, data are presented as mean $\pm$ S.E.M. Statistical analysis was performed with GraphPad Prism 4.0 (San Diego, CA, USA). Statistical significance ($P < 0.05$) for each variable was estimated by one-way ANOVA followed by Bonferroni *post-hoc* tests.

Results

General features of experimental animals

Six weeks after Ovx, the female rats exhibited significant reductions in serum E_2 ($P < 0.001$) and total testosterone ($P < 0.001$) level accompanied by a significant increase in body weight ($P < 0.001$) and a significant reduction in heart weight/body weight ratio ($P < 0.001$). E/T combination reversed the effects. Compared with Ovx group, E_2 replacement alone significantly reduced body weight ($P < 0.001$), increased heart weight/body weight ratio ($P < 0.001$) and the serum E_2 level ($P < 0.001$), but further decreased the total serum testosterone level ($P < 0.05$); testosterone replacement alone markedly increased serum total testosterone level ($P < 0.01$), whereas it had no significant effect on body weight, heart weight/body weight ratio and serum E_2 level (Table 1).

E/T combination reduced injury and increased viability

Cell injury was monitored after I/R by assaying LDH release in the culture medium. Rod-shaped cells are the viable cells and the percentage of rod-shaped cells is representative of the viability of ventricular myocytes. The baseline level of LDH release was the same in any of the Ovx groups. I/R induced a marked increase in the release of LDH (Fig. 1A) and reduction in percentage of rod-shaped cells (Fig. 1B).

The LDH release was further elevated (Fig. 1A; $P < 0.001$) and percentage of rod-shaped cells (Fig. 1B; $P < 0.001$) was further declined, in Ovx+I/R group than Sham+I/R group. E/T combination replacement restored the values to those of the Sham+I/R group (Fig. 1A and B). Both E_2 and testosterone replacement alone reduced the LDH release (Fig. 1A; $P < 0.001$ vs Ovx+I/R) and enhanced percentage of rod-shaped cells (Fig. 1B; $P < 0.001$ vs Ovx+I/R), but did not restore the values to those of the Sham+I/R group.

E/T combination reduced apoptotic cells (%)

After Hoechst 33258 staining, apoptotic cell nuclei appeared either shrunken and irregularly shaped or degraded with aggregation and fragmentation of chromatin; normal nuclei have blue chromatin with organized structure (Fig. 2A). I/R markedly increased the apoptotic cells (%; Fig. 2B), but the Ovx+I/R group had the highest

Figure 2 E/T combination inhibited apoptosis. (A) Fluorescence images of Hoechst 33258-stained. The bright parts (arrows) were the aggregation and fragmentation of chromatin. The shrunken and irregularly shaped nuclei indicate apoptosed cells. a, Sham group; b, Sham+I/R group; c, Ovx+I/R; d, Ovx+E+I/R; e, Ovx+T+I/R; and f, Ovx+E/T+I/R (original magnification $\times 100$). (B) Apoptotic cells (%). Ventricular myocytes were subjected to I/R. Data shown are mean $\pm$ S.E.M. $n = 8$ hearts in each group. # $P < 0.001$ vs Sham, * $P < 0.001$ vs Sham+I/R, # $P < 0.001$ and # $P < 0.01$ vs Ovx+I/R, and + $P < 0.001$ vs Ovx+E/T+I/R.

apoptotic cells (%; Fig. 2A and B). E/T combination returned to that of the Sham+I/R group (Fig. 2A and B). The apoptotic cells (%) were decreased in Ovx+E+I/R and Ovx+T+I/R groups, compared with Ovx+I/R group (Fig. 2B; $P<0.01$), but it is higher than the Ovx+E/T+I/R group (Fig. 2B; $P<0.001$).

ICI 118 551 (55 nM) abolished the effects of hormone replacement on injury and viability

To determine the roles of crosstalk between β_2 -ARs and combination supplementation, a selective β_2 -AR antagonist ICI 118 551 (55 nM) was administered to ventricular myocytes, which had been isolated from Ovx, Ovx+E, Ovx+T, and Ovx+E/T rats, and subjected to I/R. Ovx markedly increased the release of LDH, declined the percentage of rod-shaped cells and increased the percentage of apoptotic cells. The effects were reversed by E/T combination, β_2 -AR antagonist ICI 118 551 abolished the reversed effects of E/T combination. Both E_2 and testosterone replacement alone reduced the LDH release, enhanced percentage of rod-shaped cells and decreased the percentage of apoptotic cells, and β_2 -AR antagonist ICI 118 551 also abolished the effects. However, in ventricular myocytes from Ovx rats, there was no significant difference in LDH release, percentage of rod-shaped and apoptotic cells (%) with or without β_2 -AR antagonist ICI 118 551 (Fig. 3).

