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RESEARCH PAPER

Testosterone protects rat hearts against ischaemic insults by enhancing the effects of α_1-adrenoceptor stimulation

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Background and purpose: Testosterone alleviates symptoms in patients with ischaemic heart disease. Androgen receptors are present in the heart, and testosterone upregulates gene expression of cardiac β_1-adrenoceptors. We hypothesize that testosterone may confer cardioprotection by interacting with adrenoceptors.
Experimental approach: In isolated perfused hearts and ventricular myocytes from orchidectomized rats without or with testosterone (200 μg/100 g) replacement, we first determined the effect of ischaemia/reperfusion in the presence of noradrenaline (10^{-7} M). Then we determined the contribution of interactions between testosterone and α_1- or β_1-adrenoceptors in cardiac injury/protection (infarct size, release of lactate dehydrogenase, viability of myocytes, recovery of contractile function and incidence of arrhythmias) upon ischaemia/reperfusion by pharmacological manipulation using selective adrenoceptor agonists (α_1-adrenoceptor agonist: phenylephrine 10^{-6} M; non-selective β-adrenoceptor agonist: isoprenaline 10^{-7} M) and antagonists (α_1: prazosin or benoxathian 10^{-6} M; β_1: CGP 20712A 5×10^{-7} M). We also determined the expression of α_1 and β_1-adrenoceptor in the hearts from rats with and without testosterone.
Key results: Testosterone reduced injury induced by ischaemia/reperfusion and noradrenaline. This was achieved by enhancing the beneficial effect of α_1-adrenoceptor stimulation, which was greater than the deleterious effect of β_1-adrenoceptor stimulation (also enhanced by testosterone). The effects of testosterone were abolished or attenuated by blockade of androgen receptors. Testosterone also enhanced the expression of α_{1A} and β_1-adrenoceptor.
Conclusions and implications: Testosterone conferred cardioprotection by upregulating the cardiac α_1-adrenoceptor and enhancing the effects of stimulation of this adrenoceptor. The effect of testosterone was at least partly mediated by androgen receptors.
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Keywords: testosterone; ischaemia–reperfusion; α/β-adrenoceptors

Abbreviations: CGP, CGP 20712A; Cyp, cyproterone acetate; ICI, ICI 118, 551; IHD, ischaemic heart disease; LDH, lactate dehydrogenase; MIA/R, metabolic inhibition, anoxia and reperfusion; ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 μg per 100 g); PVB, premature ventricular beats; VT, ventricular tachycardia

Introduction

Men with heart disease or ischaemic heart disease (IHD) have lower levels of androgens than men with normal coronary angiograms (English *et al*., 2000a) and testosterone, the predominant androgen, improves ischaemic threshold and quality of life in hypogonadal men with angina (English *et al*., 2000b; Malkin *et al*., 2004). These observations suggest that androgens may confer cardioprotection against ischaemic insults. Androgen receptors are present in the heart (McGill *et al*., 1980) and mediate hypertrophy in cardiac myocytes (Marsh *et al*., 1998). More importantly, testosterone replacement in orchidectomized (ORX) rats improves recovery of contractile function and ischaemia/reperfusion-induced injury, and this is associated with attenuation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) overload (Callies *et al*., 2003). These findings prompted us to postulate that androgen may confer cardioprotection by direct action on the myocardium.

The sympathetic nervous system is one of the most important extrinsic mechanisms regulating cardiac function. Noradrenaline released from the nerve terminals of the sympathetic nervous system activates both α- and β-adrenoceptors. Activation of α_1- (Iwai-Kanai *et al*., 1999), and β_2- (Communal *et al*., 1999; Patterson *et al*., 2004) adrenoceptors reduces apoptosis, whereas activation of

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β_1-adrenoceptors is proapoptotic (Communal *et al*., 1998, 1999; Iwai-Kanai *et al*., 1999; Zhu *et al*., 2003). During myocardial ischaemia, there is a marked increase of noradrenaline discharge, which contributes significantly to cardiac injury (Waldenstrom *et al*., 1978; Schomig and Richardt, 1990; Schomig, 1990). It is now known that oestrogen, the female sex hormone, suppresses the expression of the β_1-adrenoceptor (Kam *et al*., 2004) and protein kinase A (Kam *et al*., 2005), thus, in turn, reducing cardiac responses to β_1-adrenoceptor stimulation. Similarly, there is evidence that testosterone increases the expression of the β_1-adrenoceptor (Golden *et al*., 2004), suggesting that like oestrogen, testosterone may also interact with the β_1-adrenoceptor and its signalling pathway.

We therefore hypothesized that testosterone interacts (by cross-talk) with adrenoceptors during myocardial ischaemia. Cross-talk between testosterone and adrenoceptors that are antiapoptotic may result in cardioprotection against cardiac injury in response to ischaemic insults, whereas cross-talk between testosterone and adrenoceptors that are proapoptotic may lead to increased injury. If the effect of the former is greater than that of the latter, the overall result is cardioprotection. To test the hypothesis that the male sex hormone confers cardioprotection against ischaemic insult by direct action on the myocardium, we determined the effects of ischaemic insults and reperfusion on isolated perfused hearts and isolated ventricular myocytes from sham control, orchidectomized rats without (ORX) or with testosterone replacement (ORX + T).

To mimic the increased sympathetic activation during myocardial ischaemia, 10^{-7} M noradrenaline was administered during ischaemic insults. This concentration was chosen based on the fact that the circulating level of noradrenaline is 10^{-9}–10^{-10} M (Engelhard *et al*., 2002) and there may be an up to 1000-fold increase during myocardial ischaemia (Schomig and Richardt, 1990; Schomig, 1990). Secondly, we determined the effects of testosterone on ventricular myocytes subjected to simulated ischaemia with activation of either α_1- or β_1-adrenoceptors. In the third series of experiments, we determined the injury/survival induced by simulated ischaemia and reperfusion in ventricular myocytes exposed to noradrenaline, with blockade of the β_2-adrenoceptor and either the α_1- or β_1-adrenoceptors, which allowed us to compare the contributions of cross-talk between testosterone and either α_1- or β_1-adrenoceptors. We also determined the expression of adrenoceptor subtypes in the three groups of rats hoping to obtain useful information on signalling mechanism at receptor level. The results showed that testosterone conferred cardioprotection by enhancing the beneficial action of α_1-adrenoceptor stimulation. Preliminary results have been communicated previously (Tsang *et al*., 2005, 2007).

Methods

Animal model

The study was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong. Male Sprague–Dawley rats weighing 300–350 g were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA) and randomly divided into two groups. One group was sham operated and served as normal control (sham control). The other group underwent bilateral orchidectomy (ORX) and was divided into two subgroups. One week after ORX, one subgroup was supplemented with a physiological dose of testosterone (200 μg per 100 g, s.c.) daily for 8 weeks (ORX + T) according to a previous study (Banu *et al*., 2001), and another subgroup was treated with vehicle. All surgical procedures were performed under anaesthesia with sodium pentobarbital (60 mg kg^{-1}, i.p.; Abbott Laboratory, Chicago, IL, USA).

Rat isolated heart preparation

Nine weeks after orchidectomy, rats were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and given heparin (200 IU, i.v.) before decapitation. Hearts were excised immediately and, placed in ice-cold Krebs–Henseleit perfusion buffer before mounting on the Langendorff apparatus for perfusion. Isolated hearts were perfused retrogradely with Krebs–Henseleit buffer (in mM: 118 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$ and 11 glucose) equilibrated with 95% O_2 + 5% CO_2 at a constant pressure of 80 cm H_2O (Waldenstrom *et al*., 1978) and a temperature of 37°C. The hearts were allowed to stabilize for 15 min and then subjected to 30 min regional ischaemia with modified Krebs–Henseleit buffer supplemented with 10^{-7} M noradrenaline and 120 min reperfusion as described previously (Wang *et al*., 2001). Hearts exhibiting arrhythmias during stabilization were discarded. Regional ischaemia was achieved by ligation of the left anterior descending coronary artery as described previously (Wang *et al*., 2001). Briefly, a fine silk thread was passed below the left anterior descending coronary artery. The ends of the thread were passed through a propylene tube to form a snare. Pulling the snare produced ischaemia. Release of the ligature allowed reperfusion. The effectiveness of ischaemia was confirmed by regional cyanosis and a substantial decrease in coronary flow. In the series of experiments that determined the effect of α- and β-adrenoceptors, 10^{-6} M prazosin (PZ) a selective α_1-adrenoceptor antagonist, shown to effectively block the receptor (Gallego *et al*., 2005) or 5×10^{-7} M CGP 20712A (CGP), a selective β_1-adrenoceptor antagonist, known to block the β_1-adrenoceptor (Zhang *et al*., 2000), and a selective β_2-adrenoceptor antagonist, ICI 118, 551 (ICI) at 5×10^{-7} M (Communal *et al*., 1999; Zhang *et al*., 2000), were administered.

Measurement of infarct size

At the end of reperfusion, the coronary artery was re-occluded, and the hearts were perfused with Evans blue (10%). The ischaemic risk zone and the infarct size were determined as described previously (Wang *et al*., 2001). The areas of infarct (unstained by 2,3,5-triphenyl-tetrazolium chloride) and the risk area (unstained by Evans blue) were determined by a computerized planimetric technique (ImageJ, NIH). The severity of infarct was expressed as the percentage of infarct size over risk area.

LV pressure recordings

Cardiac variables of left ventricular developed pressure (LVDP), velocity of contraction and relaxation ($\pm dP/dt_{max}$), and heart rate were monitored continuously by a PowerLab/4SD analogue-to-digital converter (AD instruments, Castle Hill, Australia). A latex balloon inserted through left atrium into left ventricle was adjusted to a mean left ventricular end-diastolic pressure (LVEDP) to 6–10 mm Hg. The hearts were paced at 5 Hz (300 beats per minute). After stabilization of mechanical function, regional ischaemia was initiated. Pacing was discontinued 5 min after the initiation of ischaemia. Hearts were reperfused after 30 min of ischaemia. Transient ventricular fibrillation occurred in all groups. Pacing was reinitiated 5 min after reperfusion.

