

## EXPERIMENTAL STUDY

# Acute actions of testosterone on contractile function of isolated rat ventricular myocytes

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## Abstract

Variation between the sexes in cardiac function have been established. The extent to which sex hormones are responsible for these differences is unclear. The current study was designed to determine whether testosterone acts acutely to enhance contractility of cultured rat ventricular myocytes. Following a 24-h treatment with testosterone (1  $\mu$ M), isolated rat ventricular myocytes display a 21% increase ( $P < 0.01$ ) in peak shortening and an 18% decrease ( $P < 0.02$ ) in time to peak shortening. In accordance with this change, testosterone treatment produced an 18% decline ( $P < 0.002$ ) in the time to relengthening when compared to vehicle-treated controls. These results provide the first evidence that short-term androgen exposure acts directly to stimulate contractility of isolated rat ventricular myocytes and thus may play a role in regulating cardiac performance in males and thereby contribute to sex differences in cardiac function.

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## Introduction

Gender disparities in cardiac function have been well established. For example, women have significantly higher peak- and end-systolic pressures when compared to men (1). Sex differences exist in the control of myocardial adaptations in response to changes in afterload (2). Gender differences in myocardial mechanical properties also have been reported in rodents (3, 4). Sex variances in myocyte calcium handling occur after puberty, suggesting that sex hormones may play a role in cardiac function (5).

Studies examining the impact of androgens on the function of cardiac myocytes are limited. A prerequisite for direct myocyte effects of androgens is the expression in myocytes of the androgen receptor protein. Evidence demonstrating androgen-binding proteins in heart come from Kreig *et al.* (6) and McGill *et al.* (7), who showed the presence of high-affinity androgen-binding proteins in whole-heart cytosol from both rats and primates. However, these studies stopped short of demonstrating whether these androgen-binding proteins were androgen receptors. Furthermore, because the heart is comprised of a heterogeneous population of cells including fibroblasts, nerve cells, and smooth muscle cells, it is still uncertain whether androgen receptor proteins exist in cardiac myocytes.

Cardioregulatory actions of androgens have been documented. Our laboratory has provided the first evidence that gonadectomy and testosterone replacement alters expression of calcium-regulatory proteins and contractile properties of isolated male rat ventricular

myocytes (8). Following a 16-week castration period, isolated cardiac myocytes from male rats display a significant reduction in contraction and relaxation velocities. Testosterone replacement completely restored contractile function. These data, along with those published by Scheuer *et al.* (9, 10), who used an isolated rat heart perfusion model to show enhancement of cardiac function in gonadectomized animals following androgen delivery, provide important insights into the potential role of androgens as cardioregulatory hormones. However, it is uncertain whether androgens exert direct beneficial effects on cardiac myocytes or whether androgens enhance contractile function via activation of other humoral or neural variables. The aims of the current investigation are to first determine whether androgen receptor proteins are detectable in cardiac myocytes and to examine the acute effects of androgens on the contractility of isolated adult rat ventricular myocytes. Understanding the influence of androgens as cardioregulatory hormones in males has important clinical implications. A loss of androgen receptor signaling in males during aging or because of therapeutic intervention (e.g. treatment for prostate cancer) may have important consequences on cardiac function.

## Materials and methods

### Cell-isolation procedures

The animal care committee at the Wayne State University School of Medicine approved this study. Four 60-day-old male Sprague–Dawley rats were housed

