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Castration Reduces mRNA Levels for Calcium Regulatory Proteins in Rat Heart

Kish L. Golden, James D. Marsh, and Yang Jiang

Department of Physiology, and Internal Medicine, Wayne State University and John D. Dingell VA Medical Center Detroit, MI 48201

Sex-related differences in the cardiac phenotype have been well established. This study was designed to determine whether androgens regulate myocardial gene expression and play a role in the sex-related differences in the myocardial phenotype. Gonadectomized male rats were treated with testosterone, and myocardial gene expression was examined in whole heart using quantitative real-time PCR. Gonadectomy produced a substantial decrease in mRNA levels for the androgen receptor, Na^+/Ca^{2+} exchanger, L- type calcium channel, and β_1-adrenergic receptor (β_1AR). Supplementation of testosterone in castrates produced a fivefold increase in androgen receptor mRNA levels. Testosterone treatment of castrates produced almost a sixfold increase in Na^+/Ca^{2+} exchanger mRNA, a tenfold increase in L-type calcium channel mRNA accumulation, and a fourfold increase in β_1AR mRNA levels. Increased calcium channel expression, β_1AR expression, and Na^+/Ca^{2+} exchanger expression together may alter cytosolic calcium. These results provide the first evidence that testosterone regulates expression of myocardial calcium regulating genes and thus may play a role in modulating the cardiac phenotype in males.

Key Words: Testosterone; calcium channel; androgen receptor; Na^+/Ca^{2+} exchanger; β_1-adrenergic receptor.

Introduction

Several aspects of cardiac phenotype are different between men and women, and these differences have physiological and pathological importance. For instance, premenopausal women have a lower incidence of cardiovascular diseases than men of the same age *(1,2)*. Furthermore, even when adjusted for body weight, the female heart of many species is smaller than that of males. Sex-related differences in the heart's ability to adapt to strenuous exercise also have been reported *(3)*. Sex-specific variances in cardiac function may be due to regulatory mechanisms induced by sex-related biological factors, since gonadectomy of rats has a profound effect on cardiac contractile performance. In heart perfusion studies, gonadectomized male and female rats display reduced cardiac function that can be reversed with hormone replacement *(3)*. The cellular mechanisms by which testosterone modulates cardiac function has not been clearly defined. Hypogonadism as a result of therapeutic interventions or a reduction in circulating steroid hormones during male andropause may have negative effects on cardiac performance.

Androgen-receptor messenger RNA has been detected in male adult rat cardiac myocytes, neonatal rat myocytes, dog heart, and juvenile human heart *(4)*. Furthermore, both testosterone and dihydrotestosterone stimulate hypertrophy of cardiac myocytes as determined by indices of protein synthesis and ANP secretion. Specificity of the response was established by blocking the hypertrophic response with an androgen receptor antagonist. This demonstrates that the hypertrophic effects of the androgenic steroids are mediated specifically by hormone binding to the androgen receptor. The mechanism by which androgens stimulate myocardial hypertrophy is unclear. Agonists that increase intracellular calcium have positive inotropic effects and activate transcriptional pathways that induce cardiac hypertrophy *(5)*. The sarcolemma and intracellular organelles ferry calcium into and out of the cytosol and play a central role in maintaining cytosolic calcium concentration. The L-type calcium channel provides the primary calcium influx pathway in cardiac myocytes, while the Na^+/Ca^{2+} exchanger is the predominate mechanism for calcium extrusion. Furthermore, variations in cardiac β_1-adrenergic-receptor expression may also promote alterations in intracellular calcium handling.

The purpose of these studies is to determine whether testosterone regulates the expression of calcium regulating proteins and β_1 adrenergic receptors in hearts of adult male rats. Our hypothesis is that testosterone-induced alterations in the expression of these proteins may lead to anomalous regulation of intracellular calcium and provide a potential pathway for androgenic stimulation of cardiac hypertrophy and altered cardiac performance.