Arrhythmia analysis

With the use of a lead II ECG tracing, the episodes of premature ventricular beats (PVB), ventricular tachycardia (VT) and ventricular fibrillation were recorded and monitored during the 30 min of ischaemia and 2 h of reperfusion. The signal electrode was superficially connected to the left ventricular wall and the reference electrode to the aorta. Arrhythmias were defined according to the Lambeth Conventions (Walker *et al*., 1988). PVBs were defined as discrete and identifiable premature QRS complexes, whereas VT was defined as a run of four or more consecutive ventricular premature beats (VPBs). An episode of ventricular fibrillation was defined as a signal where individual QRS deflections could not easily be distinguished from each other and where rate could no longer be measured (Walker *et al*., 1988).

Preparation of isolated ventricular myocytes

Ventricular myocytes were isolated from the hearts of sham control, ORX and ORX + T rats, using the collagenase perfusion method as described previously (Wu *et al*., 1999; Wang *et al*., 2001). Preliminary experiments in our lab showed that there was no difference in the viability of isolated myocytes between all the groups immediately after isolation, indicating that testosterone deficiency as a result of removal of the testes did not affect the viability of heart muscle cells. After isolation, they were allowed to stabilize for at least 30 min before experiments. The yield of myocytes was determined microscopically using a haemocytometer. Myocyte viability was assessed by both the Cell Titer Blue (CTB) reagent (Promega, Madison, WI, USA) and Trypan blue exclusion (Wu *et al*., 1999; Pei *et al*., 2003). Preparations were considered satisfactory only if rods accounted for >80% of the counted cells at the beginning of each experiment. Myocytes were re-suspended in minimal essential medium containing 1.25 mM Ca^{2+}, 5% fetal bovine serum, 5 μM insulin, 5 μM apo-transferrin, 100 U ml^{-1} penicillin G and 100 μM streptomycin and seeded at a density of 3×10^5 cells per well on laminin-coated (1 μM, Sigma, St Louis, MO, USA) six-well plates. To mimic ischaemia, ventricular myocytes were incubated with a modified glucose-free Krebs solution supplemented with 10 mM 2-deoxy-D-glucose (2-DOG), an inhibitor of glycolysis, and 10 mM sodium dithionite, an oxygen scavenger, that induce metabolic inhibition and anoxia (MIA). The cells were subjected to MIA for 10 min with or without adrenoceptor agonists at 37 °C under a 5% CO_2 atmosphere and then transferred back to normal Krebs buffer for another 10 min to simulate reperfusion (MIA/R). In the series of experiments that determined and compared the effect of α- and β-adrenoceptors, the same batch of myocytes isolated from ORX rats incubated without or with a physiological concentration of testosterone at 10^{-8} M (Phillippe *et al*., 1991; Weidemann and Hanke, 2002) for 24 h were used. In pilot studies, we showed consistent results with myocytes obtained from ORX + T rats (200 μg per 100 g, s.c.) and from ORX rats incubated with 10^{-8} M testosterone for 24 h.

Lactate dehydrogenase assay

The lactate dehydrogenase (LDH) release assay was performed using a cytotoxicity detection kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Release of the enzyme, which indicates cytotoxicity, was measured after MIA/R. Background release was assessed by measuring LDH release from untreated cells and was subtracted from the experimental value. Maximum release of LDH was obtained by adding 2% Triton X-100 to untreated cells. For measurements, each sample was transferred to a 96-well microtitre plate. LDH reagent was added to each well and incubated for 30 min at room temperature in the dark. The absorbance of samples was then measured at 490 nm. No significant LDH release was detected in the tissue culture medium alone or in drug-containing incubation medium.

Viability assay

Cell viability was determined using Cell Titer Blue reagent (Promega) according to the manufacturer's instructions. The Cell Titer Blue assay is based on determining the number of viable cells in culture, using an indicator dye, resazurin (dark

Table 1 Animal characteristics and hormone determination

	Total serum testosterone (ng ml^{-1})	*Body weight (g)*	*Heart weight (g)*	*Heart/body weight (%)*
Sham control (12)	5.22 ± 0.96	542 ± 13.7	2.14 ± 0.05	0.39 ± 0.01
ORX (14)	<0.4***, ###	489 ± 6.65*	1.73 ± 0.04**, #	0.35 ± 0.01*
ORX + T (14)	5.78 ± 1.49	516 ± 15.2	1.94 ± 0.06*	0.37 ± 0.02

Each value represents the mean ± s.e.mean. The figures in parentheses indicate the number of animals.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs sham control; #$P<0.05$, ###$P<0.001$ vs ORX with testosterone replacement (ORX + T) rats.

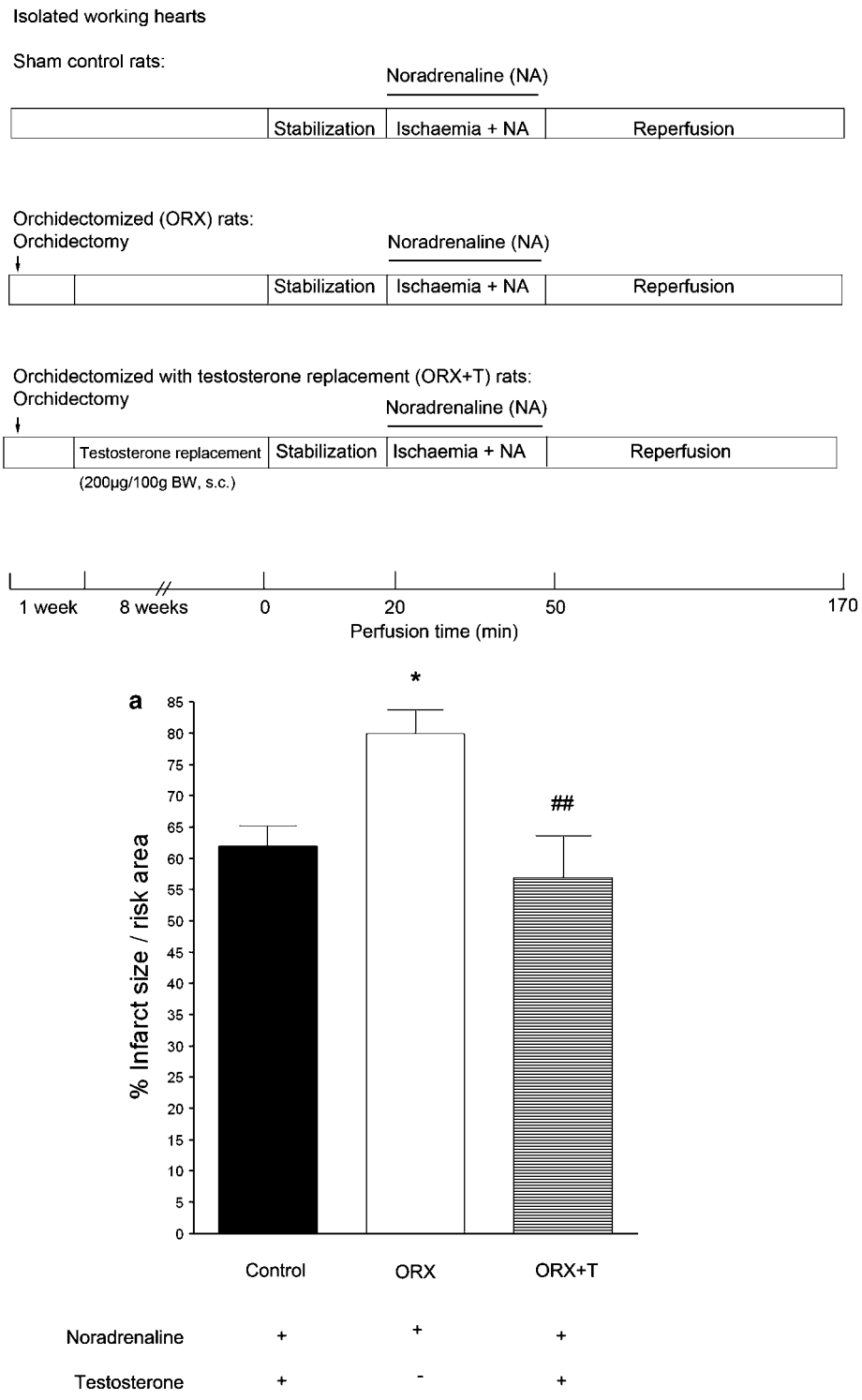


Figure 1 Effects of ischaemic insults and administration of noradrenaline (NA) on (**a**) infarct size in isolated hearts, (**b**) LDH release and (**c**) viability of isolated ventricular myocytes from sham, ORX and ORX + T rats. Isolated perfused hearts were subjected to regional ischaemia by occlusion of the coronary artery for 30 min followed by release of the occlusion for 2 h. This produced reperfusion shown in the protocol (**a**). Infarct size was determined at the end of reperfusion. Noradrenaline (NA; 10^{-7} M) was administered during ischaemia. As shown in (**b**) and (**c**), left ventricular myocytes were superfused for 10 min with a medium containing 10 mM 2-deoxy-D-glucose, an inhibitor of glycolysis, to induce metabolic inhibition, and 10 mM sodium dithionite, an oxygen scavenger, to produce anoxia. This simulated ischaemic insult and was followed by superfusion with the normal Krebs buffer for 10 min to simulate reperfusion. Values are mean ± s.e.mean from 12 hearts in each group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs sham control rats; $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.01$, $^{\#\#\#}$ $P<0.001$ vs ORX rats. ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 µg per 100 g).

