Receptor-Mediated Suppression of Cardiac Heat-Shock Protein 72 Expression by Testosterone in Male Rat Heart

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The impact of testosterone on cardiac expression of heat-shock protein 72 (HSP72) remains to be elucidated. Male Sprague Dawley rats 10 wk of age (adult) were castrated. Four weeks later, testosterone (10 mg/kg, ip) was administered as a single dose, followed by the application of hyperthermia (HT) (43 C) at 6 h after testosterone administration. Twenty-four hours later, each heart was isolated. Cardiomyocytes were prepared from 3- to 5-d-old Wistar rats and male Sprague Dawley rats 10 wk of age. Testosterone (0.1–10 μM) was added to the medium, followed by the application of HT (42 C). Twenty-four hours later, cells were collected. We observed the following: 1) Exogenous testosterone suppressed HT-induced HSP72 expression, but castration alone had no influence. 2) HT resulted in better reperfusion-induced cardiac performance in castrated rats comparable with sham-operated rats, which was inhibited by testosterone. The number of apoptotic cells after ischemia/reperfusion was also increased by testosterone. 3) HT-induced HSP72 expression in cultured cardiomyocytes was suppressed by testosterone. 4) HT resulted in less damage to cells, including apoptosis, in response to hypoxia/reoxygenation, which was inhibited by testosterone. 5) Flutamide, a testosterone receptor blocker, cancelled the suppressive effects of testosterone on HSP72 expression. 6) The HT-induced increase in heat-shock factor 1 activity to bind to heat-shock element DNA was suppressed by testosterone, and this was reversed by flutamide. Our results indicate that testosterone potentially has inhibitory effects on cardiac HSP72 expression by modulating transcription, through testosterone receptor-mediated genomic mechanisms. (Endocrinology 148: 3148–3155, 2007)

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Abbreviations: CPP, Coronary perfusion pressure; DAPI, 4',6-diamino-2-phenylindole; DHT, dihydrotestosterone; FAPBSA, fatty acid-free BSA; HSE, heat-shock elements; HSF1, heat-shock factor 1; HSP72, heat-shock protein 72; HT, hyperthermia; KHB, Krebs-Henseleit buffer; LDH, lactate dehydrogenase; L min, left ventricular; LVDP, left ventricular developed pressure; NT, normothermia; released CK, creatine kinase content; siRNA, small interference RNA; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick-end labeling.

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Materials and Methods
All animals were treated in accordance with Oita University Guidelines for the Care and Use of Laboratory Animals.

Materials
Antibody to HSP72 (mouse) was purchased from Stressgen Biotechnologies (San Diego, CA). Horseradish peroxidase-tagged secondary antibodies and enhanced chemiluminescence reagents were purchased from GE Healthcare Biosciences (Piscataway, NJ). Bradford protein assay kits were purchased from Bio-Rad (Richmond, CA). Testosterone and flutamide were purchased from Sigma (St. Louis, MO). Testosterone by estrogen. Because the heart can accumulate testosterone at higher concentrations than other testosterone target organs (10) and testosterone receptors are present in isolated cardiomyocytes (11), it is therefore interesting to know the effects of testosterone on HSP72 expression. In this regard, however, little information is available.

Because HSP72 expression is up-regulated by the activation of its transcriptional factor, heat-shock factor 1 (HSF1), to bind to heat-shock element (HSE) DNA in the promoter region of the HSP gene, confirmation of its increased activity is required when HSP72 expression is observed (12, 13). The isolated protective effects of HSP72 can be clarified by small interfering RNA (siRNA) technique (14, 15). In the present study, using rat heart and rat neonatal and adult cultured cardiomyocytes, we investigated whether testosterone would positively or negatively regulate HSP72 expression in relation to its cardioprotective effects against ischemic insults. In experiments using rat neonatal cultured cardiomyocytes, the activation of HSF1 was assessed using EMISA, and the effects of siRNA targeting HSP72 were also evaluated.
and estradiol enzyme immunoassay kits were purchased from Cayman Chemicals (Ann Arbor, MI).