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Author to whom all correspondence and reprint requests should be addressed: Kish L. Golden, PhD, Wayne State University School of Medicine, 421 E. Canfield Ave., Detroit, MI 48201. E-mail: kgolden@med.wayne.edu

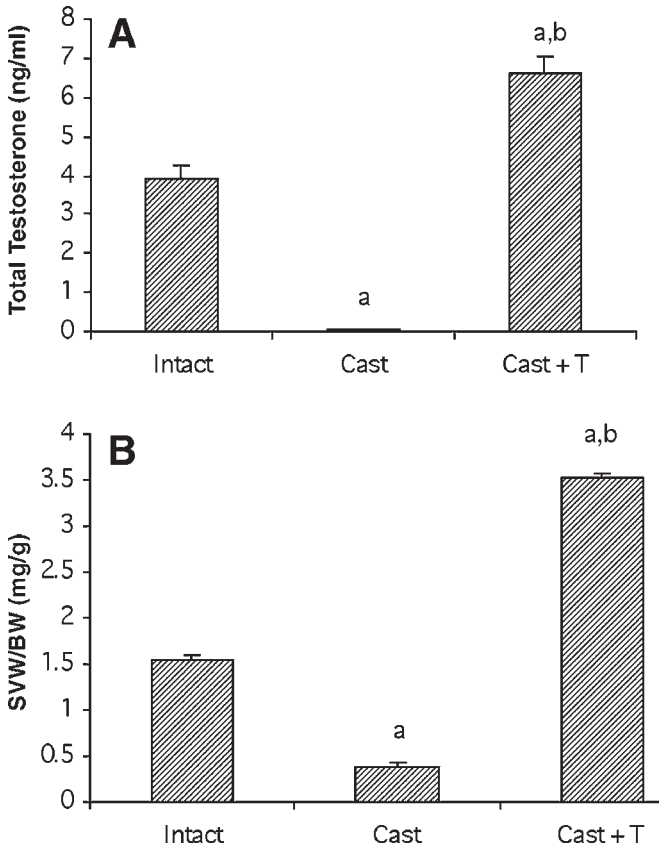

Fig. 1. Serum testosterone concentrations (panel A) and seminal vesicle weights (SVW) (panel B) from castrated (Cast) rats treated with and without testosterone propionate (T) for 14 d. Data are shown as mean ± standard error of the mean. a, $p < 0.01$ vs intact; b, $p < 0.01$ vs castrates; $n = 5$/group.

Results

To determine the level of testosterone released from silastic capsules, a radioimmunoassay (RIA) was performed on serum obtained from castrates treated with and without testosterone. As expected, testosterone levels in castrated animals were below detectable limits (Fig. 1). Supplementation of testosterone in castrated animals significantly increased serum testosterone to levels approx twofold higher than concentrations observed in normal male rats (Fig. 1).

Seminal vesicle weight in relationship to total body weight is shown in panel B, Fig. 1. This served as an additional bioassay for the efficacy of testosterone treatment to complement the RIA data. Castration significantly reduced seminal vesicle weights. Testosterone treatment of castrated animals produced approx sevenfold increase in seminal vesicle weights.

To assess whether testosterone treatment of castrated animals produces cardiac hypertrophy, we determined heart weight to body weight ratios for each group (Fig. 2). Following a 2-wk treatment, testosterone-treated castrates displayed a significant increase (16%, $p < 0.01$) in heart weight to body weight ratios when compared to non-treated castrates. The absolute heart weights were 1.19 ± 0.01 g for castrates and 1.42 ± 0.05 g for castrates treated with testosterone. Pilot studies indicated no significant changes in heart weight to body weight ratios following a 1-wk treatment (data not shown).

To determine whether testosterone regulates expression of its own receptor, androgen-receptor (AR) mRNAs in heart were measured using quantitative real-time PCR (Fig. 3). Castrated animals displayed low levels of AR expression, which increased fivefold with testosterone treatment.