E/T combination improved contractile function of isolated ventricular myocytes subjected to I/R and ICI 118 551 (55 nM) abolished the effects

Ovx markedly increased shortening amplitude, prolonged TTP contraction and shortened R_{100} . Inhibition of β_2 -AR with ICI 118 551 did not change the effects in Ovx rats. However, combination supplementation decreased shortening amplitude, shortened TTP, prolonged R_{100} to the levels of Sham group. Inhibition of β_2 -AR with ICI 118 551 abolished the effects in the Ovx+E/T group. E_2 replacement alone reduced shortening amplitude, but had no significant effect on TTP and R_{100} . However, testosterone replacement alone had no significant effect on shortening amplitude, but shortened TTP and prolonged R_{100} . Inhibition of β_2 -AR with ICI 118 551 increased shortening amplitude in the Ovx+E group, prolonged TTP and shortened R_{100} in the Ovx+T group, respectively, to the level of the Ovx group (Fig. 4).

E/T combination upregulated the expression of β_2 -AR

Ovx downregulated the expression of β_2 -AR. Compared with Ovx, E_2 replacement alone, testosterone replacement alone, and combination supplementation all increased the expression of β_2 -AR. There was no significant difference in the expression of β_2 -AR between Ovx+E and Ovx+T groups. However, compared with Ovx+E and Ovx+T groups, the expression of β_2 -AR was higher in Ovx+E/T group (Fig. 5).

Discussion

To our knowledge, this study is the first to address that addition of physiological dose of testosterone to estrogen therapy enhances the cardioprotection of E_2 in Ovx rat cardiomyocytes subjected to I/R. Our data showed that

Figure 3 ICI 118 551 (55 nM) abolished the effects of hormone replacement on injury and viability. (A) LDH release. (B) Percentage of rod-shaped cells. (C) Apoptotic cells (%). Ventricular myocytes were subjected to I/R. Data shown are mean $\pm$ S.E.M. $n=8$ hearts in each group. [#] $P<0.001$ vs Sham, ^{*} $P<0.001$ and ^{**} $P<0.05$ vs Ovx, ^{\$} $P<0.001$, ^{\$\$} $P<0.01$, and ^{\$\$\$} $P<0.05$ vs Ovx+E, ⁺ $P<0.001$, ⁺⁺ $P<0.01$, and ⁺⁺⁺ $P<0.05$ vs Ovx+T, and [^] $P<0.001$ vs Ovx+E/T.

Figure 4 E/T combination improved contractile function and ICI 118 551 (55 nM) abolished the effects. (A) Shortening amplitude. (B) Time-to-peak (TTP) contraction (ms). (C) Time-to-100% relaxation (R_{100} ; ms). Ventricular myocytes were subjected to I/R. Data shown are mean $\pm$ S.E.M. $n=8$ hearts in each group, 10–15 cells were measured in each heart. [#] $P<0.001$ and ^{**} $P<0.05$ vs Sham, ^{*} $P<0.001$ and ^{**} $P<0.01$ vs OvX, ^{\$} $P<0.001$ and ^{\$} $P<0.05$ vs OvX+E, [&] $P<0.001$ vs OvX+T, and ⁺ $P<0.001$ vs OvX+E/T.

E_2 or testosterone replacement alone or combination supplementation reduced myocardial injury, increased viability, improved contractile function. Combination treatment appeared to be more effective than either E_2 or testosterone replacement alone. We also found that the benefits of E/T combination may be associated with β_2 -AR.

A number of clinical and experimental studies confirmed that estrogen has beneficial effects on the cardiovascular system, particularly in myocardial I/R injury, atherosclerosis, and arrhythmia (Booth & Lucchesi 2008). Xin *et al.* (2002) found that estrogen plays a protective role in the hypertrophic response of the heart to Ca^{2+} dysregulation. Pretreatment of male rabbits with E_2 before *in vivo* coronary artery ligation significantly reduced infarct size (Das *et al.* 2006). Physiological estrogen replacement reduced cardiomyocyte apoptosis after myocardial infarction *in vivo* in OvX female mice (Patten *et al.* 2004). In this study, compared with the OvX group, E_2 replacement alone increased the percentage of rod-shaped cells, reduced apoptotic cells (%), improved contractile function, and decreased the LDH release. This is in agreement with the result of our previous studies in which E_2 has been shown to play a cardioprotective role in female rat hearts subjected to I/R injury, and the effects of E_2 are associated with decreased cardiomyocyte contraction (Wu *et al.* 2008).