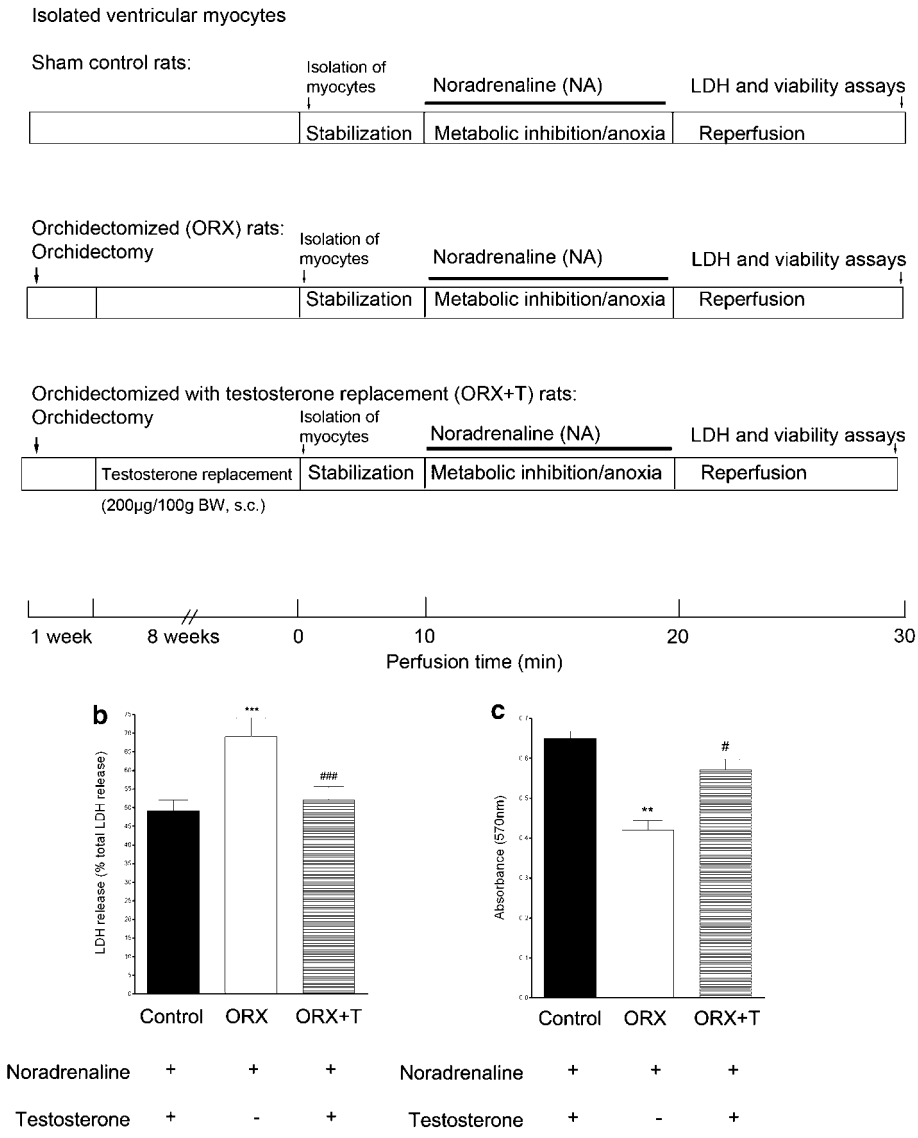


Figure 1 Continued.

blue). Viable cells retain the ability to reduce resazurin into resorufin, which is pink and highly fluorescent. Nonviable cells, which rapidly lose metabolic capacity, do not reduce the indicator dye. Cells were transferred to a 96-well microtitre plate with Cell Titer Blue reagent. The absorbance of samples was measured at 570 nm using 600 nm as a reference wavelength. No change in absorbance was detected in tissue culture medium alone or in drug-containing medium.

Immunoassays

Nine weeks after orchidectomy, rats were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) before decapitation. Blood samples were allowed to clot and sera were separated by centrifugation and stored at −80 °C until assayed. Total serum testosterone was determined by a commercially available RIA (Diagnostic Products Corp., Los Angeles, CA, USA; sensitivity 0.4 nM) according to the manufacturer's instructions.

Protein extraction and western blot

Membrane protein from ventricular myocardium was extracted as described previously (Shen *et al.*, 2000; Pei *et al.*, 2003). Briefly, left ventricular tissue was homogenized followed by centrifugation at 10 000 *g* for 10 min. The supernatant was centrifuged again at 100 000 *g* for 1 h at 4 °C, and the pellet was collected as the membrane fraction. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Membrane proteins (60 μg) were loaded onto 10% sodium dodecyl sulphate-polyacrylamide gels. Separated proteins were transferred to polyvinylidine difluoride membrane, blocked and probed with goat polyclonal antibodies SC-568 (V-19) for the β_1-adrenoceptor and SC-28982 (H-136) for the

α_{1A}-adrenoceptor (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (O'Connell *et al*., 2006). Monoclonal antibody against β-actin (Sigma) was chosen as an internal control (supplementary information). Bands were detected by enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were stripped and reblotted (Re-Blot Plus solution, Chemicon, Temecula, CA, USA) with anti-β-tubulin monoclonal antibody (Sigma) to ensure equal amount of protein loading.

Statistical analysis

Data were expressed as mean ± s.e.mean. Between-group comparisons were performed using ANOVA. The nonparametric Kruskal–Wallis test was used to analyse drug effects. A difference of $P<0.05$ was considered statistically significant. χ^2 or Fisher exact test was used for percentages of VPBs and VT.

Drugs and chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) except benoxathian HCl (Research Biochemical International, USA). Drugs were dissolved in double-distilled H_2O or Krebs buffer unless otherwise stated. Stock solutions of prazosin and propranolol were dissolved in methanol and ethanol, respectively. Testosterone was dissolved in ethanol/DMSO. The final concentration of methanol/ethanol was <0.01% (vol/vol), which itself had no effect on the heart.

Results

Animal characteristics and hormone determination

The total serum testosterone level in the sham control group was 5.22 ng ml^{-1} (18.1 nmol l^{-1}). Nine weeks after orchidectomy (ORX), the level fell below the detection limit of 0.4 ng ml^{-1} (Table 1). The decrease in testosterone level was accompa-

Table 2 Incidence of premature ventricular beats (PVB) and ventricular tachycardia (VT) in perfused hearts from sham control, ORX and ORX + T rats over first 30 min of reperfusion period after 30 min of regional ischaemia

	PVB (%)	*VT (%)*
Sham control ($n=12$)	50	25
ORX ($n=12$)	100*	50
ORX + T ($n=9$)	56$^{\#}$	44

Values shown are the percentage of rats exhibiting PVBs and VT (mean ± s.e.mean rounded to the nearest whole number) over the first 30 min reperfusion period.
*$P<0.05$ relative to sham control; $^{\#}P<0.05$ relative to ORX by χ^2 test for percentages.

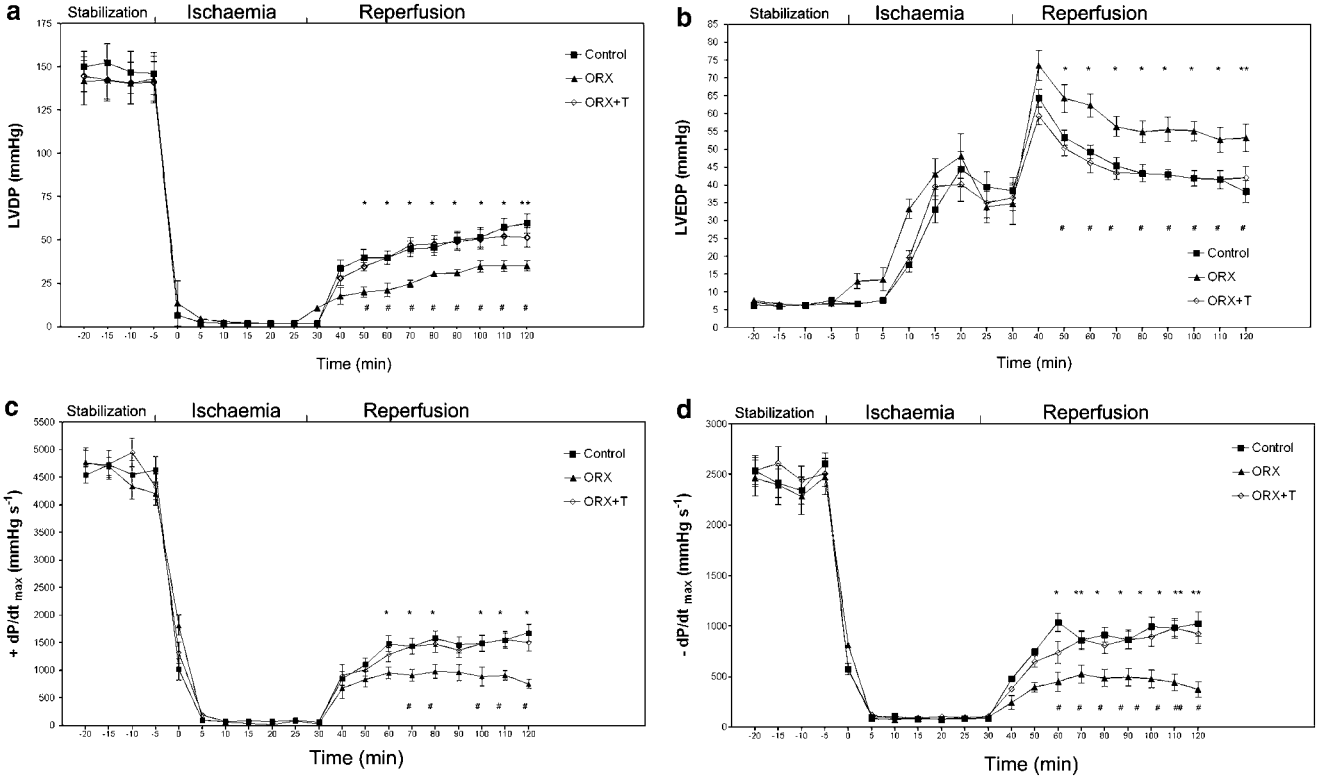


Figure 2 Effects of ischaemic insults and administration of noradrenaline (NA) on contractile variables in isolated hearts (**a–d**) from sham, ORX and ORX + T rats. (**a**) LVDP, (**b**) LVEDP, (**c**) $+dP/dt_{max}$ and (**d**) $-dP/dt_{max}$ obtained from perfused hearts in pre-ischaemic conditions (stabilization), during ischaemia and then during reperfusion. Values are mean ± s.e.mean from 12 hearts from sham control, 12 hearts from ORX and 9 hearts from ORX + T group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs sham control rats; *$P<0.05$, **$P<0.01$ vs ORX rats. ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 μg per 100 g).

nied by significant reductions in body and heart weights, and heart weight/body weight ratio in the ORX rats.

Effects of adrenergic stimulation and ischaemia/reperfusion on injury/viability in the isolated perfused heart and ventricular myocytes from sham control, ORX and ORX + T rats

When isolated perfused rat hearts were subjected to regional ischaemia/reperfusion in the presence of 10^{-7} M of noradrenaline to mimic sympathetic over-activity during ischaemia, myocardial infarction occurred. The infarct size was significantly greater in the ORX group, and testosterone replacement (ORX + T: 200 μg per 100 g, s.c.) restored the value to that of the sham control (Figure 1a).