In Fig. 4, we analyzed the effects of testosterone on the expression of genes encoding the Na^+/Ca^{2+} exchanger, the α_{1c}-subunit of the L-type calcium channel (DHP receptor) and the β_1-adrenergic receptor. Castration produced a dramatic decrease in the levels of all mRNAs studied. Testosterone treatment of castrated animals produced a sixfold increase in Na^+/Ca^{2+} exchanger mRNA abundance (Fig. 4, panel A), a tenfold increase in DHP receptor mRNA abundance (Fig. 4, panel B), and a sixfold increase in β_1-adrenergic receptor message levels (Fig. 4, panel C). Testosterone treatment was not sufficient to restore normal message levels of any genes measured (Figs. 3 and 4). It is conceivable that the testosterone analog used in this study was adequate to regulate seminal vesicle weight but insufficient to completely restore the levels of gene expression.

Discussion

Altered circulating testosterone levels occur frequently under physiological and pathophysiological conditions in human males. In addition to the dramatic fall in androgens that occur in males after gonadectomy as part of treatment for some tumors, a substantial fall in plasma androgen concentration occurs as a part of the normal male aging process *(6)*. Thus, a reduction in circulating androgens may alter functional expression of calcium regulatory proteins and thereby influence cardiac contractile function.

In this article we note that gonadectomized animals treated for 2 wk with high levels of testosterone showed a significant increase in heart weight to body weight ratios compared to castrates. These results are consistent with in vitro studies published by Marsh et al., who were the first to demonstrate that cultured cardiac myocytes display an increase in size following treatment with testosterone *(4)*. Although there was no statistical difference in cardiac size between intact animals and castrates receiving testosterone, there was a tendency for the latter group to have greater heart weight to body ratios (Fig. 2). It is possible that a longer exposure to high levels of testosterone could further augment cardiac size and also possibly increase ventricular stiffness *(7,8)*. Published studies have shown that athletes using illicit anabolic-androgenic steroids for long periods develop pathological cardiac hypertrophy and an increase in collagen synthesis *(9)*. The biological activity of testosterone employed in this study was confirmed by noting an increase in semi-

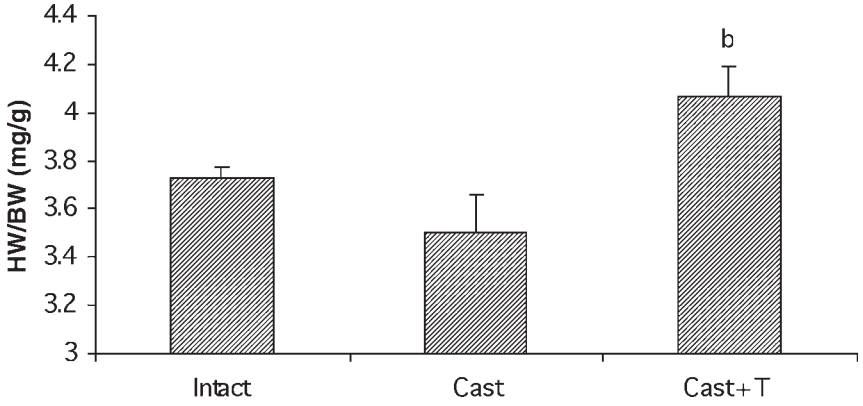

Fig. 2. Heart weight-to-body weight ratios of castrated animals treated with and without testosterone propionate (T) for 14 d. Data are shown as mean ± standard error of the mean. b, $p < 0.01$ vs castrates; $n = 5$/group.