**Animals and thermo treatments**

Male Sprague Dawley rats 10 wk of age (referred to as “adult” in the present study) were housed in a room illuminated daily from 0700 to 1900 h (12-h light, 12-h dark cycle) with temperature maintained at 21 ± 1 C. All animals were allowed free access to tap water and standard pellet rat chow (Clea Japan, Tokyo, Japan). Male rats of 10 wk were castrated (castrated group) or sham operated (sham-operated group). Four weeks later, the serum concentrations of testosterone and estradiol were quantified using enzyme immunoassay kits. Testosterone at a single dose of 10 mg/kg in 15% of (2-hydroxypropyl)-β-cyclodextrin (testosterone-treated castrated group) or vehicle alone (placebo-treated castrated group) was exogenously administered ip to castrated rats. Our preliminary experiments estimating time-dependent change of serum testosterone levels revealed that, after administration at a single dose of 10 mg/kg, mean serum testosterone concentrations were 446.8 ng/ml at 0.5 h, 314.0 ng/ml at 1 h, 116.3 ng/ml at 3 h, and 29.5 ng/ml (â– 4 times the level observed in sham-operated group) at 6 h after administration and were below detectable levels at 12 and 24 h. Accordingly, 6 h after the administration of testosterone or vehicle, HT (43 C for 20 min), or normothermia (NT) (37 C for 20 min) was applied, as described previously (6, 9). Briefly, rats were anesthetized with pentobarbital (20 mg/kg, ip) and placed, with their heads on a pillow to avoid aspiration of water, for 20 min in a bath in which the water temperature was maintained at 43 C for 20 min. Temperature was monitored throughout the thermo-treatment experiments. Application of HT at 43 C elevated the rectal temperature to 41 C within 10 min, and the temperature was maintained between 41 and 42 C during HT application. Twenty-four hours later, each heart was isolated and prepared for Western blot analysis and for isolated perfused heart experiments.

**Western blotting analysis**

Western blotting was performed as described previously (6, 9). Briefly, rats were heparinized (500 IU/kg, ip) and anesthetized with pentobarbital (50 mg/kg, ip) and placed, with their heads on a pillow to avoid aspiration of water, for 20 min in a bath in which the water temperature was maintained at 43 C for 20 min. Temperature was monitored throughout the thermo-treatment experiments. Application of HT at 43 C elevated the rectal temperature to 41 C within 10 min, and the temperature was maintained between 41 and 42 C during HT application. Twenty-four hours later, each heart was isolated and prepared for Western blot analysis and for isolated perfused heart experiments.

**Isolated-perfused heart experiments**

To examine the reperfusion-induced left ventricular (LV) functional recovery, isolated-perfused experiments using Langendorff apparatus were performed as described previously (6, 9). Twenty-four hours after whole-body HT (n = 8 for each group) or NT (n = 8 for each group), each rat was heparinized and anesthetized. Subsequently, the heart was isolated and perfused retrogradely with Krebs-Henseleit buffer (KHB) [in mM: (pH 7.4) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, and 11.0 glucose] equilibrated with a 95% O2-5% CO2 gas mixture at 37 C with a constant pressure of 75 mm Hg. The coronary effluent during the 30-min reperfusion period was stored on a PCM data recorder (RD-111T; Teac, Tokyo, Japan) for later analysis.