In ventricular myocytes subjected to MIA/R in the presence of 10^{-7} M noradrenaline, the release of LDH increased, whereas the viability decreased (Figures 1b and c). The LDH release from isolated cardiomyocytes (Figure 1b)

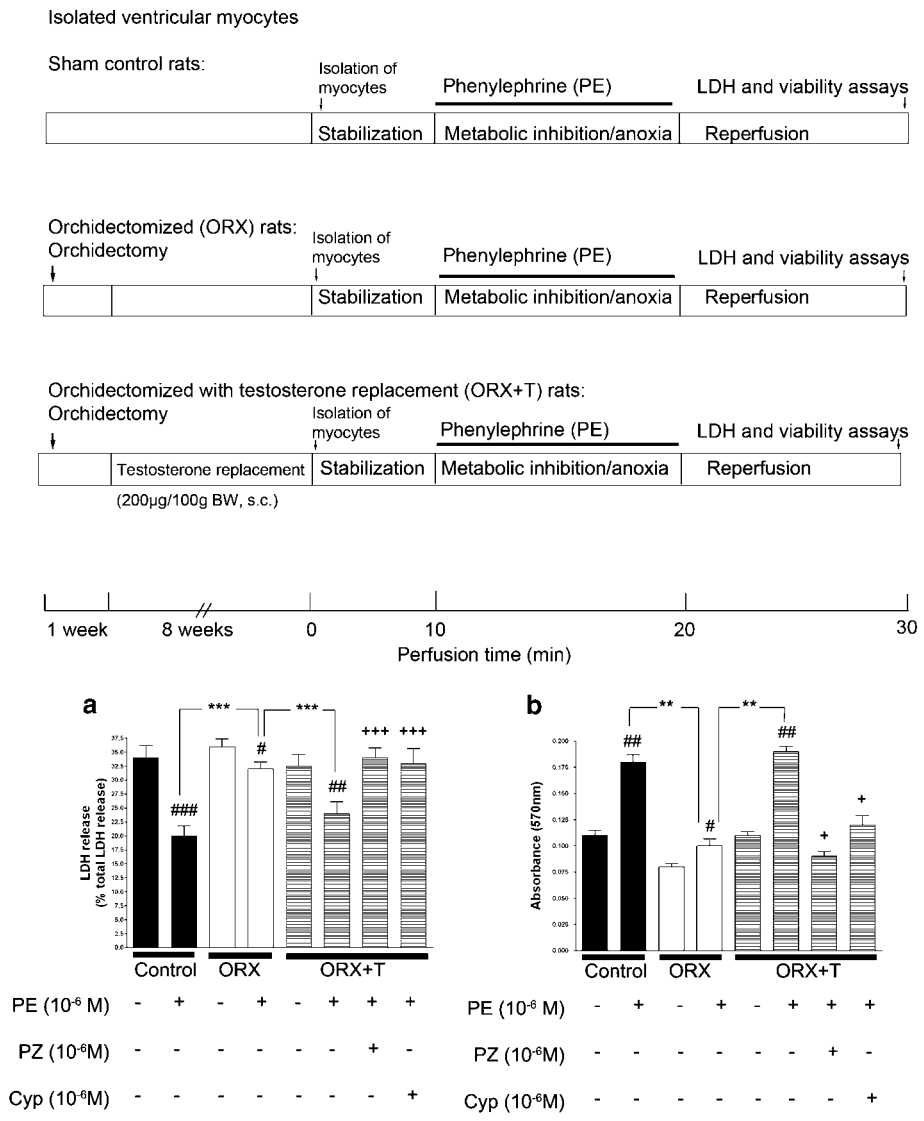


Figure 3 Effects of MIA/R in the presence of α_1 (**a**, **b**) or β_1-adrenoceptors (**c**, **d**) stimulation on LDH release and viability of left ventricular myocytes from sham, ORX and ORX + T groups. For stimulation of α_1-adrenoceptors, 10^{-6} M phenylephrine (PE) in the presence of 10^{-6} M propranolol, a β-adrenoceptor blocker, and 5×10^{-7} M yohimbine, an α_2-adrenoceptor blocker, was administered during MIA. Cyproterone acetate (Cyp), 10^{-6} M, prazosin (PZ), 10^{-6} M, was used for blockade of the androgen receptor and α_1, adrenoceptor, respectively. (**a**) % LDH release resulting from stimulation of α_1-adrenoceptors. (**b**) Viability resulting from stimulation of α_1-adrenoceptors. Values represent mean ± s.e.mean of triplicate determinations from six hearts in each group. *, **, *** $P<0.05$, 0.01, 0.001 vs corresponding sham control, respectively. #, ##, ### $P<0.05$, 0.01, 0.001 vs ORX rats, respectively. +, ++, +++ $P<0.05$, 0.01, 0.001 vs ORX + T group with phenylephrine, respectively. For stimulation of β_1-adrenoceptors, 10^{-7} M isoprenaline (ISO) in the presence of 10^{-6} M phentolamine, an α-adrenoceptor antagonist, and 5×10^{-7} M ICI, a β_2-adrenoceptor antagonist, were administered during MIA. (**c**) % LDH release resulting from stimulation of β_1-adrenoceptors. (**d**) Viability resulting from stimulation of β_1-adrenoceptors. Values are mean ± s.e.mean of triplicate determinations from six hearts in each group, respectively. *, **, *** $P<0.05$, 0.01, 0.001 vs corresponding sham control, respectively. #, ##, ### $P<0.05$, 0.01, 0.001 vs ORX group, respectively. +, ++, +++ $P<0.05$, 0.01, 0.001 vs ORX + T group with isoprenaline, respectively. ICI, ICI 118, 551; MIA/R, metabolic inhibition and anoxia/reperfusion; ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 μg per 100 g).

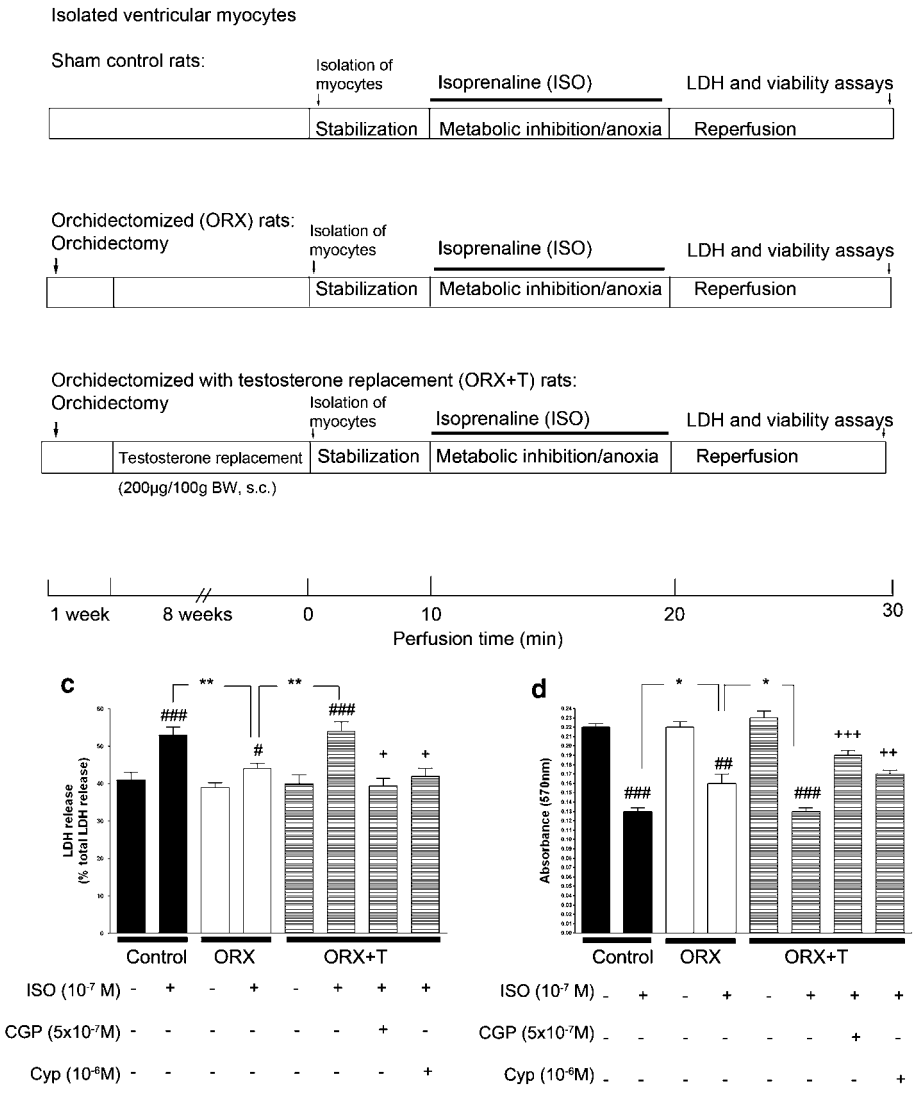


Figure 3 Continued.

was significantly greater, whereas the viability (Figure 1c) was significantly lower, in the ORX group than the sham control group. Testosterone replacement (ORX + T) restored the values to those of the sham control.

Effects of adrenergic stimulation and ischaemia/reperfusion on cardiac variables and reperfusion arrhythmias in the isolated heart from sham control, ORX and ORX + T rats

There were no significant differences in contractile function up to the end of ischaemia between sham control, ORX and ORX + T. During ischaemia, the LVDP and $\pm dP/dt_{max}$ were markedly reduced, whereas the LVEDP was elevated. Upon reperfusion, the LVDP and $\pm dP/dt_{max}$ were partially restored towards the base-line level, whereas the LVEDP was further increased. The post-ischaemic LVDP and $\pm dP/dt_{max}$ were higher, whereas LVEDP was lower, at the end of reperfusion in sham control and ORX + T rats than in ORX rats (Figure 2). PVB and VT were observed during reperfusion in all groups. The incidence of both PVB and VT was higher in the hearts of the ORX rats (Table 2). Testosterone replacement restored the incidence of PVB to that of the sham control (Table 2).

