Regulation of Myosin Heavy Chain Expression in the Hearts of Hypertensive Rats by Testosterone

Ingo Morano, Jutta Gerstner, Johann Caspar Rüegg, Ursula Ganten, Detlev Ganten, and Hans Peter Vosberg

**Methods**

Stroke-prone spontaneously hypertensive rats were used for our investigation of the influence of prepubertal gonadectomy and testosterone substitution on blood pressure, cardiac hypertrophy, and the expression of ventricular myosin heavy chain (MHC) isoenzymes at different developmental stages. Blood pressure and the degree of cardiac hypertrophy were decreased by castration and increased by testosterone substitution. We found the same relative distributions of MHC isoforms on the protein level (investigated by pyrophosphate electrophoresis) and on the messenger RNA level (investigated by the polymerase chain reaction). Castration favored the expression of the β-MHC form, and testosterone substitution enhanced the expression of the α-MHC form. These effects were more pronounced in 8-week-old than in 14–16-week-old animals. We conclude that testosterone regulates cardiac MHC expression on a pretranslational level. This regulation is independent of hemodynamic load or cardiac hypertrophy. (Circulation Research 1990;66:1585–1590)

**Materials and Methods**

**Animal Model**

Six-week-old male spontaneously hypertensive rats of the stroke-prone strain (SHRSPs) were gonadectomized (G group), gonadectomized with 3 mg/day dihydrotestosterone substitution (G+T group), or sham-operated (control group). Blood pressure was measured by the tail-cuff technique. Serum testosterone levels were determined by standard radioimmunoassay.

Eight- and 14–16-week-old animals (2 weeks and 10–12 weeks of treatment, respectively) were killed by cervical dislocation. The hearts were excised, blotted, and weighed. Left ventricles were divided into two pieces (one for mRNA and the other for protein analysis) and quickly frozen in liquid nitrogen.

**Pyrophosphate Polyacrylamide Gel Electrophoresis**

MHC isoenzymes were analyzed by pyrophosphate polyacrylamide gel electrophoresis as described. Coomassie-stained MHC bands were evaluated...
densitometrically at 550 nm. The relative amount of the isoenzymes was expressed as percentage of the total sum of areas under the entire tracing. The percent of α-MHC in total MHC then is represented by %V₁ + 0.5 · %V₂.

Quantitative Polymerase Chain Reaction

Relative distribution of MHC-mRNA isoforms was studied by the polymerase chain reaction (PCR). The first step was to synthesize single-stranded complementary DNA (cDNA) to define segments of the two MHC mRNAs. Total DNA-free RNA was prepared according to the method of Chomczynski and Sacchi. RNA (1 μg/ml) was incubated with reverse transcriptase and two different forward primers (25 pmol each) specific for the 3' part of α-MHC (oligo 2) and β-MHC (oligo 4) mRNA. The sequence of α- and β-MHC cDNA was taken from Mahdavi et al. In the PCR, complementary strands were synthesized on the cDNA by adding two distinct specific 5'-labeled oligonucleotides, 77 bp (oligo 1) and 99 bp (oligo 3), to the initial primer for the α- and β-MHC mRNA, respectively (Figure 1). This led to the amplification of two distinct double-stranded cDNA products specific for the α- and β-MHC in the same test tube. cDNA products were amplified using 2 units Taq-polymerase (AGS, Heidelberg, FRG) in a medium containing 67 mM Tris-HCl, 1.5 mM MgCl₂, 16.6 mM ammonium sulfate, 0.2 mM nucleotide triphosphate mix, 0.2 mM oligonucleotides 01–04, 10% dimethyl sulfoxide, and 4 mg/ml bovine serum albumin at pH 8.8. Two different temperatures, 90°C (1 minute) for denaturation and 57°C (4 minutes) for both annealing and synthesis, were used per cycle.