**Culturing of rat neonatal and adult cardiomyocytes, thermo treatments, and hypoxia reoxygenation**

Neonatal cardiomyocytes were prepared from 3- to 5-d-old Wistar rats as described previously (9). Briefly, cardiomyocytes were placed onto 35-mm culture dishes at a density of 5 × 105/cm2 and cultured in DMEM supplemented with 10% fetal bovine serum at 37 C under 5% CO2 and 95% room air. On d 3, the medium was switched to serum-free DMEM supplemented with 5 μg/ml transferrin and 100 μg/ml insulin, and the cells were incubated for 24 h. Testosterone (0.1–10 μM) was applied to the culture medium. Thirty minutes after the testosterone application, the cultured myocytes were incubated at 42 C (HT) or 37 C (NT) for 30 min. HSP72 expression was evaluated 24 h after HT or NT. In some dishes, 10 μM flutamide was added to the culture medium immediately before testosterone application for testosterone receptor blockade. Exposure to HT per se caused few deaths of the cultured cardiomyocytes. To evaluate the tolerance to hypoxia/reoxygenation, HT or NT was applied to cells, which were then incubated for 24 h in the presence or absence of testosterone. The cardiomyocytes were incubated in serum-free DMEM without glucose under a hypoxic gas mixture (95% N2 and 5% CO2) at 37 C for 3 h. The cells were reoxygenated for 1 h with a normoxic gas mixture, and the supernatant was carefully collected for determination of lactate dehydrogenase (LDH) using a LDH assay kit (Eiken Chemical, Tokyo, Japan). Adult rat LV cardiomyocytes were isolated from 10-wk-old male Sprague Dawley rats. Excised hearts were sequentially perfused with KHB, KHB containing 0.5% fatty acid-free BSA (FAMBSA) (Sigma), and KHB containing 0.04% collagenase (type II; Worthington Biochemical, Lakewood, NJ) and 0.23% FABSA. After mechanical dissection, cardiomyocytes were washed in KHB with 0.5% FABSA and then plated on culture dishes coated with 10 μg/ml laminin. Cells were cultured in media M199 Earle (Biochrome, Berlin, Germany) supplemented with 0.2% BSA, 15 μg/ml insulin, 5 mM creatinine, 2 mM carnitine, 5 mM taurine (Sigma), and 100 U/ml penicillin (Invitrogen, Carlsbad, CA).

**EMSA**

Whole-cell extracts from the neonatal cultured cardiomyocytes were assayed by EMSA kit from Panomics (Redwood City, CA) (16). Because HSF1 is normally present in the cell in an inactive form, we were able to use whole-cell lysates. Cells were harvested 1 h after the application of heat shock treatments with protein extracts (10 μg) were performed for 30 min at room temperature in a solution of 2.0 μl of the 5× binding buffer, 1.0 μg poly(dl-dC)-poly(dl-dC), 10 ng of the biotin-labeled probe, and 50 μl distilled water. The samples were then electrophoresed on a 6.0% polyacrylamide gel and transferred to the Biodyne B membrane, and the membranes were exposed to Hyperfilm ECL for 5–20 min. For the competition experiments, the binding reaction mixtures contained a 66-fold molar excess of unlabeled HSE oligonucleotides. For antibody supershift experiments, 1.0 μl HSF1 (Affinity BioReagents, Golden, CO) was added to whole-cell extracts before the binding reaction.

**Apoptosis assays**

In isolated-perfused heart experiments, at the end of the reperfusion period, the hearts were fixed by 4% polyformaldehyde solution in 0.1 M NaH2PO4 for 12 h at room temperature, embedded in paraffin, cut into serial sections 5-μm thick, and stained. Apoptotic cells were detected with the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method using an apoptosis kit (Medical Biological Lab, Nagoya, Japan). Sections were treated with proteinase K and then treated with a mixture of terminal deoxynucleotidyl transferase (TdT), fluorescein isothiocyanate-dUTP, and TdT buffer II at 37 C for 1 h. After washing these slides with PBS, they were mounted with mounting medium using 4',6-diamidino-2-phenylindole (DAPI).
Neonatal and adult rat cultured cardiomyocytes were plated in chamber slides (Nalge Nunc, Naperville, IL). Cells were fixed after reoxygenation in 4% poly paraformaldehyde solution in 0.1 M NaH2PO4 and permeabilized with 0.5% Triton X-100 (Sigma). Apoptotic cells were detected with the TUNEL method using the same apoptosis kit. Slides were treated with a mixture of TdT, fluorescein isothiocyanate-dUTP, and TdT buffer II at 37°C for 1 h. After washing these slides with PBS, they were mounted with mounting medium using DAPI.