Effects of MIA/R and stimulation of α_1- or β_1-adrenoceptors on injury/viability of isolated ventricular myocytes from sham control, ORX and ORX + T rats

To determine the roles of cross-talk between testosterone and adrenoceptor subtypes, an agonist of α_1- or β_1-adrenoceptors was administered to ventricular myocytes, which had been isolated from ORX and ORX + T rats, and subjected to MIA/R. Phenylephrine (10^{-6} M), an α_1-adrenoceptor agonist, with a selective β-adrenoceptor antagonist, propranolol significantly decreased LDH release (Figure 3a) and increased viability (Figure 3b) of myocytes from control, ORX and ORX + T rats. These effects were abolished by 10^{-6} M prazosin, a selective α_1-adrenoceptor antagonist. The reduction in LDH release and increase in viability were significantly greater in the control and ORX + T rats than the corresponding values in the ORX rats (Figures 3a and b).

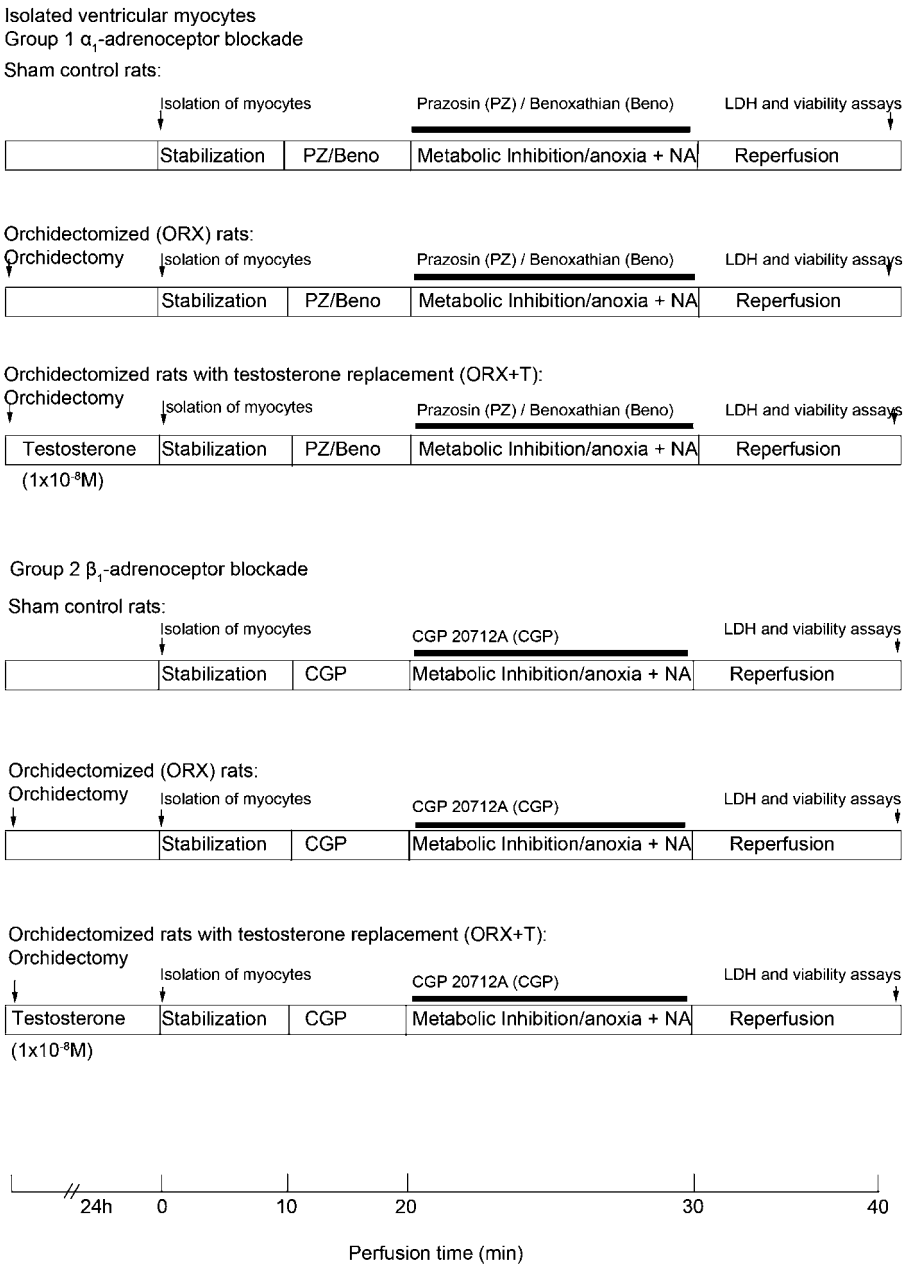
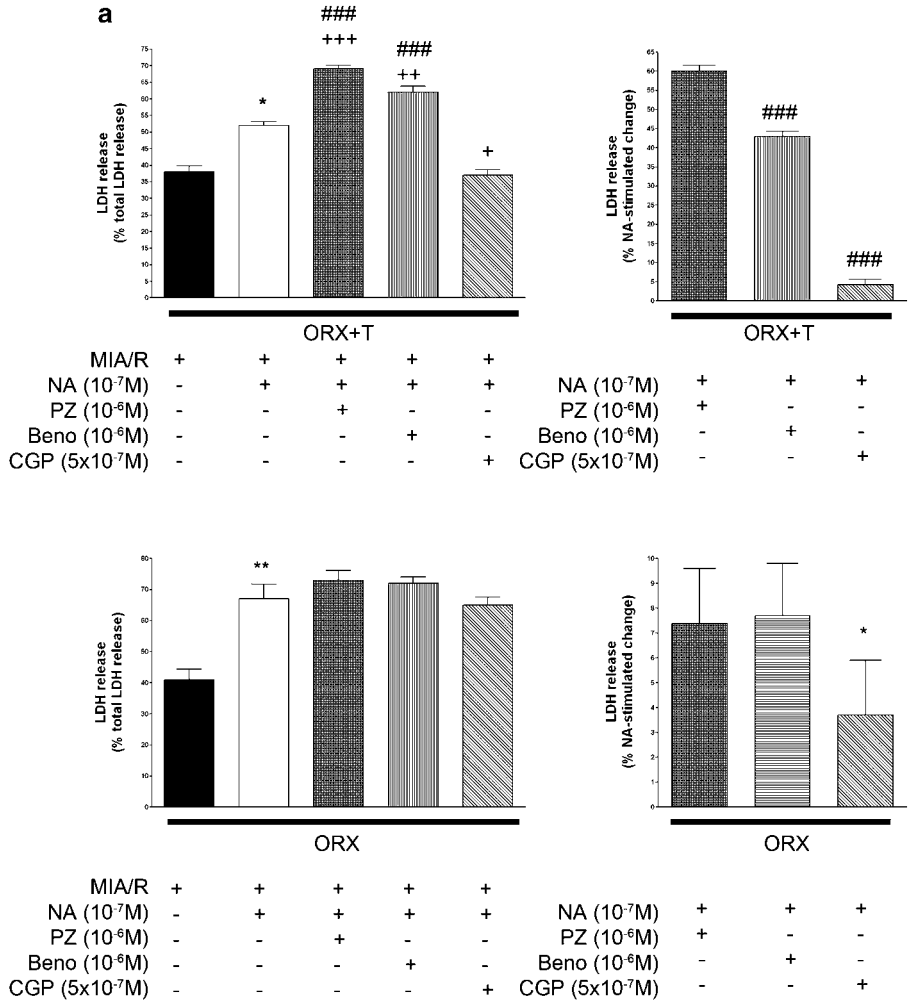


Figure 4 Effects of MIA/R in the presence of noradrenaline (NA) on the release of LDH and viability of left ventricular myocytes from ORX rats upon blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. For blockade of α_1-adrenoceptors, two selective α_1-adrenoceptor antagonists, 10^{-6} M prazosin (PZ) or 2×10^{-6} M benoxathian (Beno), were administered. The β_1-adrenoceptor-selective antagonist CGP at 5×10^{-7} M in the presence of ICI at 5×10^{-7} M was used to block both β-adrenoceptor subtypes. NA (10^{-7} M) was administered. (**a**) LDH release from myocytes with or without testosterone replacement. Left panels indicate % release after different treatments and right panels indicate net changes in LDH release after blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. (**b**) Viability of myocytes with or without testosterone replacement. Left panels indicate viability after different treatments and right panels indicate net changes in viability after blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. The results are expressed as percentage of noradrenaline and antagonist-stimulated change in LDH release/viability. Where appropriate, data are expressed as percentage of baseline (i.e., 100% × induced value/value before stimulation. Data represent mean ± s.e.mean of triplicate determinations in eight independent experiments using cell preparations from different rats. Differences between multiple groups were determined by one-way ANOVA followed by *post hoc* Neuman–Keuls multiple comparison tests. *$P<0.05$, **$P<0.01$ vs MIA/R. $^{+}$, $^{++}$, $^{+++}$ $P<0.05$, 0.01, 0.001 vs MIA/R with NA, respectively.$^{\#\#\#}$ $P<0.001$ vs CGP. CGP, CGP 20712A; ICI, ICI 118, 551; MIA/R, metabolic inhibition and anoxia/reperfusion; ORX, orchidectomized male rats.