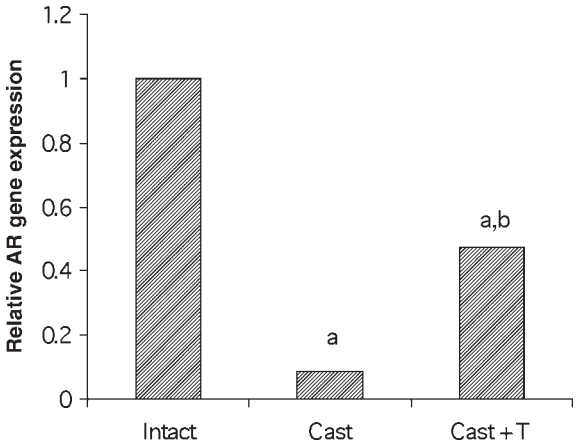

Fig. 3. Relative quantity of androgen receptor mRNA abundance in castrated animals treated with and without T for 14 days. a, $p < 0.01$ vs intact; b, $p < 0.01$ vs castrates; $n = 5$/group (SEM bars are too small to be visible).

nal vesicle weights, which is a target tissue for androgens. Thus, the increase in heart size in castrated animals treated with testosterone can be attributed to testosterone per se.

The effects of androgens are mediated through its interaction with the AR; therefore, any effect of testosterone on myocardial gene expression will be influenced by AR density. Here we provide the first evidence that testosterone treatment of castrated animals produced a substantial increase in AR mRNA accumulation in heart. This is consistent with several reports that have documented the ability of androgens to up-regulate their receptor levels. Mora and Mahesh showed that testosterone could increase expression of AR by sequestering it in polyribosomes, thereby enhancing the stability of AR mRNA *(10)*. Chronic pharmacological treatment of rats with androgens has been shown to increase AR expression in motor neurons and alter neuromuscular function *(11)*. The lack of specific AR antibodies has prevented us from accurately measuring AR protein levels using immunoblot analysis. Nevertheless, our results suggest that testosterone-induced changes in myocardial gene expression of calcium regulatory proteins may be, in part, mediated through an increase in AR transcript abundance.

Myocardial relaxation occurs when calcium is extruded from the cytoplasm by the Na^+/Ca^{2+} exchanger and sequestered into the sarcoplasmic reticulum *(12–14)*. Our results provide the first evidence that testosterone-induced hypertrophy of castrated animals results in an increase in Na^+/Ca^{2+} exchanger message abundance. These data are consistent with various models of hypertrophy and heart failure where functional expression of the Na^+/Ca^{2+} exchanger has been shown to be up-regulated *(15,16)*. Reinecke et al. found an increase in Na^+/Ca^{2+} exchanger transcript abundance in a model of phenylephrine induced hypertrophy *(17)*. Weinberg et al. demonstrated an increase in cardiac Na^+/Ca^{2+} exchanger mRNA levels in animals following aortic banding *(18)*. The specific transcriptional processes by which testosterone can regulate Na^+/Ca^{2+} exchanger expression are unclear. However, it is possible that hormone-bound AR may interfere with transcriptional repressors either by direct protein–protein interactions or through competition for mutual cofactors *(19,20)*. Nevertheless, alterations in functional expression of the Na^+/Ca^{2+} exchanger in response to gonadectomy have been shown previously to modulate relaxation properties of cardiac myocytes *(21)*.

There is substantial disparity in the levels of expression of L-type calcium channels in various models of cardiac hypertrophy. The effects range from no change to significant increases in DHP receptor numbers *(22–25)*. Our laboratory has recently demonstrated an increase in L-type calcium channel expression in hypertrophy following chronic norepinephrine treatment *(26)*. In accordance with the change in expression levels, we also detected an increase in calcium transient amplitude and contractility, which is consistent with increased L-type calcium channel current density. Additional studies are needed to determine whether the amplitude of the calcium transient is modified in our current studies. Testosterone has been shown to potentiate voltage-dependent L-type calcium channel stimulation of calcium