### siRNA transfection

The siRNA targeting HSP72 were purchased from Ambion (Austin, TX) and transfected to cells at a concentration of 10 nM with siPORT NeoFX Transfection (Ambion) (14, 15). The control cells were transfected with negative control siRNA. Then, 24 h after transfection, cells were incubated at HT (42°C) or NT (37°C) for 30 min in the presence or absence of testosterone or testosterone/flutamide in the culture medium. HSP72 expression was evaluated 24 h after HT or NT.

### Statistical analysis

Data are expressed as mean ± SEM. Serial changes in LVDP, dP/dt, CPP, and heart rate were analyzed by two-way ANOVA, followed by the Bonferroni-Dunn test. Comparison of physiological and serum parameters, the relative intensity of each protein, the ratio of released CK, and heart rate were analyzed by two-way ANOVA, followed by the Bonferroni-Dunn test. A P value < 0.05 was considered statistically significant.

### Results

#### Basic characteristics

Table 1 summarizes the basic characteristics including serum concentrations of sex hormones of both the sham-operated gonadally intact group and castrated group. Body weight and heart weight were lower in the castrated group than in the sham-operated group (P < 0.01 for each), whereas the ratio of heart weight to body weight was not significantly different. The indices of glucose and lipid were not significantly different between the groups. Regarding sex hormones, the serum concentration of estradiol was not significantly different between the groups, whereas serum testosterone was not detected in the castrated group.

### Effects of testosterone treatment on cardiac HSP72 expression

Figure 1 shows the representative bands of cardiac HSP72 and its relative density in sham-operated, castrated, and testosterone-treated castrated groups. NT resulted in low levels of HSP72 expression without significant differences among the three groups. When compared with NT, HT resulted in a 2.0-fold increase in HSP72 expression in the sham-operated group and castrated group (P < 0.01 for each). However, the HT-induced HSP72 expression was attenuated by treatment with exogenous testosterone (P < 0.01).

#### Effects of testosterone on postischemic LV functional recovery and myocardial damage

At baseline, none of LVDP, dP/dt, CPP, or heart rate showed a significant difference among the six experimental groups, and, during no-flow global ischemia, LVDP in all six groups rapidly decreased to zero (data not shown). Figure 2 shows the serial changes in LVDP during the 30-min reperfusion period. The HT, when compared with NT, resulted in

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**TABLE 1.** Basic characteristics of the two groups of rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham-operated (n = 8)</th>
<th>Castrated (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>373 ± 3</td>
<td>330 ± 3*</td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.03 ± 0.17</td>
<td>0.89 ± 0.18*</td>
</tr>
<tr>
<td>HW (mg/BW g)</td>
<td>2.76 ± 0.53</td>
<td>2.65 ± 0.54</td>
</tr>
<tr>
<td>Blood glucose (mmol/liter)</td>
<td>11.4 ± 0.4</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>Plasma insulin (nmol/liter)</td>
<td>1.25 ± 0.10</td>
<td>1.20 ± 0.14</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>52.3 ± 3.0</td>
<td>50.8 ± 3.2</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>90.3 ± 12.3</td>
<td>95.5 ± 13.6</td>
</tr>
<tr>
<td>Free fatty acid (mg/dl)</td>
<td>1322 ± 201</td>
<td>1384 ± 221</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>9.43 ± 0.64</td>
<td>8.85 ± 0.58</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>6.7 ± 0.7</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. BW, Body weight; HW, heart weight; n.d., not detected.

\*P < 0.01 vs. sham-operated group.
enhanced postischemic LV functional recovery during reperfusion in both the sham-operated group and castrated group ($P < 0.05$ for each by ANOVA). However, the HT-induced improved LV functional recovery was attenuated by treatment with testosterone ($P < 0.05$). With respect to the released CK relative to ventricular weight during the 30-min reperfusion period, HT reduced the amount of released CK in the sham-operated group and castrated group compared with corresponding NT groups ($163 \pm 44$ vs. $356 \pm 62$ IU/g, $P < 0.01$ and $176 \pm 20$ vs. $340 \pm 62$ IU/g, $P < 0.05$, respectively). However, the reduction in released CK in the testosterone-treated castrated group did not reach statistical significance ($248 \pm 47$ vs. $331 \pm 44$ IU/g; $P = \text{NS}$).