On the contrary, the non-selective β-adrenoceptor agonist isoprenaline at 10^{-7} M increased LDH release (Figure 3c) and decreased viability (Figure 3d) upon blockade of α- and β_2-adrenoceptors in all three groups. The effects were abolished by 5×10^{-7} M CGP, a selective β_1-adrenoceptor antagonist. Similar to α_1-adrenoceptor stimulation, the changes in LDH release and viability were also significantly greater in the control and ORX + T groups than in the ORX group



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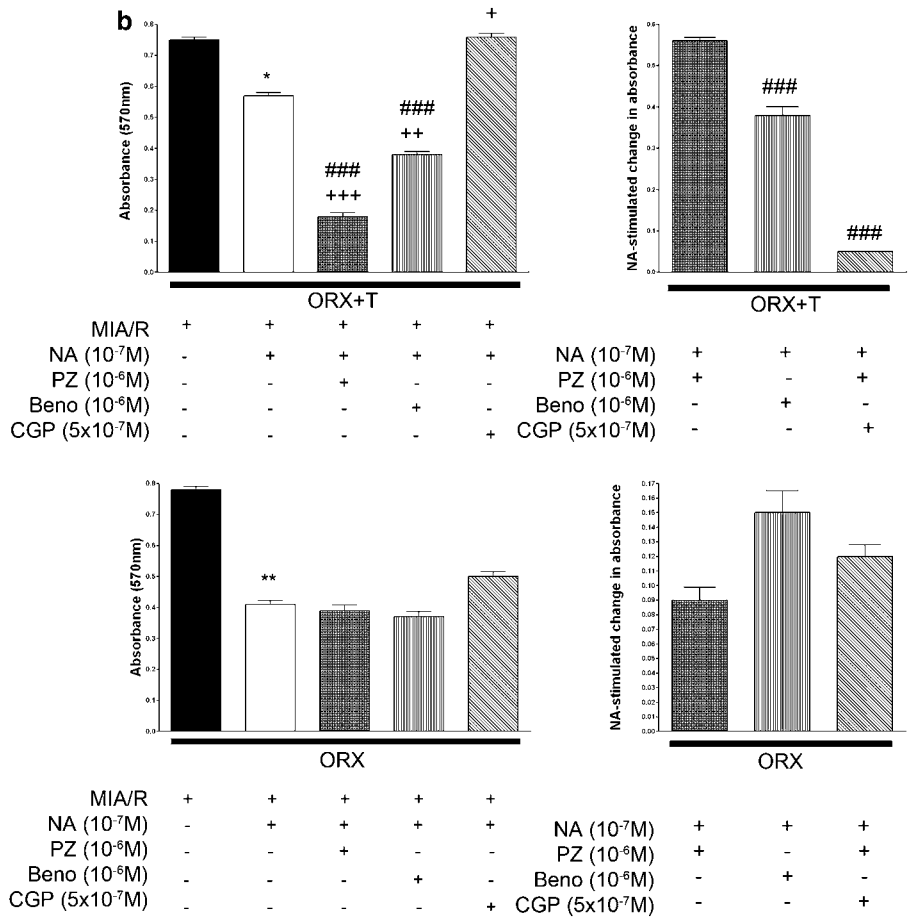
(Figures 3c and d). The enhancing effects of testosterone on adrenoceptor stimulation were abolished by 10^{-6} M cyproterone acetate, an androgen receptor antagonist (Figure 3).

Effects of MIA/R and adrenergic stimulation on injury/viability of ventricular myocytes from ORX rats with or without testosterone replacement in the presence of either α_1- and β_2-adrenoceptor or β_1- and β_2-adrenoceptor blockades

To compare the beneficial effects of cross-talk between testosterone and the α_1-adrenoceptor and the harmful effects of cross-talk between testosterone and the β_1-adrenoceptor, we determined the effects of blocking β_1- and β_2-adrenoceptors, and effects of blocking α_1- and β_2-adrenoceptors in ventricular myocytes subjected to MIA/R in the presence of 10^{-7} M noradrenaline. We eliminated the effects of the β_2-adrenoceptor (which may also be beneficial) based on the marked effect of testosterone on α_1-adrenoceptor stimulation (Figures 3a and b) by using a selective β_2-adrenoceptor antagonist, ICI at 5×10^{-7} M. Noradrenaline significantly increased the LDH release (Figure 4a) and reduced the viability (Figure 4b) induced by MIA/R in ventricular myocytes exposed to testosterone at a physiological concentration 10^{-8} M (ORX + T) for 24 h. The LDH release (Figure 4a) was enhanced and viability (Figure 4b) was attenuated upon blockade of α_1- and β_2-adrenoceptors. On the contrary, LDH release (Figure 4a) was attenuated and the viability (Figure 4b) was enhanced upon blockade of β_1- and β_2-adrenoceptors. The injury caused by blockade of the α_1-adrenoceptor was markedly greater than the beneficial effect of blockade of the β_1-adrenoceptor (Figures 4a and b). This was in contrast to the findings in myocytes not incubated with testosterone: a much smaller difference in LDH release between α_1-adrenoceptor blockade in the presence of benoxathian and blockade of β_1-adrenoceptors with CGP (Figure 4c), and no significant difference in viability between α_1- and β_1-adrenoceptor blockades (Figure 4d).

Effects of adrenergic stimulation and ischaemia/reperfusion on cardiac variables and reperfusion arrhythmias in the isolated heart from sham control, ORX and ORX + T rats in the presence of either α_1- or β_1-adrenoceptor blockade

To substantiate the beneficial effects of cross-talk between testosterone and the α_1-adrenoceptor and the harmful effects of cross-talk between testosterone and the β_1-adrenoceptor,



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we compared the post-ischaemic recovery of contractile functions and arrhythmias in presence of β_1- and β_2-adrenoceptor blockers, and effects of blocking α_1- and β_2-adrenoceptors in the isolated hearts. The post-ischaemic recovery of LVDP and $\pm dP/dt_{max}$ was attenuated, whereas that of the LVEDP was enhanced upon blockade of α_1- and β_2-adrenoceptors (Figure 5a upper panel). On the contrary, the post-ischaemic recovery of LVDP and $\pm dP/dt_{max}$ was enhanced, whereas that of the LVEDP was attenuated upon blockade of β_1- and β_2-adrenoceptors (Figure 5a upper panel). The changes in LVDP and $+dP/dt_{max}$ were greater, whereas the change in LVEDP was smaller after α_1-adrenoceptor blockade than after β_1-adrenoceptor blockade (Figure 5a lower panel). This was in contrast to the isolated hearts not incubated with testosterone: smaller and insignificant differences in post-ischaemic recovery of LVDP, $\pm dP/dt_{max}$ and LVEDP upon blockade of either α_1- or β_1-adrenoceptors (Figure 5b). There was a slight, but significant, greater change in $+dP/dt_{max}$ upon α_1-adrenoceptor blockade than β_1-adrenoceptor blockade (Figure 5b lower panel). As seen with injury and contractile recovery, arrhythmias during reperfusion were significantly enhanced upon blockade of α_1-adrenoceptor blockade but significantly increased upon β_1-adrenoceptor blockade (Figure 6a). More importantly, the increase in incidence of VT following α_1-adrenoceptor blockade was 23%, which was significantly greater than that following β_1-adrenoceptor blockade (Figure 6a). In the absence of testosterone, blockade of either adrenoceptor did not lead to any significant difference in arrhythmias (Figure 6b).

Expression of α_1- and β_1-adrenoceptors in the isolated heart from sham control, ORX and ORX + T rats

The expression of α_{1A}- and β_1-adrenoceptors was lower in hearts from ORX rats than sham control (Figure 7), whereas that of α_{1B} and α_{1D}-adrenoceptors was unchanged (data not shown). Testosterone replacement (ORX + T) restored the expression of α_{1A}- and β_1-adrenoceptors (Figure 7).

Discussion

The first important finding of the present study was that testosterone reduced injury and arrhythmias and improved contractile recovery during ischaemia and reperfusion and upon increased sympathetic stimulation *in vitro*, a situation that mimics myocardial ischaemia *in vivo*, when there is an increased sympathetic discharge to the heart. The observation indicates that testosterone confers cardioprotection against ischaemic insult. The protective effects were observed in both the isolated perfused heart, a model that is

close to the *in vivo* situation, and cardiomyocytes, showing that the effects were directly on the myocardium. This finding is also in agreement with a previous observation that testosterone is associated with a reduced susceptibility to myocardial ischaemia (Callies *et al*., 2003). The advance in the present study is that we showed that the protective effect of testosterone is due to a direct action on the myocardium in addition to the beneficial effects of androgen, such as dilation of the coronary artery (Weidemann and Hanke, 2002). The cardioprotective action of testosterone observed previously (Callies *et al*., 2003) and in the present study provides an explanation of the observation that administration of testosterone improves the angina threshold in men (English *et al*., 2000b; Malkin *et al*., 2004).

Another important finding was that testosterone enhanced the effects of stimulation of both α_1- and β_1-adrenoceptors, thus enhancing both cardioprotection and injury, respectively. The effects of testosterone were abolished or attenuated upon androgen receptor blockade, indicating that the effects were mainly androgen receptor mediated. Interestingly, the enhanced cardioprotective effects (reduced injury and arrhythmias, and improved contractile recovery) of cross-talk between testosterone and the α_1-adrenoceptor were greater than the deleterious effect of cross-talk between testosterone and the β_1-adrenoceptor, and therefore the overall effects were beneficial. The β_1-adrenoceptor has long been known to be the predominant receptor in the heart, mediating the sympathetic stimulation of cardiac contraction. This is in agreement with recent observations that myocytes from male mice with double knockout of the α_{1A}- and α_{1B}-adrenoceptor subtypes are more susceptible to apoptosis after oxidative and β-adrenoceptor stimulation, and survival of these mice in response to pressure overload by transverse aortic constriction is significantly reduced (O'Connell *et al*., 2006). Taken together,

