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Gonadectomy Alters Myosin Heavy Chain Composition in Isolated Cardiac Myocytes

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Sex differences in cardiac function have been identified. Studies suggest that the presence of testosterone in males may contribute to the observed differences in cardiac function. Our laboratory has shown previously that testosterone treatment of gonadectomized adult male rats enhances contractility of isolated rat ventricular myocytes. In this study we tested the hypothesis that gonadectomy and hormone replacement influences contractility by altering myosin heavy chain (MHC) composition. To test this hypothesis we analyzed myosin isoform expression in ventricular myocytes isolated from castrated rats displaying a decrease in myocyte contractile velocity and compared them to castrates treated with testosterone that displayed normal myocyte shortening velocity. Sixteen weeks after castration isolated rat ventricular myocytes displayed a 90% ($p < 0.001$) decline in MHC-α mRNA levels and over a twofold ($p < 0.01$) increase in MHC-β transcripts when compared to sham-operated controls. Consistent with these changes we also observed a substantial decline in the ratio of MHC-α to MHC-β protein expression. A reversal in myosin heavy chain composition was achieved following testosterone replacement. These studies provide the first direct evidence that testosterone replacement in gonadectomized animals enhances contractility via transcriptional and translational control of myosin heavy chain composition in isolated rat ventricular myocytes. The influence of testosterone on MHC composition in males may underlie some of the observed sex differences in cardiac function.

Key Words: Cardiac myocyte; gonadectomy; testosterone; myosin heavy chains.

Introduction

Androgens may have important cardioregulatory actions in males. Our laboratory has recently demonstrated that gonadectomy of adult male rats significantly slows contractile velocity of isolated rat ventricular myocytes *(1)*. Moreover, androgen supplementation to castrates enhances myocyte contractile velocity to rates comparable to intact controls. The importance of myosin heavy chain (MHC) composition on cardiac contractile function has been well established. Myosin hydrolyzes ATP and this released energy is utilized for muscle contraction. There are two homodimer myosin isoforms present in mammalian cardiac muscle, MHC-α and MHC-β *(2,3)*. Small amounts of hybrid heterodimer myosin that contain one MHC-α peptide and one MHC-β peptide also may be found *(2,3)*. Cardiac myocytes with more MHC-α have faster velocities of contraction compared to hearts that have more MHC-β *(4)*. The relative amounts of myosin heavy chain isoforms vary under both physiological and pathophysiological conditions. For instance, in rats, the ratio MHC-α to MHC-β has been shown to decrease during aging and this is associated with a decline in contractile velocity *(5,6)*. Other reports demonstrate that ventricular MHC isoform composition is different between diabetics and non-diabetics as well as adult nonfailing and failing hearts *(7,8)*. Because of the potential functional significance of altered MHC composition in cardiac function, we examined MHC isoform expression in isolated cardiac myocytes obtained from the same castrated animals treated with and without testosterone, which we previously reported, displayed significant alterations in contractile velocity *(1)*.

Results

Figure 1 shows myosin heavy chain transcript abundance in castrated animals treated with and without testosterone. Following a 16-wk castration period isolated rat ventricular myocytes displayed an 80% ($p < 0.01$) decrease in MHC-α transcript levels (Fig. 1, upper panel) and a twofold ($p < 0.01$) increase in MHC-β transcripts (Fig.1, lower panel). Testosterone treatment of castrates completely reversed these effects. Alterations in gene expression were similar to changes in protein expression where MHC-β levels are reduced and