**Effects of testosterone on apoptotic cell death by TUNEL staining**

Apoptotic assays in vivo were performed using LV tissue isolated from adult rat. HT reduced TUNEL-positive cells in both the sham-operated and castrated groups (data not shown). As shown in Fig. 3, low levels of TUNEL-positive cells were detected without ischemia (reperfusion only) in the HT-treated sham-operated group ($5.8 \pm 1.2\%$). Ischemia/reperfusion induced an increase in TUNEL-positive cells. Although castration alone did not have an influence, testosterone treatment enhanced the ischemia/reperfusion-induced increase in TUNEL-positive cells ($30.2 \pm 2.7\%$) when compared with the HT-treated sham-operated group ($19.7 \pm 2.4\%; P < 0.01$) and HT-treated castrated group ($17.3 \pm 2.6\%; P < 0.01$). Figure 4, A and B, shows representative photomicrographs of TUNEL staining and the percentage of apoptotic cells in neonatal rat cultured cardiomyocytes and adult rat cultured ventricular cardiomyocytes after hypoxia/reoxygenation, respectively. HT reduced TUNEL-positive cells in both neonatal and adult cultured cells (data not shown). In HT-treated neonatal cardiomyocytes, application of 10 μm testosterone increased TUNEL-positive cells than vehicle-treated cells ($38.8 \pm 5.8$ vs. $21.5 \pm 3.8\%; P < 0.05$). Similarly, in HT-treated adult rat cultured cardiomyocytes, application of 10 μm testosterone increased TUNEL-positive cells than vehicle-treated cells ($43.3 \pm 4.6$ vs. $29.7 \pm 3.2\%; P < 0.05$).

**Effects of flutamide on HSP72 expression in rat cultured cardiomyocytes**

Figure 5 shows representative bands of HSP72 protein and its relative density in rat cultured cardiomyocytes. Application of 1.0 μm testosterone alone did not influence HSP72 expression (lane 2). In the absence of testosterone, HT resulted in a 9.8-fold increase in HSP72 content (lane 3) compared with NT-treated cells (lane 1) ($P < 0.01$). Although 0.1 μm testosterone did not significantly influence expression (lane 4), its application at a concentration of 1.0 μm suppressed the HT-induced HSP72 expression (lane 5) ($P < 0.01$). The suppressive effect was more pronounced at 10 μm testosterone (lane 6). Figure 6 shows the effects of flutamide on testosterone-induced attenuation of HT-induced HSP72 expression. The testosterone at a concentration of 10 μm suppressed HT-induced HSP72 expression by 60.9% (lane 6). Pretreatment with flutamide (10 μm) reversed the suppressive effects of testosterone (lane 7). Flutamide alone showed no suppressive effects on HT-induced HSP72 expression (lane 8).

**HSF1 activity**

As shown in Fig. 7, low DNA-binding activity was observed in NT-treated cells (lane 2, arrow), whereas HT resulted in a large increase in the activated HSF1 levels (lane 3). The HT-induced increase in HSF1 activity was suppressed by treatment with testosterone (lane 4). Pretreatment with flutamide almost reversed the suppressive effects of testosterone (lane 5). Cold competition with unlabeled probe showed no gel shift changes (lane 6). Supershift was observed when an anti-HSF1 antibody was present (lane 7).

**Fig. 3.** Representative photomicrographs of TUNEL staining in LV tissue section. Nuclei with green staining indicated TUNEL-positive cells. Quantitative results of TUNEL staining for different groups are shown at the right. Sham, Sham operated; Cast, castrated; Cast+T, castrated and testosterone (10 mg/kg, ip) treatment; R only, reperfusion only. I/R, ischemia/reperfusion. n = 8 for each group. Data are mean ± SEM. **, $P < 0.01$. NS, Not significant.
Effects of HSP72-siRNA on HSP72 expression and hypoxia/reoxygenation injury