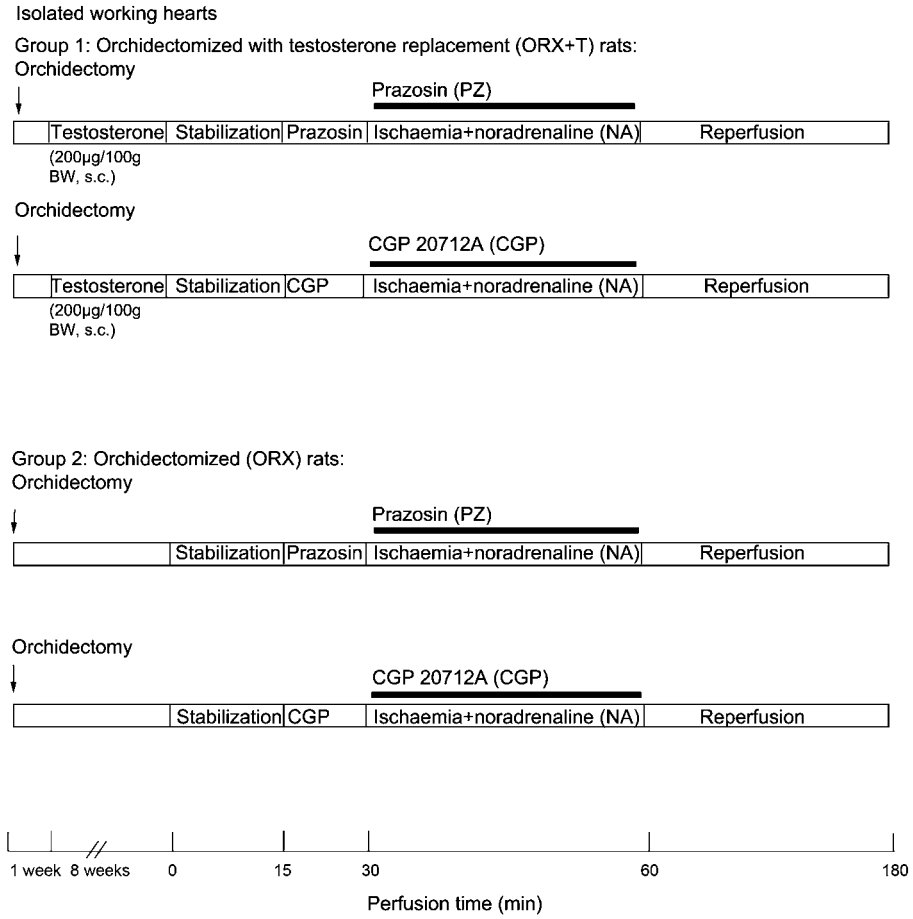
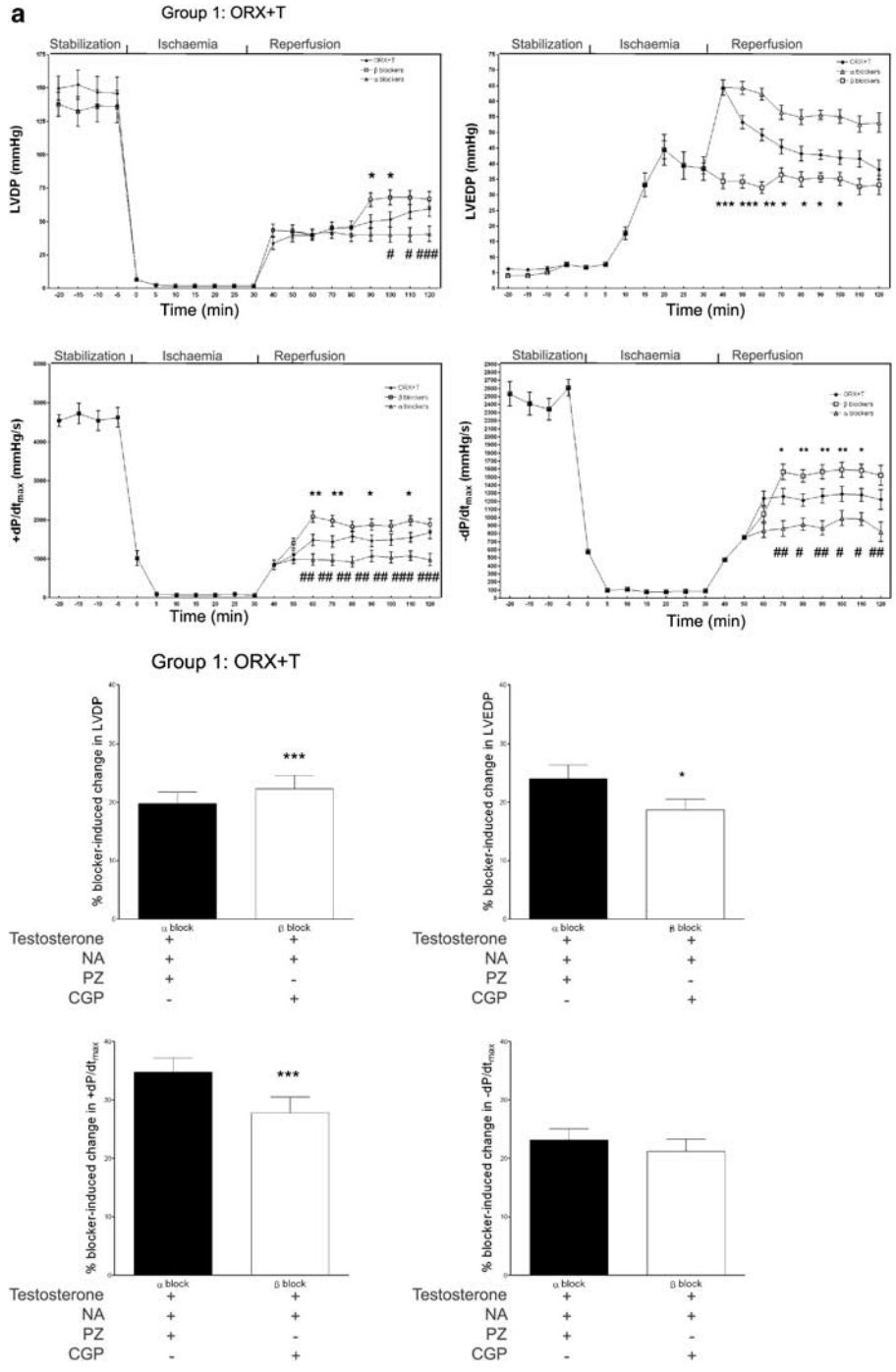


Figure 5 Effects of ischaemic insult in the presence of noradrenaline (NA) on cardiac contractile variables in the isolated hearts from ORX and ORX + T rats upon blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. For blockade of α_1-adrenoceptors, the selective α_1-adrenoceptor antagonist 10^{-6} M prazosin (PZ) was administered. The β_1-adrenoceptor-selective antagonist CGP at 5×10^{-7} M in the presence of ICI at 5×10^{-7} M was used to block both β-adrenoceptor subtypes. Noradrenaline (10^{-7} M) was administered. Analyses were made during stabilization (baseline), ischaemia and reperfusion in (**a**) ORX + T and (**b**) ORX rats. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; $\pm dP/dt_{max}$, velocity of contraction and relaxation, respectively. Lower panel: net changes in contractile variables after blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. Values are mean ± s.e.mean of 9–12 rats. $^*P<0.05$, $^{**}P<0.01$ vs ORX + T; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs CGP. CGP, CGP 20712A; ICI, ICI 118, 551; ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 μg per 100 g).



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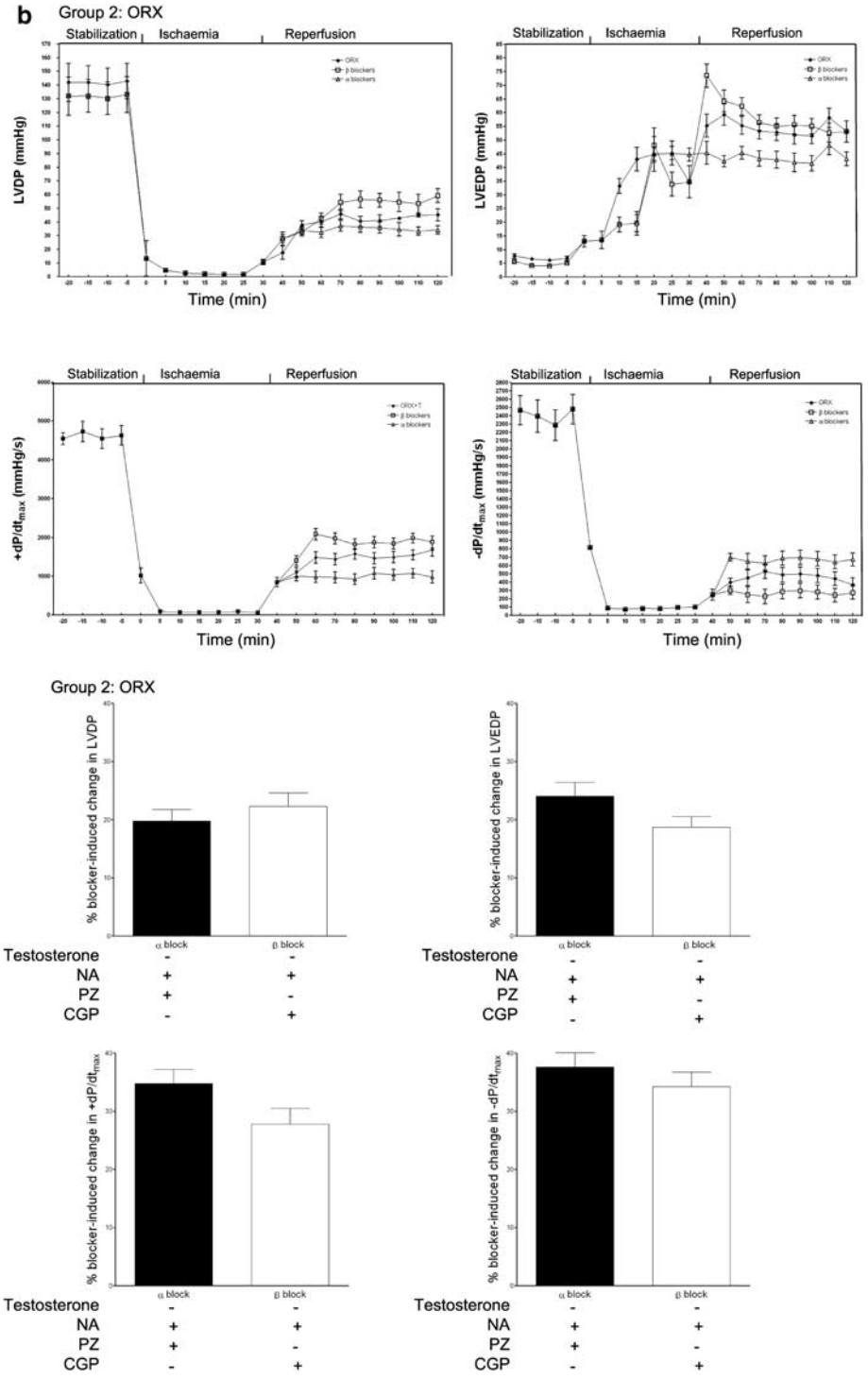
the previous findings and the present study provide evidence that the α_1-adrenoceptor may play an important role in cardioprotection, particularly in the male.

There are three α_1-adrenoceptor subtypes, namely, α_{1A}, α_{1B} and α_{1D}, having different actions. In the present study, we found that testosterone upregulated the expression of the α_{1A} subtype, suggesting that α_{1A}-adrenoceptor may be the main receptor subtype cross-talking with testosterone. In support of this, studies from transgenic mice showed that overexpression of the α_{1A}-adrenoceptor improved outcome after myocardial infarction (Rorabaugh *et al.*, 2005; Du *et al.*, 2006), enhanced cardiac contractility (Lin *et al.*, 2001) and reduced arrhythmias (Woodcock, 2007), which are in agreement with our findings from experiments with selective agonists and antagonists that activation of the α_1-adrenoceptor led to reduced cardiac injury, increased contractile recovery and reduced arrhythmias in response to ischaemic insult, whereas the opposite was observed with blockade of the receptor. In addition, a previous study showed that the α_1-adrenoceptor is antiapoptotic (Iwai-Kanai *et al.*, 1999).