Products were analyzed by polyacrylamide gel electrophoresis (8%) after 15 cycles, stained with ethidium bromide, photographed (AgfaPan), and evaluated by film densitometry using a microdensitometer (Joyce). Relative amounts of α- and β-MHC cDNA were determined from the peak areas as described above for the MHC isoenzymes.

Theoretically, amplification of cDNA products can be described by the formula P = m · 2ⁿ, where P is the number of cDNA products synthesized, m is the number of cDNA molecules at the beginning of the reaction, c is the number of PCR cycles, and 2 is the amplification factor (doubling of the cDNA products after each cycle). Thus, because there are two different cDNAs coding for α- and β-MHC, the amount of amplified cDNA products can be used to estimate the relative distribution of the two different transcription products provided that 1) the rate of amplification of both forms is identical and 2) the amplification of cDNA products is an exponential function of the number of cycles.

To check these two prerequisites of quantitative PCR, specific primers for α- and β-MHC cDNA (oligo 1 and 3) were labeled at the 5' ends with 32P using polynucleotide kinase, and the products were analyzed after 3, 6, 9, 12, 15, and 18 cycles by polyacrylamide gel electrophoresis. Radioactivity of the PCR products was evaluated by radioactive scanning and expressed as counts per minute (Figure 2). In our PCR system, the ratios between cDNA product amplification and the number of cycles were identical for both α- and β-MHC cDNAs and could be described by the formula P = m · 1.4ⁿ (an increase of cDNA products by a factor of 1.4 after each cycle) (Figure 2). This exponential amplification rate could be observed up to 18 cycles. The efficiency factor of 1.4 has been determined empirically from the radioactivity of the cDNA products.

Results

Analysis of Blood Pressure, Cardiac Hypertrophy, and Serum Testosterone Levels

Table 1 lists data for blood pressure, heart weight, body weight/heart weight ratio, and serum testosterone levels of 8-week-old (2 weeks of treatment) and 14–16-week-old (8–10 weeks of treatment) SHRSPs from the control, G, and G+T groups. After 2 weeks of treatment (8-week-old SHRSPs), hypertension and the degree of cardiac hypertrophy were lower in the G group than in the control group. Testosterone substitution reversed the castration effects. Serum testosterone levels decreased upon gonadectomy (from 12 nmol/l in the control group to 4 nmol/l in the G group), whereas testosterone substitution led to an elevated level (77 nmol/l).

Similar results were obtained after 8–10 weeks of treatment (14–16-week-old SHRSPs). Blood pre-
sure, the degree of cardiac hypertrophy, and serum testosterone levels decreased in the G group. The G+T group showed these values to be elevated compared with those observed in the control group.

**Analysis of Isoforms on mRNA and Protein Level**

**MHC-mRNA isoforms.** As shown in Figure 3, 15 PCR cycles were enough to yield a product signal in the polyacrylamide gel electrophoresis after fluorescence staining. This is well within the exponential phase of product amplification (see Figure 2). Since all prerequisites of quantitative PCR fit our conditions well, the relative distribution of α- and β-MHC-specific mRNA could be analyzed after 15 PCR cycles. Eight-week-old control SHRSPs revealed predominantly the α-MHC mRNA type (79%). Gonadectomy led to a redistribution of the MHC mRNA types to the β-MHC form, and α-MHC mRNA decreased to 54%. Testosterone substitution reestablished the control mRNA pattern (83% α-MHC) (Figures 3 and 4). A similar steroid effect could be observed in the 14–16-week-old SHRSPs (Figure 4). At this age, however, differences between control and treated groups were less pronounced. α-MHC mRNAs were 56%, 49%, and 58% in the control, G, and G+T groups, respectively (four animals per group).

**MHC isoenzymes.** Figures 3 and 4 show the different MHC isoenzymes of 8-week-old SHRSPs after separation by native pyrophosphate polyacrylamide gel electrophoresis α-MHC was 89%, 54%, and 87% in the control, G, and G+T groups, respectively. In the 14–16-week-old SHRSPs, α-MHCs were 56%, 46%, and 59% in the control, G, and G+T groups, respectively (Figure 4).