As shown in Fig. 8, the HSP72 siRNA effectively suppressed HT-induced HP72 expression irrespective of the presence or absence of testosterone or testosterone/flutamide. The LDH release in response to hypoxia/reoxygenation was reduced by HT when compared with NT-treated cells (9.0 ± 1.3 × 10⁻³ vs. 30.6 ± 2.5 × 10⁻³ IU/ml; P < 0.01). The HT-induced reduction in LDH release was partially reversed by testosterone (16.1 ± 0.9 × 10⁻³ IU/ml; P < 0.05), and the effect was abolished by pretreatment with flutamide (9.8 ± 1.2 × 10⁻³ IU/ml; P < 0.05). Irrespective of the presence or absence of testosterone and testosterone/flutamide, HSP72 siRNA inhibited the suppressive effects of HT on LDH release to comparable levels (30.3 ± 3.3 × 10⁻³ IU/ml in HT-treated cells, 31.0 ± 3.7 × 10⁻³ IU/ml in HT-treated cells in the presence of testosterone, and 28.2 ± 3.3 × 10⁻³ IU/ml in HT-treated cells in the presence of testosterone/flutamide).

Discussion

In the present study, we demonstrated the suppressive effects of testosterone on cardiac HSP72 expression induced by HT by using heart in vivo and cultured cardiomyocytes. Androgen receptor has been reported to be expressed in neonatal cardiomyocytes (11). We confirmed that mRNA for the androgen receptor was expressed in neonatal cardiomyocytes at approximately half the level as in adult cardiomyocytes by real-

Fig. 4. Representative photomicrographs of TUNEL staining in neonatal rat cultured cardiomyocytes (A) and adult rat cultured ventricular cardiomyocytes (B) after hypoxia/reoxygenation. Nuclei with green staining indicated TUNEL-positive cells. Quantitative results of TUNEL staining for different groups are shown at the right. T, Testosterone (10 μM); H/R, hypoxia/reoxygenation. n = 8 for each group. Data are mean ± SEM. **, P < 0.01.

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The nongenomic effects are characterized by rapid onset (seconds to minutes), which are faster than genomic mechanisms and were not usually blocked by testosterone receptor blockers. These two effects can work inversely. For instance, Michels et al. (18) reported that acute application of testosterone reduced the T-type calcium current (ICa,T) that was not abolished by flutamide, whereas testosterone pre-treatment for 24–30 h (long-term) enhanced ICa,T. Interestingly, enhanced ICa,T by long-term testosterone involved increased expression of T-type calcium channel subunits (18). Therefore, it is important to determine whether the effects are receptor mediated or not. In our experiments in vitro, HT was applied 30 min after the application of testosterone to culture medium. The testosterone-induced suppression of either HSP72 expression or HSF1 activation was reversed by flutamide. The mechanisms for testosterone-induced suppression of HSF1 activation are unclear. After the application, testosterone may enter the cytosol to bind and activate the testosterone receptor (17). In addition to the possibility that testosterone directly blocks the trimerization and phosphorylation of HSF1, other indirect effects of testosterone might inhibit the HSF1 activation. Although the effects of flutamide were not evaluated in experiments in vivo, the negative regulatory effect of HSP72 expression by a single ip dose of testosterone administered 6 h before HT appears consistent with the in vitro observations. Together, although the involvement of acute or nongenomic effects cannot be excluded completely, our results indicate that testosterone potentially has negative regulatory effects on HSP72 expression by modulating the transcriptional level, at least in part, by testosterone receptor-mediated mechanisms. In contrast to a single-dose administration, some investigations treated the castrated animals with testosterone or dihydrotестosterone (DHT) replacement for several weeks to maintain the serum testosterone or DHT concentration close to physiological levels (2, 19). However, testosterone and DHT enter the cell to bind the testosterone receptor, leading to the transcriptional regulation of many genes to produce long-term genomic effects during replacement. Thus, translationally regulated proteins may directly or indirectly influence the expression of targeted protein, HSP72 in our study. Our approach using a single administration of testosterone appears to make it possible to isolate the effects of testosterone, as a substance, on targeted gene regulation and subsequently its protein expression. In other words, these two approaches observe different aspects of the effects of testosterone, i.e., replacement for a longer period estimates the significance of maintaining the physiological serum testosterone concentration. In fact, Liu et al. (19) recently demonstrated that preconditioning by metabolic inhibition conferred delayed cardioprotection in association with enhanced HSP72 expression in the presence of testosterone but not in the absence of testosterone. In contrast to our observations, they demonstrated that preconditioning failed to both elevate cardiac HSP72 expression and activate HSF1 in castrated rats, which was restored by chronic testosterone supplementation. From the above viewpoints, the disparity may be explained by the different experimental approaches in terms of application periods of testosterone, because 8-wk testosterone replacement in vivo and 24-h...
incubation with testosterone in vitro were introduced in their experiments (19). In particular, our single administration of testosterone transiently increased serum testosterone levels beyond physiological range within 30 min and quickly decreased. In fact, the serum testosterone level at HT application, which was 6 h after testosterone administration, was approximately 4.4 times higher than the sham-operated group (physiological level).