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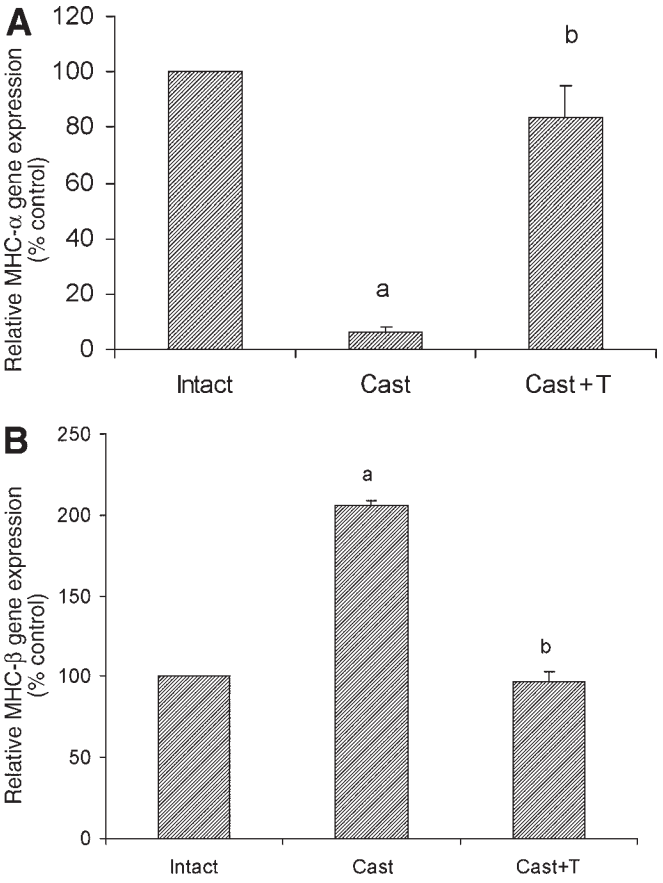

Fig. 1. Relative quantity of myosin heavy chain α (MHC-α) (upper panel) and myosin heavy chain β (MHC-β) (lower panel) mRNA levels in ventricular myocytes isolated from intact and castrated animals treated with (cast+T) and without testosterone (Cast) for 16 wk (see Methods). Each sample was run in duplicate. Data are shown as mean ± standard error of the mean. [a]$p < 0.01$ vs intact; [b]$p < 0.01$ vs castrates.