**Comparison Between MHC-mRNA Isoforms and MHC Isoenzymes**

Figure 4 shows schematically the effects of gonadectomy and testosterone substitution on MHC expression after 2 (8-week-old SHRSPs) and 10–12 (12–14-week-old SHRSPs) weeks of treatment. Effects were more pronounced in the 8-week-old than in the 12–14-week-old animals.

Hormone depletion and substitution induced the same effects on the relative distributions of MHC on both the protein and mRNA level, indicating

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**TABLE 1. Heart Weight, Body Weight/Heart Weight Relation, Serum Testosterone Levels, and Blood Pressure of 8-Week-Old and 14–16-Week-Old Sham-Operated, Gonadectomized, and Testosterone-Substituted Stroke-Prone Spontaneously Hypertensive Rats**

<table>
<thead>
<tr>
<th>SHRSP</th>
<th>HW (g)</th>
<th>BW/HW</th>
<th>ST (nmol/l)</th>
<th>BP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-week-old</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.65±0.04 (4)</td>
<td>227±20 (4)</td>
<td>12±2 (10)</td>
<td>167±7 (7)</td>
</tr>
<tr>
<td>G</td>
<td>0.58±0.02 (2)</td>
<td>259±10 (2)</td>
<td>4±0.5 (10)</td>
<td>160±5 (7)</td>
</tr>
<tr>
<td>G+T</td>
<td>0.71±0.01 (2)</td>
<td>206±12 (2)</td>
<td>77±10 (10)</td>
<td>185±9 (7)</td>
</tr>
<tr>
<td>14–16-week-old</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.95±0.05 (4)</td>
<td>229±6 (4)</td>
<td>10±2 (10)</td>
<td>192±11 (7)</td>
</tr>
<tr>
<td>G</td>
<td>0.80±0.08 (4)</td>
<td>264±10 (4)</td>
<td>3±0.1 (10)</td>
<td>160±5 (7)</td>
</tr>
<tr>
<td>G+T</td>
<td>1.15±0.17 (4)</td>
<td>190±29 (4)</td>
<td>44±8 (10)</td>
<td>204±12 (7)</td>
</tr>
</tbody>
</table>

Values are mean ± SD. The numbers of animals investigated are in parentheses. SHRSP, stroke-prone spontaneously hypertensive rats; HW, heart weight; BW, body weight; ST, serum testosterone level; BP, blood pressure; C, sham-operated (control) rats; G, gonadectomized rats; G+T, gonadectomized rats receiving testosterone substitution. The 8-week-old rats received 2 weeks of treatment; the 14–16-week-old rats received 8–10 weeks of treatment.
Figure 3. Analysis by pyrophosphate polyacrylamide gel electrophoresis (PP-PAGE) and quantitative polymerase chain reaction (PCR) of α- and β-myosin heavy chain (MHC) messenger RNA and MHC isoenzymes of 8-week-old stroke-prone spontaneously hypertensive rats (SHRSP) after 2 weeks of treatment. The rats were divided into three groups: control (sham-operated), gonadectomized, and gonadectomized with dihydrotestosterone substitution. Left panel: Analysis of the relative distributions of α- and β-MHC isoenzymes performed by PP-PAGE. Bands were stained with Coomassie blue and evaluated densitometrically. αα, αβ, and ββ correspond to V1, V2, and V3 isoforms, respectively. Right panel: Amplifications of complementary DNA (cDNA) products derived from the reverse transcriptase reaction of MHC messenger RNA performed by quantitative PCR. cDNA fragments (77 and 99 bp for α- and β-MHC, respectively) were separated by gel electrophoresis, labeled with ethidium bromide, and photographed. After 15 PCR cycles, cDNA bands were evaluated by film sensitometry.
pretranslational control of MHC gene expression by testosterone.