Using a very similar approach to the present study, we recently evaluated the effects of a single administration of estrogen on cardiac HSP72 expression in ovariectomized female rats and demonstrated that HSP72 expressions as well as HSFI phosphorylation were inhibited by estrogen (9). It is interesting that both estrogen and testosterone, when singly applied, down-regulated the cytoprotective HSP72 expression at a transcriptional level. The physiological significance of the effects of testosterone and estrogen is unclear. Aging is one of the most suppressive regulators of HSP72 expression (20) and also gradually reduces the serum concentration of testosterone and estrogen. The decrease in these two hormones with aging leads to unmasking of the suppression of HSP72 expression and may compensate the aging-induced reduction in HSP72 expression. The interaction of testosterone and estrogen for HSP72 should also be considered. In the present study, the 4-wk period after castration did not change serum estrogen levels (Table 1) as shown in a previous study (21). However, such low levels of estrogen may interact with testosterone to influence HSP72 expression. Testosterone is converted to estrogen and DHT by aromatase and 5α-reductase, respectively, both of which are present in the heart (22). The effects of DHT should be further clarified.

When applying HT for HSP72 expression, because HT induces a large number of cytoprotective proteins (23–25), it is unclear whether the protective effects were really provided by the HSP72 expression. In this regard, effective elimination of HSP72 expression by siRNA targeting HSP72 was observed in vitro in association with the elimination of protection against hypoxia/reoxygenation, strongly supporting the validity of our hypothesis that HT-induced HSP72 expression essentially provides protection.

Clinical implications

There have been a number of clinical reports regarding the gender difference in mortality and morbidity after myocardial infarction (26–28). A number of factors relate to differences in outcomes between males and females developing myocardial infarction, to which the effects of sex hormones, including estrogen and testosterone, may contribute at least in part. HSP72 expression is known to be induced in response to ischemia/reperfusion (4) and may be involved to some extent in the suppression of myocardial damage. Future studies will be required to investigate how HSP72 is involved in the protection against human ischemic insults. It should also be clarified whether induction of cardiac HSP72 in human patients is able to render protection. In the present study, we introduced HT (43 C), which may be impossible to introduce with human subjects. The other HSP72 inducers, including physical exercise (8), geranylgeranylacetone (6), or some other possible synthetic compounds, should be explored.

Limitations

There are several limitations in the present study. First, whereas neonatal cardiomyocytes were isolated from Wistar rats, experiments in vivo were performed using Sprague Dawley rats that were 10 wk old. Second, although 10-wk-old rats were not mature, we regarded them as adult rats in the present study. Different or novel findings may be obtained by using 4- to 6-month-old rats. Although the age of rats is an important factor in this area of research, previous reports also used immature animals (1, 2, 8, 19). The difference in age may explain some of the differences among the studies.

Conclusions

Our results indicate that testosterone potentially has suppressive effects on cardiac HSP72 expression by modulating gene transcription, at least in part, through receptor-mediated genomic mechanisms.

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The authors have nothing to disclose.

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