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Further study is urgently needed to determine the signalling mechanisms of the α_1-adrenoceptor subtypes in cardioprotection conferred by testosterone.

In the present study, our focus on α_1- and β_1-adrenoceptors was based on the following considerations. Firstly, we believed that α_1- and β_1-adrenoceptors were mainly responsible for cardioprotection and injury, respectively. Secondly, α_2-adrenoceptors are not present in the heart (Porter *et al.*, 2003) and the β_3-adrenoceptor is not expressed in the rat heart (Evans *et al.*, 1996; Gauthier *et al.*, 1999). It should, however, be noted that the expression of the β_2-adrenoceptor, which is also antiapoptotic (Communal *et al.*, 1999; Patterson *et al.*, 2004), was downregulated in rats without testosterone (data not shown), indicating that testosterone may also interact with this receptor subtype. In the present study, the β_2-adrenoceptor was blocked, thus allowing us to

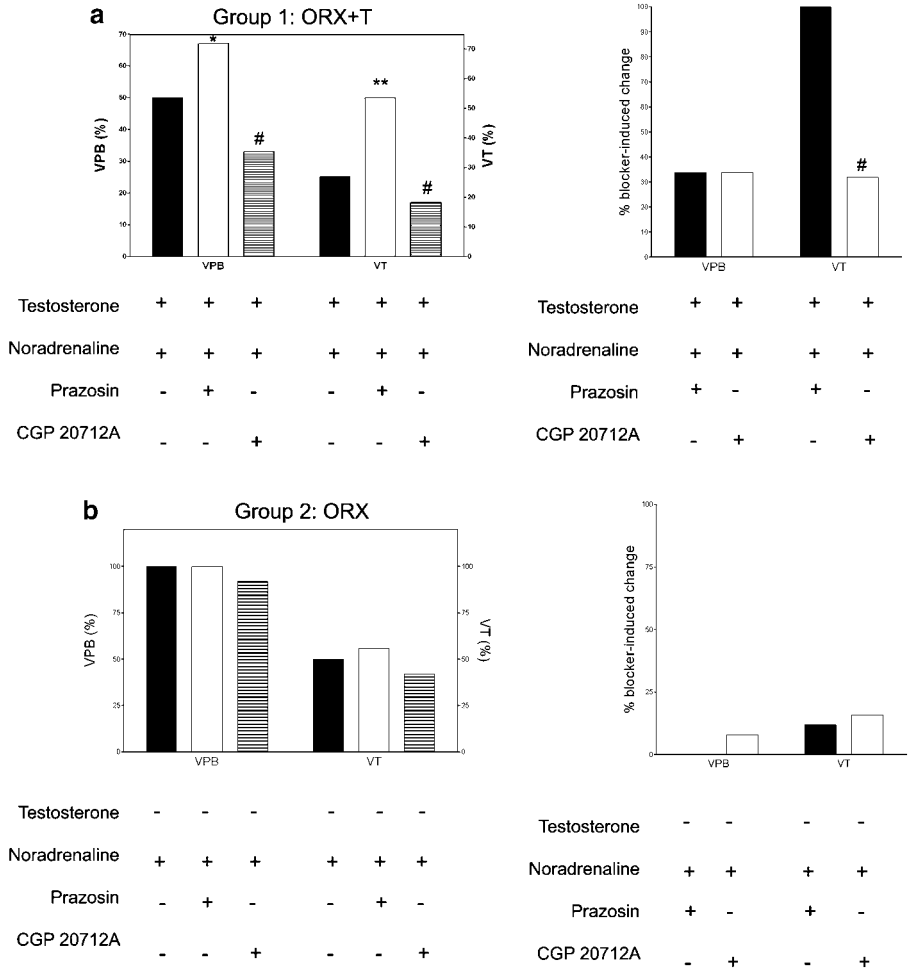

Figure 6 Effects of ischaemic insult in the presence of noradrenaline on reperfusion arrhythmias in the isolated hearts from ORX and ORX + T rats upon blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. The protocol shown in Figure 5 was adopted. Values shown are the percentage of hearts exhibiting VPBs and VT in (**a**) ORX + T and (**b**) ORX rats over the first 30 min reperfusion period. Values are mean ± s.e.mean rounded to the nearest whole number. Right panels: net changes in VPBs and VT after blockade of either α_1- and β_2-adrenoceptors or β_1- and β_2-adrenoceptors. Values are mean ± s.e.mean of 9–12 rats. *$P<0.05$, ** $P<0.01$ relative to vehicle control and $^{\#}P<0.05$ relative to prazosin by χ^2 test for percentages. ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 μg per 100 g); VPBs, ventricular premature beats; VT, ventricular tachycardia.

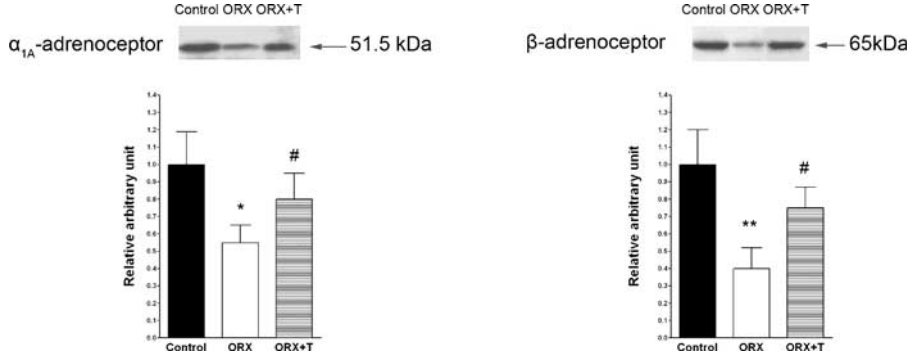

Figure 7 Effect of orchidectomy (ORX) on the expression of α_{1A}- and β_1-adrenoceptors in the rat heart. The expression of subtypes of α_1- and β_1-adrenoceptors was evaluated by western blotting from sham control, ORX and ORX + T as described in Methods. The relative arbitrary unit for sham control group was assigned as 1. Data are normalized to average value of sham control obtained from the same blot. Values are mean ± s.e.mean of 5–6 rats. *$P<0.05$ vs control; **$P<0.01$ vs control; $^{\#}P<0.05$ vs ORX. ORX, orchidectomized male rats; ORX + T, orchidectomized male rats with testosterone replacement (200 μg per 100 g).

compare the contribution of the α_1-adrenoceptor, which was beneficial, and the β_1-adrenoceptor, which was deleterious. Interestingly, even without β_2-adrenoceptors, the beneficial effect of testosterone on cardioprotection was already greater than the deleterious effect, suggesting that testosterone may be even more beneficial than demonstrated in the experimental conditions of the present study.

We found that the enhancing effects of testosterone on α_1-adrenoceptor stimulation were completely abolished by overnight incubation with cyproterone acetate, indicating that these were genomic, androgen receptor-mediated events. On the other hand, the enhancing effect of testosterone on α_1-adrenoceptor action on cell viability was only attenuated by the androgen receptor antagonist, indicating that testosterone may have non-genomic actions. It has been shown that endogenous testosterone induces cytoprotection via activating cardiac mitochondrial ATP-sensitive K^+ channels, an effect not mediated by the androgen receptor (Er *et al*., 2004). These effects are acute, suggesting that they are non-genomic actions and independent of the androgen receptor. This may explain, at least in part, why blockade of its receptor did not abolish the effect of testosterone on cell survival (Er *et al*., 2004).

Two recent studies have shown that chronic administration of testosterone at physiological concentrations enhances Ca^{2+} influx via the L-type Ca^{2+} channel in neonatal (Michels *et al*., 2006) and adult (Er *et al*., 2007) rat ventricular myocytes. In the adult ventricular myocyte, testosterone also increases the Ca^{2+} spark, indicating an increase in Ca^{2+} release from the sarcoplasmic reticulum. These effects are abolished upon androgen receptor blockade, indicating receptor-mediated events. It has also been shown that testosterone increases the Na^+–Ca^{2+} exchange mRNA level in the heart (Golden *et al*., 2004), suggesting increased activity of the exchanger, which is responsible for Ca^{2+} removal. So testosterone may alter Ca^{2+} homeostasis, thus attenuating the $[Ca^{2+}]_i$ overload in response to ischaemic insult and conferring cardioprotection.

It is believed that conversion of testosterone to oestrogen by aromatization may also be responsible for the protective action of testosterone. In the present study, the effects of testosterone were attenuated by androgen receptor blockade, indicating that its actions are mainly mediated via its receptor. So it is unlikely that oestrogen contributes significantly to the protective action of testosterone.

We found that the heart weights differed among groups, which may reflect differences in many parameters such as wall thickness and ventricular volume. So, the cardiac responses to ischaemic insult may not have been due to the presence or absence of testosterone only, but rather the differences in heart weight. However, the cardiac responses to ischaemic insult were similar in the sham and ORX + T groups, which had different heart weights, indicating that the responses to ischaemic insult were not directly correlated with heart size. In support of this, we found in a previous study that after ovariectomy, the heart weight was the same in the sham and ovariectomy groups, but the cardiac responses to ischaemic insult were significantly different (Kam *et al*., 2004, 2005). So, it is unlikely that the different responses to ischaemic insults in different groups were due to heart weight.

IHD is a leading cause of mortality, particularly in the aged, who have low testosterone levels. That testosterone enhances the cardioprotective effect of α-adrenoceptor stimulation makes testosterone particularly useful for the treatment of IHD in patients with low testosterone, particularly aged patients.

In conclusion, the present study has provided the first evidence that testosterone upregulated the expression of α_1-adrenoceptors and enhanced the cardiac responses to their stimulation, thus reducing cardiac injury. Testosterone also improved contractile recovery and reduced arrhythmia upon ischaemia and reperfusion. The effects of testosterone were mediated by androgen receptors. Further studies are warranted to delineate the signalling mechanisms and to explore the possibility of using testosterone and α_1-adrenoceptor activators in the aging male population with IHDs.

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Conflict of interest

The authors state no conflict of interest.

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