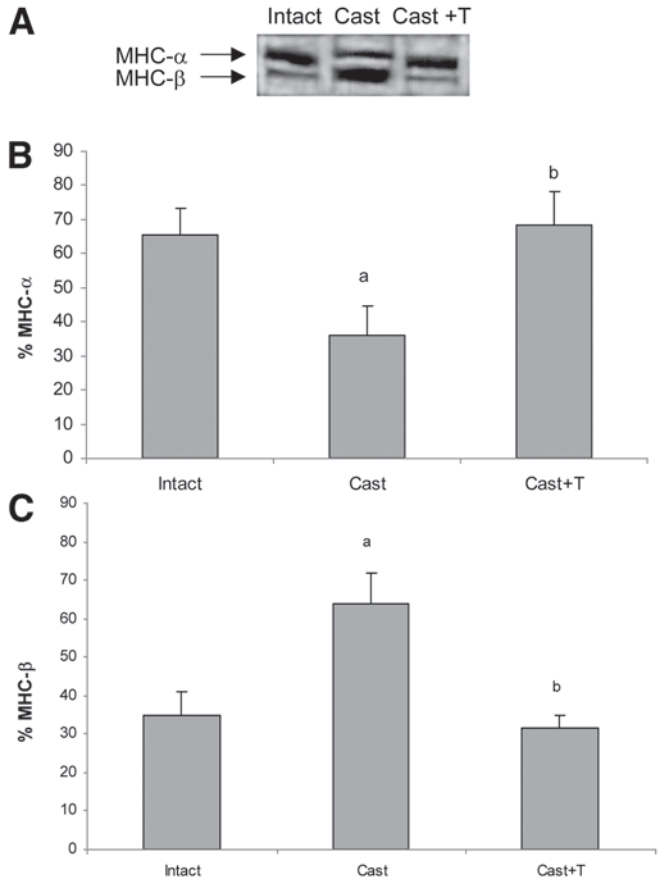

Fig. 2. The effects of castration (cast) and androgen replacement (cast+T) on myosin heavy chain composition in isolated rat ventricular myocytes. (**A**) Myosin heavy chain location on a silver-stained SDS gel (see Methods). (**B** and **C**) Graphic representation of MHC-α and MHC-β isoform expression in isolated rat ventricular myocytes from castrates treated with and without testosterone propionate. Each sample was run in duplicate. Data are shown as mean ± standard error of the mean. [a]$p < 0.05$ vs intact; [b]$p < 0.05$ vs castrates.

MHC-α abundance are increased in castrated animals (Fig. 2). Androgen replacement to castrates completely reversed these effects.

Discussion

Results from the current study provide the first evidence that gonadectomy and androgen replacement elicits coordinate independent regulation of MHC gene and protein expression in isolated ventricular myocytes. As stated earlier, the velocity of cardiac myocyte shortening in gonadectomized animals is increased following androgen replacement *(1)*. Thus, the enhanced rate of contractile velocity is partially explained by a profound switch in myocyte MHC isoform expression from MHC-α to MHC-β. This switch reverses the proportions of MHCs to that seen in intact controls. Studies show that androgens can act directly on isolated cardiac myocytes to coordinate transcriptional alterations in MHC isoforms (our unpublished results). Thus, it is apparent that androgens are capable of controlling MHC isoform expression in cardiac myocytes independent of other humoral and neuronal factors. The presence of androgens in males may account for differences in MHC isoform expression observed in male diabetic hearts when compared to females *(9)*. Nevertheless, our results are consistent with Morano et al. and Schaible et al., who demonstrated similar changes in MHC isoform expression following castration and hormone replacement in whole heart *(10,11)*.

The mechanism(s) by which gonadectomy and hormone replacement affects MHC isoform gene expression are unclear but likely involve activation of dynamic signaling cascades via myocyte androgen receptors *(12)* with consequential alterations in the expression of several genes. Published reports demonstrate that the levels of mRNAs for certain calcium regulatory proteins are affected by gonadectomy and androgen replacement, which may contribute to the observed functional changes *(1)*.

In summary, we have demonstrated that gonadectomy of male rats leads to transcriptional alterations of MHC genes with subsequent changes in myosin protein isoform expression. These effects of androgen deprivation are rescued by testosterone replacement. A fall in plasma androgen concentration occurs as part of the normal male aging process *(13)*. Furthermore, advancing age is associated with changes in MHC composition and slower contraction times *(5,6)*. The extent to which the age-related reduction in myocardial contractility and changes in myosin isoform composition is related to the decline in circulating androgens levels is unclear. However, the current studies suggest that androgens may contribute to the age-associated change in myocardial MHC composition. The ability of testosterone to regulate MHC composition and contractility of isolated rat ventricular myocytes points to a regulatory mechanism of action of androgens on the function of ventricular myocytes. We speculate that the present observation on androgen replacement restoring the youthful MHC phenotype, as well as other salutary effects of androgens on excitation–contraction coupling, may have important therapeutic implications for the aging heart.

Materials and Methods

Animal Castration

The animal care committee at the Wayne State University School of Medicine approved this study. The methods for gonadectomy are as previously described *(1)*. Briefly, six age-matched 60-d-old male Sprague Dawley 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 scrotal sac. The spermatic cord was tied with 4.0 silk suture and the testes were removed. The incision was closed with 4.0 silk sutures and the animal was allowed to recover. Of these six animals, three animals were implanted with silastic capsules containing testosterone propionate (see below), while the remaining three animals were implanted with empty capsules. Three additional age-matched intact animals served as sham-operated controls.