Studies demonstrated that estrogen may create an iatrogenic testosterone deficiency state (Casson *et al.* 1997, Shifren *et al.* 2007). In this study, 6 weeks after OvX, the serum levels of E_2 and total testosterone significantly fell; administration of E_2 alone to OvX rats markedly increased serum E_2 concentration and decreased total testosterone concentration. CVD is rare in women before menopause compared with men of the same age. However, women do not always fare better than men with respect to CVD (Luczak & Leinwand 2009). The incidence of CVD in women increases after menopause; when comparing men and women over 65 years of age, this sexual dimorphism disappears and mortality is higher in postmenopausal women compared with age-matched men (Bhupathy *et al.* 2010). Postmenopausal women have approximately the same estrogen levels as do men, but approximately tenfold lower circulating testosterone levels than do men (Luczak & Leinwand 2009). Testosterone circulates at concentrations that are an order of magnitude greater than those of E_2 in the blood of postmenopausal women (Simpson 2002). In addition, androgen receptors are expressed in female hearts (Nordmeyer *et al.* 2004, Lizotte *et al.* 2009). An implication of the above is that androgens have an important role in the higher risk of CVD in postmenopausal women. Increased cardiovascular events induced by estrogen replacement therapy may be associated with the decline of testosterone concentrations in postmenopausal women.

A large number of studies have demonstrated that testosterone replacement benefits cardiac recovery from I/R injury in male rodent models. Callies *et al.* (2003) found that administration of testosterone reduced susceptibility to myocardial I/R injury, improved the recovery of cardiac contractile function. Tsang *et al.* (2008) demonstrated that testosterone reduced cardiac injury induced by I/R and noradrenaline, in a model close to the *in vivo* situation in orchidectomized rats.

In this study, physiological dose of testosterone significantly reduced cardiac injury and apoptosis, increased survival, and

Figure 5 E/T combination upregulated the expression of β_2 -AR. The expression of β_2 -adrenoceptors was evaluated by western blotting. The expression of β -actin was detected as an internal standard. The relative arbitrary unit for Sham group was assigned as 1. Each value represents the mean $\pm$ S.E.M. $n=6$ hearts in each group. # $P<0.05$ vs Sham, * $P<0.05$ vs Ovx and Ovx+E/T, and + $P<0.001$ vs Ovx.

to some extent, improved contractile function by shortening TTP and prolonging R_{100} . Shortened TTP and prolonged R_{100} allow cardiomyocytes to rest for a longer time. Thus, the work time of myocytes was reduced and myocardial oxygen consumption was decreased and more energy was saved. These results are consistent with the observations that testosterone reduced apoptosis induced by hyperosmotic stress in the embryonic rat heart cell line H9c2 (Sánchez-Más *et al.* 2010) and reduced cardiac injury induced by I/R and noradrenaline in ovariectomized rats (Tsang *et al.* 2008). Combined with our findings, results indicated that physiological dose of testosterone replacement has a direct protective effect on cardiomyocytes.

Taken together, although E_2 plays a useful role in the cardiovascular system, it can cause the increase of cardiovascular events (Rossouw *et al.* 2002, Vickers *et al.* 2007). E_2 -only replacement results in testosterone deficiency, and testosterone replacement plays a protective role in cardiomyocytes. Therefore, addition of testosterone to E_2 replacement therapy is reasonable in the prevention and treatment of CVD among postmenopausal women. In this study, addition of physiological dose of testosterone to E_2 therapy also had beneficial effects on myocardial survival and contractile function, and was significantly more effective than either E_2 or testosterone supplementation alone. However, Cavin *et al.* (2003) found that E_2 and testosterone play different and opposing roles in the development of heart failure and long-term remodeling after myocardial infarction in mice; E_2 prevents maladaptive chronic remodeling and

further deterioration of cardiac performance, whereas testosterone adversely affects myocardial healing, degrades cardiac dysfunction and remodeling, and exerts pronounced effects when E_2 levels are reduced. Nevertheless, it remains clear that the role of testosterone in myocardial injury requires further study.