individually, fed and watered *ad libitum* and maintained on a 12-h light/dark cycle at 23°C. Single ventricular myocytes were isolated enzymatically as described previously (11). Briefly, hearts were rapidly removed and perfused (at 37°C) with Krebs–Henseleit bicarbonate (KHB) buffer containing 118 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 10 mM Hepes and 11.1 glucose. The KHB was equilibrated with 5% CO<sub>2</sub>/95% O<sub>2</sub>. Hearts were subsequently perfused with a nominally Ca<sup>2+</sup>-free KHB buffer for 2–3 min until spontaneous contractions ceased. This was followed by a 20-min perfusion with Ca<sup>2+</sup>-free KHB containing 223 U/ml collagenase (Worthington Biochemical Corp., Freehold, NJ, USA) and 0.1 mg/ml hyaluronidase (Sigma). After perfusion, ventricles were removed and minced, under sterile conditions, and incubated with the above enzymatic solution for 3–5 min. The cells were further digested with 0.02 mg/ml trypsin (Sigma) before being filtered through a nylon mesh (300 µm) and subsequently separated from the enzymatic solution by centrifugation (60 g for 30 s). Myocytes were resuspended in a sterile filtered, Ca<sup>2+</sup>-free Tyrode's buffer that contained 131 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM Hepes and 10 mM glucose, supplemented with 2% BSA, pH 7.4, at 37°C. Cells were initially washed with Ca<sup>2+</sup>-free Tyrode's buffer to remove remnant enzyme and extracellular calcium was added incrementally back to 1.25 mM. Following the isolation procedure rat ventricular myocytes were treated with either testosterone propionate (1 µM; Sigma) or vehicle for 24 h and mechanical properties were analyzed.

### Immunoblot analysis

Ventricular myocytes were isolated from four 60-day-old male rats (see above) and proteins were solubilized in SDS sample buffer containing 1% 2-mercaptoethanol at 100°C for 10 min. Proteins were separated by SDS/PAGE and then transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% BSA for 30 min in buffer containing 9 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl and 0.08% Tween-20. The membranes were then washed three times for 15 min and probed with a polyclonal anti-androgen receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times for 15 min the androgen receptor protein was detected using enhanced chemiluminescence (ECL) reagent (Amersham).

### Immunohistochemistry

Tissue sections from three 60-day-old male rats were incubated for 1 h in 1.5% blocking serum in PBS (ABC Staining System; Santa Cruz Biotechnology). Sections were incubated with an androgen receptor polyclonal antibody (1:500 dilution; Santa Cruz

Biotechnology) for 30 min overnight at 4°C. Sections were washed and then incubated for 30 min with biotin-conjugated secondary antibody as provided (ABC Staining System) in PBS with 1.5% blocking serum. Detection was accomplished using avidin-biotinylated horseradish peroxidase.

### Cell shortening/relengthening

Mechanical properties of ventricular myocytes isolated from four 60-day-old male rats were assessed using a SoftEdge video-based edge-detection system (IonOptix Corporation, Milton, MA, USA) (11). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (approximately 1 ml/min at 37°C) with a buffer containing 131 mM NaCl, 4 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose and 10 mM Hepes (pH 7.4). The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz and 3-ms duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a MyoPacer stimulator (IonOptix Corporation, Milton, MA, USA). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. The SoftEdge software was used to capture changes in cell length during shortening and relengthening. Myocytes with obvious sarcolemmal blebs or spontaneous contractions were not used; only rod-shaped myocytes with clear edges were selected for recording of mechanical properties.

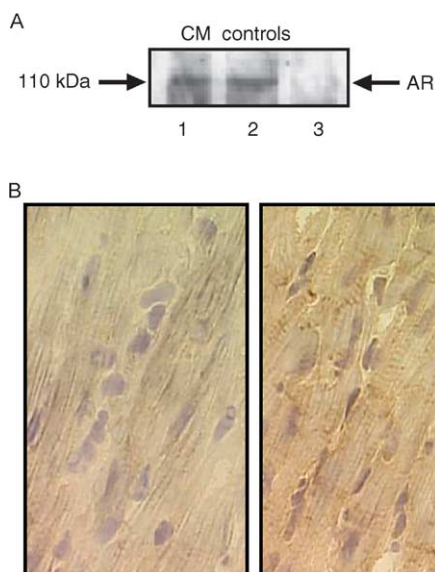
### Data analysis

Data were analyzed using GB-STAT software (Dynamic Microsystems, Silver Springs, MD, USA). Differences between variables were analyzed by the nonparametric Kruskal–Wallis one-way analysis of variance. Data are shown as means ± S.E.M.