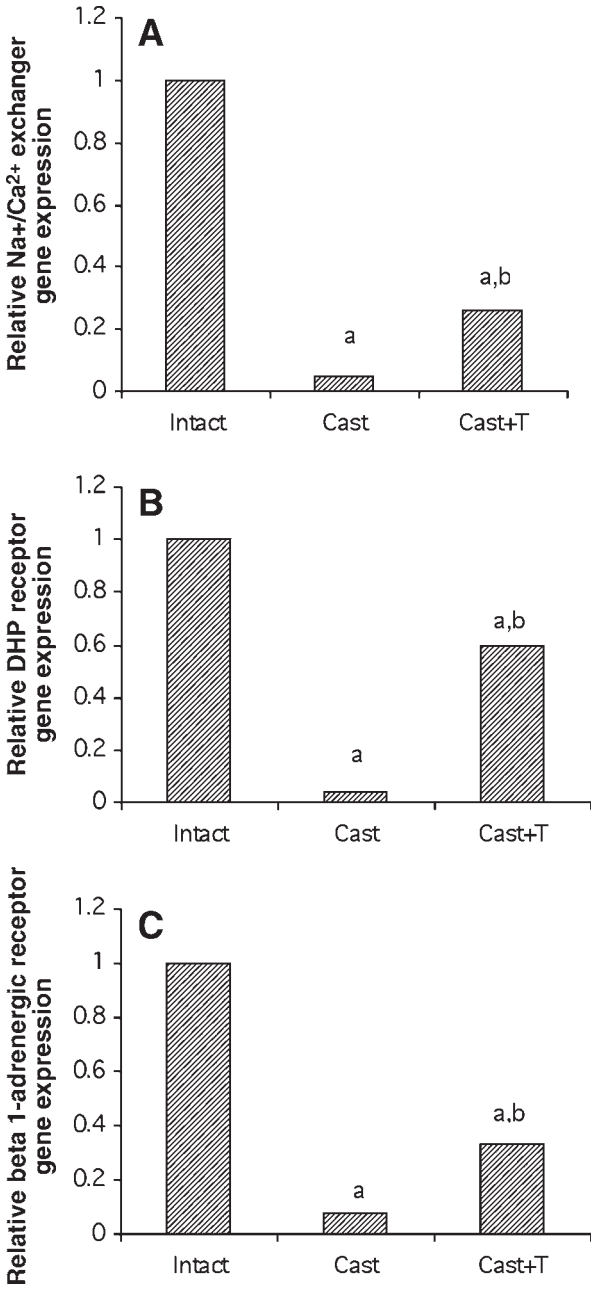

Fig. 4. Relative quantity of Na^+/Ca^{2+} exchanger (panel A), DHP receptor (α_{1c}-subunit of L-type calcium channel) (panel B), and β_1-adrenergic receptor (panel C) mRNA levels in castrated animals treated with and without T for 14 d. a, $p < 0.01$ vs intact; b, $p < 0.01$ vs castrates; $n = 5$/group (SEM bars are too small to be visible).

uptake in vascular smooth muscle cells *(27)*. Decreased expression of the L-type calcium channel in gonadectomized animals may embody the observed reduction in left ventricular function. Work from the Marsh laboratory has shown that the 5' untranslated region of the gene encoding the α_{1c} subunit of the L- type calcium channel contains a consensus HRE *(28)*. When cardiac myocytes are transfected with a reporter gene construct containing the 5' flanking region of the calcium channel α_{1c} gene, there is a marked increase in reporter gene expression following testosterone treatment. These results are consistent with Koenig et al., who demonstrated that acute androgen treatment results in an increase in calcium flux in rat cardiac myocytes *(29)*.

A decrease in functional expression of cardiac β_1-adrenergic receptor appears to be a general phenomenon in various models of decompensated hypertrophy and failure *(30–32)*. In our studies we observed an increase in β_1-adrenergic receptor gene expression. The reasons for these discrepant findings are unclear, but it may be due to the relatively short time course of our studies compared to those employed to induce failure. Consistent with our findings, testosterone-induced increase in lipolytic response to catecholamines in rat white adipocytes is mediated through an increase in beta-adrenergic receptor density *(33)*. Furthermore, published studies demonstrate male myocytes have a higher β_1-adrenergic receptor density when compared to females *(34)*. It is likely that higher β_1-adrenergic receptor density in males may be due to the presence of testosterone.