In this study, in addition to the superior benefits on cardiac injury, survival and contractile function, E/T combination supplementation restored body weight and heart weight/body weight ratio compared to those of the Sham group. However, in Ovx+T group, body weight and heart weight/body weight ratio had no significant changes. Moreover, combination supplementation counteracted the fall of serum testosterone in Ovx+E group and the lower level of serum E_2 in Ovx+T group. Therefore, E/T combination supplementation may be the best hormone therapy, compared with either E_2 or testosterone replacement alone – it not only plays a protective role in cardiomyocytes, but may also be better at simulating the levels of premenopausal physiological state of hormone, causing few side effects.

The mechanisms through which combination supplementation would affect Ovx rat cardiomyocytes subjected to I/R are still unclear, and this study, by its design, cannot directly answer this question. However, some tentative explanations can be advanced. Our previous studies indicated (Wu *et al.* 2008) that incubation of Ovx ventricular myocytes with E_2 for 24 h significantly reduced the amplitude of myocellular shortening, reduced the protein expression of β_1 -AR and increased the protein expression of β_2 -AR. The effect of E_2 was abolished by co-incubation with ICI 182 780, a high-affinity estrogen receptor antagonist. These data indicated that the action of E_2 on the myocardial cells is mediated by estrogen receptors.

As to testosterone, most of the studies support the view that the cardiovascular effects of testosterone are androgen receptor-independent. Zhang *et al.* (2011) found that testosterone suppresses oxidative stress via androgen receptor-independent pathway in murine cardiomyocytes. In cultured cardiac myocytes, testosterone induced a rapid and nongenomic intracellular Ca^{2+} release through activation of a plasma membrane androgen receptor associated with the PTX-sensitive G protein-phospholipase C/IP3 signaling pathway (Vicencio *et al.* 2006). Bourghardt *et al.* (2010) found that testosterone atheroprotection was androgen receptor-dependent as well as androgen receptor-independent in male mice.

The conversion of testosterone to estrogen by aromatization may also be responsible for the protective action of testosterone, because of the presence of the aromatase enzyme within cardiac tissue. In this study, testosterone replacement alone increased the serum E_2 level slightly, but there was no statistical significance, compared with Ovx group. So it is unlikely that E_2 contributes significantly to the protective action of testosterone.

Liou *et al.* (2010) indicated that chronic E₂ treatment prevented bilateral ovariectomy-induced Fas receptor-dependent apoptotic pathways in rat models. In addition, studies found that estrogen reduced apoptosis of cardiomyocytes *in vivo* and *in vitro* by activating the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway (Ren *et al.* 2003, Patten *et al.* 2004), which is a downstream pathway activated by β_2 -AR stimulation (Xiao *et al.* 2006). Our previous study showed that E₂ may protect heart via β_2 -AR and subsequent signaling (Xu *et al.* 2010). Tsang *et al.* (2008) showed that β_2 -AR was downregulated in rats without testosterone, indicating that testosterone may also interact with this receptor subtype. The cardiac protective effect of β_2 -AR signaling is largely mediated by activating the cell survival pathway Gi-G $\beta\gamma$ -PI3K-AKT, which not only counteracts apoptosis but also protects cardiomyocytes against other apoptotic stimuli (Zhu *et al.* 2005). It is likely that E₂ and testosterone exert a cardiac protective effect through activating common signaling PI3K-AKT via β_2 -AR. In this study, a selective β_2 -AR antagonist ICI 118 551 abrogated the protective effects of either E₂ or testosterone alone or E/T combination on cardiomyocytes subjected to I/R. Moreover, the expression of β_2 -AR was upregulated by E₂ or testosterone or E/T combination supplementation, especially by the latter. The results suggested that the cardioprotective effects of combination supplementation are associated with β_2 -AR.

In conclusion, this study demonstrated that testosterone enhances E₂'s cardioprotection in Ovx rats, E/T combination protects cardiomyocytes against I/R injury, and the protective effects are at least partly mediated by β_2 -AR. Further studies are warranted to delineate the potential mechanisms and to explore the possible therapeutic application of E/T combination in postmenopausal women with CVD.

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.

Funding

This work was supported by the research and innovation project of Xuzhou Medical College (XYCX201011) and Research Subjects of Xuzhou Medical College (2010KJ15).

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Youjian Qi.

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Received in final form 17 September 2011

Accepted 30 September 2011

Made available online as an Accepted Preprint
30 September 2011