### Results

Figure 1A shows immunoblot analysis of androgen receptor protein in isolated ventricular myocytes. As a positive control, protein samples isolated from rat prostate were conducted in parallel (Fig. 1A, lane 2). Figure 1A (lane 1) shows that isolated rat cardiac myocytes are strongly immunoreactive for androgen receptor. We further confirmed the presence of AR by immunohistochemical analysis of adult male heart (Fig. 1B, right-hand panel). Immunostaining of whole heart revealed uniform expression of androgen receptor protein in both cytosolic and nuclear compartments. Sarcomeric striation patterns, a well-known characteristic of cardiac muscle, were evident.

To determine whether androgens are capable of acute actions on cardiac myocyte function, we examined the effects of testosterone (1 µM) on contractile

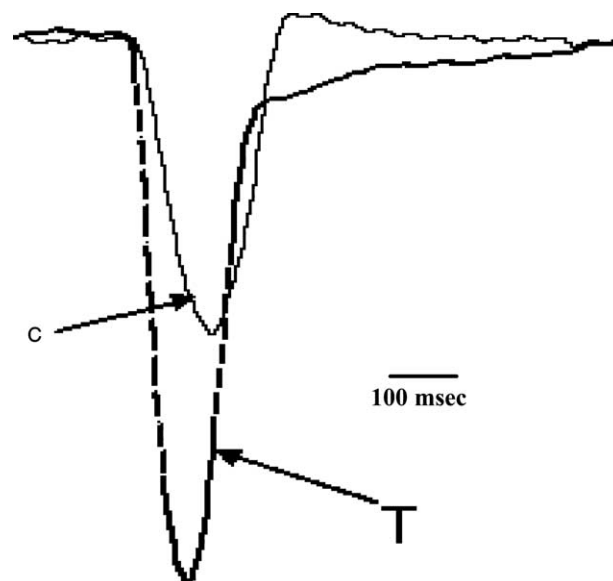


**Figure 1** (A) Detection of androgen receptor protein in isolated rat ventricular myocytes. Cell lysates from isolated cardiac myocytes (CM; lane 1) and rat prostate (lane 2) were probed with an anti-androgen receptor mouse polyclonal antibody. Lane 3 represents a negative control exposed to secondary antibody alone. Protein samples were subjected to SDS/PAGE, electrotransfer and immunoblotting. A representative experiment out of four with similar results is shown. (B) Immunohistochemical staining of rat ventricular myocardium with an anti-androgen receptor antibody shows strong expression of androgen receptor (right-hand panel) within myofibrils. Negative control sections were incubated with secondary antibody alone (left-hand panel).

properties of isolated cardiac myocytes following a 24-h exposure (Fig. 2). This time point was selected to eliminate the confounding influence of androgen-induced hypertrophy on contractile performance that is produced by longer-term treatment (12). The concentration of testosterone was chosen to examine the functional consequences of altered expression of cardiac calcium regulatory genes in response to  $1 \mu\text{M}$  testosterone treatment, which has been published previously (13). Testosterone treatment produced a 21% ( $P < 0.01$ ) increase in peak shortening as normalized to cell length (Fig. 3). Figure 4 shows the effects of testosterone treatment on myocyte contractile velocity. Myocytes exposed to testosterone also displayed an 18% decrease ( $P < 0.02$ ) in time to peak shortening. When the effect of testosterone on myocyte time to relaxation was analyzed, testosterone exposure resulted in a 17% increase ( $P < 0.002$ ) in myocyte relaxation velocity (Fig. 5).