In summary we have provided the first evidence that testosterone modulates gene expression of AR and calcium regulatory proteins in adult rat heart. Alterations in the levels of expression of calcium-regulating proteins in castrated animals treated with testosterone may influence cytosolic calcium and provide a potential mechanism by which androgens modulate cardiac size and performance. Further studies are needed to determine whether androgens influence the expression of other important calcium regulating proteins including the calcium release channel and the sarcoplasmic reticulum calcium pump.

Materials and Methods

Animal Castration

The animal care committee at the Wayne State University School of Medicine approved this study. Age-matched 60-d-old male Sprague Dawley rats were housed individually, fed and watered ad libitum, and maintained on 12-h light/dark cycle, at 23ºC. Rats were anesthetized and the posterior tip of each scrotum was swabbed with alcohol and betadine solution. A small incision was made into the posterior tip of each scrotum sac. The spermatic cord was tied with 4.0 silk suture and the testes were removed. The incision was closed with 4.0 silk suture and the animal was allowed to recover. Intact animals were sham-operated.

Androgen Replacement

Steroid capsules were prepared by cutting silastic tubing (0.62 id × 0.125 od in.) (Dow Corning, Midland, MI) into 15-mm lengths, sealing one end with silastic adhesive, and filling the capsule with testosterone propionate (Sigma Chemical, St. Louis, MO), a synthetic androgen that cannot be reduced to dihydrotestosterone. The tubes were then sealed with silastic adhesive. Immediately before implantation, capsules were rinsed using 70% ethanol and washed

with sterile saline *(35)*. For capsule implantation, a small lateral incision was made on the back of the neck. The skin was bluntly dissected to form a pocket where the silastic capsule was inserted immediately following castration. Castrated control males were implanted with empty silastic capsules. Two weeks after implantation, the animals were weighed and sacrificed. Blood samples were collected and hearts were frozen on dry ice for subsequent analysis of mRNA levels. Serum testosterone levels were determined using a commercially available radioimmunoassay kit (Coat A Count Total testosterone kit, Diagnostic Products). The assay can detect as little as 4 ng/dL. The assay has a broad working range where the coefficient of variation is low and uniform. Seminal fluid was expressed, and the seminal vesicles were weighed to gauge the efficacy of the exogenous androgen treatments. There were no differences in body weights among groups (data not shown).

Real-Time Quantitative PCR

Total RNA was extracted from rat heart with guanidium thiocyanate-phenol-chloroform by the single-step method as previously described *(26)*. Real-time quantitative RT-PCR was performed on cDNA generated from 300 ng of total RNA using murine Moloney leukemia virus reverse transcriptase (Invitrogen) and random hexamers *(36)*. For the PCR, we used 200 n*M* of both sense and antisense primers (Genset); 30 ng of cDNA and SYBR Green PCR Master Mix (PE Applied Biosystems) in a final volume of 25 µL, and a ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Sense and antisense primers were CGAAGGCAGCAGCAGCGTGAGA and GCGAGCGGA AAGTTGTAGTAGT for AR; GATGGGATCATGGCTT ATGG and GGCCAGCTTCTTTCTCTCCT for α_{1c}–subunit of the L-type calcium channel (DHP receptor); GCCCAGA AACAGGTGAAGAA and GATGATGCCCAGTGTCTTGA for β_1-adrenergic receptor; GTTCGTCGATTGCTGCA TTA and ATTTCCCTCACACCTTGCTG for the Na^+/Ca^{2+} exchanger CGGCTACCACATCCAAGGAA and GCTCG AATTACCGCGGCT for 18S). Fluorescent signals were normalized to an internal reference (ΔR_n) and the threshold cycle (C_t) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR C_t values are normalized by subtracting the 18S C_t value, which gives the ΔC_t value. From this value the relative expression level to 18S for each target PCR was calculated using the following equation: Relative gene expression = $2^{-\Delta C_t}$. Data are represented as relative gene expression versus control.

Data Analysis

Data were analyzed using Sigma-STAT software (Dynamic Microsystems, Inc., Silver Springs, MD). Differences among variables were analyzed by Kruskal–Wallis one-way analysis of variance.

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