Androgen Replacement

Steroid capsules were prepared as previously described *(1)*. Silastic tubing (0.62 id × 0.125 od in.) (Dow Corning, Midland, MI) was cut into 15-mm lengths, sealing one end with silastic adhesive, and filling the capsule with testosterone propionate (Sigma Chemical, St. Louis, MO). The tubes were then sealed with silastic adhesive. Immediately before implantation, capsules were rinsed using 70% ethanol and washed with sterile saline. 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 into three animals immediately following castration. New capsules were implanted every two weeks to maintain constant hormone levels. Three castrated control males were implanted with empty silastic capsules. Sixteen weeks after implantation, the animals were weighed and sacrificed *(1)*. At the time of sacrifice serum testosterone levels were determined using a radioimmunoassay (RIA) *(1)*. As expected testosterone levels in gonadectomized animals were below detectable limits. Testosterone levels in intact and testosterone-treated gonadectomized animals were 2.1 ± 0.051 ng/mL and 265 ± 0.31pg/mL, respectively.

Cell Isolation Procedures

Single ventricular myocytes were enzymatically isolated from three animals in each group *(1)*. Briefly, hearts were rapidly removed and perfused (at 37°C) with Krebs–Henseleit bicarbonate (KHB) buffer containing (in m*M*): 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 *N*-[2-hydro-ethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 11.1 glucose. The KHB was equilibrated with 5% CO_2–95% O_2. Hearts were subsequently perfused with a nominally Ca^{2+}-free KHB buffer for 2–3 min until spontaneous contractions ceased. This was followed by a 20 min perfusion with Ca^{2+}-free KHB containing 223 U/mL collagenase (Worthington Biochemical Corp., Freehold, NJ) and 0.1 mg/mL hyaluronidase (Sigma Chemical). 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 mm) and subsequently separated from the enzymatic solution by centrifugation (60*g* for 30 s). Myocytes were resuspended in a sterile-filtered, Ca^{2+}-free Tyrode's buffer that contained (m*M*): 131 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, and 10 glucose, and was supplemented with 2% bovine serum albumin, and had a pH of 7.4 at 37°C. Cells were initially washed with Ca^{2+}-free Tyrode's buffer to remove remnant enzyme, and extracellular calcium was added incrementally back to 1.25 m*M*. Following myocyte isolation, gene and protein expression of MHC isoforms were analyzed.

Real-Time Quantitative PCR

Total RNA was extracted from isolated rat ventricular myocytes with guanidium thiocyanate–phenol–chloroform by the single-step method as previously described *(1)*. 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. 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 TCCAGAGGCCAGTACACATTT and AACAGCTG GGGTTGAATTTG for MHC-α; AGTTCAAGGGCCAGT

GCTAC and GCGTCTGCTCCAGGTACTGT for MHC-β and CGGCTACCACATCCAAGGAA and GCTCGAAT TACCGCGGCT for 18S. For the PCR reaction control, samples lacking reverse transcriptase were run to confirm that our PCR product was not a result of DNA contamination. 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. Specificity of each pair of primers was confirmed by subjecting them to melting curve analysis and by subsequent analysis on agarose gels (data not shown). 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 can be calculated using the following equation: Relative gene expression = $2^{-\Delta Ct}$.

Quantification of MHC Isoforms

Myosin isoforms were separated according to previously published procedures *(14,15)*. Preparation of gels were modified from those described by Talmadge and Roy *(16)*. Briefly, the stacking and separating gels are composed of 4 and 8% acrylamide, respectively, containing 5% glycerol *(15)*. The gel and electrode buffers are identical to those in Talmadge and Roy *(16)*, except that 2-mercaptoethanol is added to the upper buffer at a final concentration of 10 m*M* *(16)*. Following electrophoresis, the gels are fixed and silver-stained as in Blough et al. *(14)*, with slight modifications *(14,15)*. The gels are then dried and scanned with a laser densitometer (Molecular Dynamics, Sunnyvale CA).

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. Data are shown as mean ± standard error of the mean.

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