## Discussion

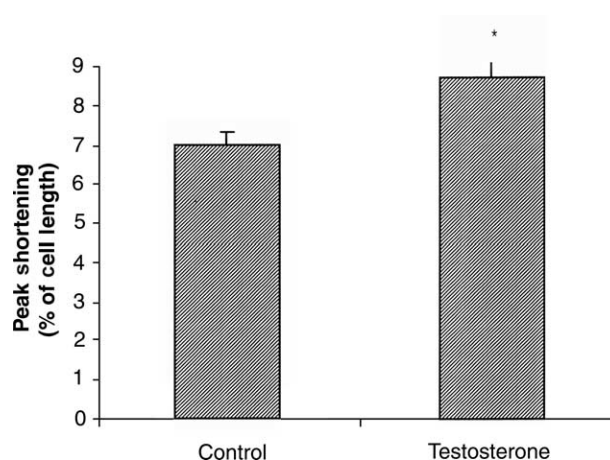
There are two major findings of this investigation. First, we demonstrate for the first time that androgen receptor protein is present in cardiac myocytes. Secondly, we demonstrate a positive inotropic effect of testosterone in



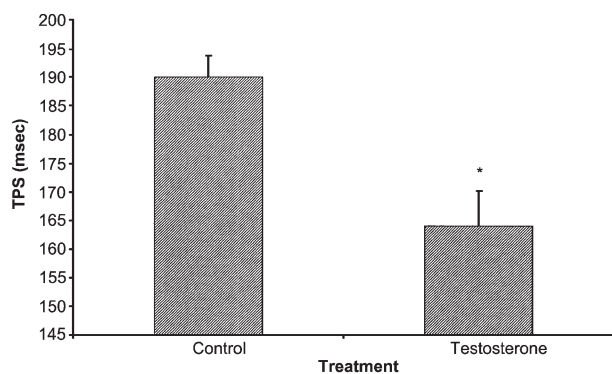
**Figure 2** Testosterone augments contractility of isolated ventricular myocytes. Representative traces showing contractility of adult cardiac myocytes treated with testosterone propionate (T;  $1 \mu\text{M}$ ) or vehicle control (C) for 24 h.

cardiac myocytes that is clearly distinct from systemic effects or paracrine effects of other cell types. Furthermore, it demonstrates that myocytes from a testosterone-rich *in vivo* milieu can respond to pharmacological doses of testosterone *in vitro*. Of note, shorter treatment times of myocytes with testosterone (5 min) had no effect on contraction and relaxation properties (results not shown).

Androgens exert their actions on target tissues by binding to a nuclear receptor which operates as a transcriptional regulator (14). In this study, we have provided novel data demonstrating the presence of androgen receptor protein in isolated cardiac myocytes.



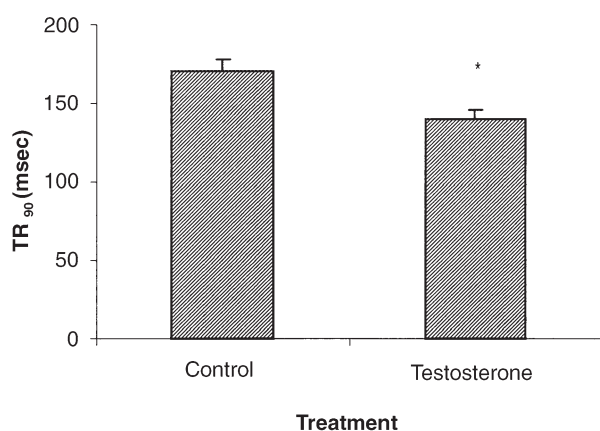
**Figure 3** Testosterone enhances cell peak shortening of isolated cardiac myocytes following a 24 h treatment;  $*P < 0.02$ ,  $n = 55-63$ /group.



**Figure 4** Testosterone (1  $\mu$ M) decreases the time to peak shortening (TPS) of isolated rat ventricular myocytes; \* $P < 0.001$ ,  $n = 55-63$ /group.