Discussion

We estimated the relative amount of mRNA coding for cardiac α- and β-MHC using the PCR to amplify cDNA products derived from the reverse transcriptase reaction. Efficiency of cDNA product amplification in our assay system was lower than expected from theoretical considerations: instead of doubling the cDNA products after each cycle (efficiency of 2^2), apparent amplification efficiency of cDNA products was 1.4. Nonetheless, the basic requirements for quantitative PCR (exponential product amplification, identical amplification efficiencies of the different cDNA isoforms, and cDNA product evaluation within the exponential amplification rate) were given in our experimental conditions. Thus, the relative distribution of both cDNA isoforms could be determined after 15 cycles by simple fluorescence labeling and film sensitometry.

Our results suggest that testosterone regulates cardiac MHC expression of SHRSPs on a pretranslational level due to the similar distributions of α- and β-MHC protein and mRNA isoforms. Gonadectomy caused a redistribution of the MHC isoforms to the β-MHC form, whereas testosterone substitution shifted the MHC isoenzyme pattern to the α-MHC form. This finding is in accordance with our earlier study but is not restricted to genetically hypertensive rats since it could be observed in normotensive rats, too.

Gonadectomy did not completely abolish serum testosterone levels. The remaining concentrations could be sufficient to maintain an α-MHC expression of approximately 50–60% of the total (see “Results”). Differences between the G and control groups became smaller with aging (compare with steroid effects on 8-week-old and 14–16-week-old animals). This may be due to the developmentally induced decreased expression of the α-MHC in the rat heart. Testosterone substitution did not exactly balance the removal of the hormone caused by the surgical procedure. Although plasma testosterone levels were approximately four to six times higher in the G+T than in the control group, redistribution of the MHC isoenzyme pattern always led to the control values. Therefore, testosterone seems to be part of a regulatory system that determines the developmental expression of the different cardiac MHC isoforms. Testosterone is required for the normal run of a fixed genetic program; it is modified by decreased levels rather than by a large excess of the hormone.

In addition to aging, artificially or genetically induced development of hypertension and cardiac hypertrophy is accompanied by a shift of the cardiac MHC isoenzymes to the β-MHC form. Steroid hormones, however, may act independent of these secondary determinants of MHC expression. Gonadectomy decreased both blood pressure and the degree of cardiac hypertrophy while MHC isoenzymes shifted to the β-MHC form. Testosterone substitution increased blood pressure and cardiac hypertrophy but favored the expression of the α-MHC form. Interestingly, the thyroid hormones regulate MHC expression in a comparable manner and are also able to dissociate cardiac MHC isoenzyme pattern from elevated blood pressure. Indeed, the 5’ flanking promoter region of the rat cardiac α-MHC gene contains binding regions for thyroxine and, probably, steroid hormones. Taking the consensus sequence for steroid hormone receptor binding sites (GGTACANNTGTTTC; compare with Reference 15), we observed a putative binding region for steroid receptors at position −1270 to −1295 at the α-MHC gene by sequence comparison. Therefore, both thyroxine and testosterone may regulate cardiac MHC gene expression first by binding the ligands to their specific intracellular receptors and then by direct interaction of the hormone-receptor complexes with specific responsive elements on the α-MHC promoter region.

References


FIGURE 4. Schematic representation of the amount of cardiac α-myosin heavy chain (α-MHC) (percent of total MHC) on both the protein and messenger RNA (mRNA) levels of 8-week-old and 14–16-week-old stroke-prone spontaneously hypertensive rats. Animals were sham-operated (C), gonadectomized (G), and gonadectomized with testosterone substitution (G+T). Values are mean ±SD. Two to four animals per group in the 8-week-old groups and four animals per group in the 14–16-week-old groups were investigated.
14. Flink IL, Morkin E: Thyroid hormone receptors bind to specific DNA sequences in the 5’ flanking region of the rat alpha-myosin heavy chain gene (abstract). Clin Res 1989;37:258A

**KEY WORDS** • testosterone • myosin heavy chain • cardiac muscle • polymerase chain reaction • hypertension
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