These results support those of Marsh *et al.* (12) who showed the existence of androgen receptor transcripts in isolated cardiac myocytes from animals and humans. The androgen receptor mediates dramatic effects of androgens on cardiac morphology and gene expression (12, 13). Although it is difficult to be certain whether the aggrandized cardiac response to androgen treatment in the current study is due to direct signaling via myocyte androgen receptors, it is hypothesized given that androgen receptors are required for androgenic stimulation of cellular and nuclear hypertrophy (12, 13). These conclusions are based on data showing that androgen-induced structural alterations of isolated cardiac myocytes are inhibited by the androgen receptor antagonist cyproterone acetate. The presence of androgen receptor protein in ventricular myocytes supports a direct regulatory role of androgens on cardiac function.

Consistent with our previously published results, cultured cardiac myocytes display a significant increase in contractile velocity following acute testosterone treatment (8). Surprisingly, the magnitude of this effect is



**Figure 5** Testosterone reduces the time to 90% relengthening (TR<sub>90</sub>) of isolated ventricular myocytes; \* $P < 0.002$ ,  $n = 55-63$ /group.

identical when comparing cultured myocytes treated *in vitro* to freshly dissociated myocytes from gonadectomized animals treated chronically with and without androgens. These results suggest that androgens are capable of directly enhancing cardiac myocyte function in the presence and/or absence of other cardioregulatory hemodynamic and humoral factors. *In vivo* studies show that augmented contractile velocity in orchietomized animals administered testosterone is associated with a profound switch in myocyte myosin heavy-chain isoform expression from the slower myosin heavy chain  $\beta$  to the faster myosin heavy chain  $\alpha$  and enhanced L-type calcium-channel mRNA accumulation (8). In support of the latter, Liu *et al.* (15) have shown that a consensus hormone-response element within the 5'-untranslated region of the gene encoding the  $\alpha_{1c}$  subunit of the L-type calcium channel alone is capable of driving reporter gene expression in response to testosterone treatment. These data suggest that testosterone may contribute to the larger intracellular calcium transients reported in male myocytes when compared to females (16). The relative contribution of myosin isoform switching and enhanced calcium-channel expression to the overall increase in contractile velocity and peak shortening are unclear but it demonstrates that testosterone regulates the expression of key proteins that affect myocyte contractility.

Similar to the changes in shortening velocity, acute testosterone treatment produced a coordinate comparable increase in the rate of relaxation of isolated cardiac myocytes. Our results are consistent with reports of increased relaxation rates in isolated papillary muscles isolated from gonadectomized animals administered testosterone (10). The intracellular mechanism by which testosterone enhances relaxation of cardiac myocytes is unclear but may involve a faster rate of calcium removal. In support of this hypothesis, Hintz *et al.* (17) showed that cardiac myocytes isolated from gonadectomized animals display a prolonged calcium-transient decay rate when compared to cardiac myocytes isolated from sham-operated controls. Additionally, our laboratory has shown that castration of male rats reduces cardiac myocyte Na/Ca-exchanger gene expression. Androgen replacement completely restores Na/Ca-exchanger mRNA levels, most likely via complex protein-protein interactions involving the androgen receptor and a number of *cis*- and *trans*-acting factors (18).

In summary, this is the initial demonstration of androgen receptor protein in isolated cardiac myocytes, which provides initial mechanistic insights into androgen signaling in heart. Furthermore, it is clear that androgens act acutely to stimulate contractility of cardiac myocytes independent of other factors which can influence myocyte function *in vivo*. The extent to which a decline in androgens, as a result of aging or as part of treatment for some tumors (e.g. prostate), impacts cardiac function has not been explored.

However, androgen-replacement therapy has gained considerable attention over the past few years as a means to prevent or reverse aging. The ability of testosterone to enhance contractility of ventricular myocytes may account for some of the observed sex differences in cardiac function.

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