Brief exposure to exogenous testosterone increases death signaling and adversely affects myocardial function after ischemia

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Submitted 29 November 2005; accepted in final form 17 January 2006

Crisostomo, Paul R., Meijing Wang, George M. Wairiuko, Eric D. Morrell, and Daniel R. Meldrum. Brief exposure to exogenous testosterone increases death signaling and adversely affects myocardial function after ischemia. Am J Physiol Regul Integr Comp Physiol 290: R1168–R1174, 2006. First published January 26, 2006; doi:10.1152/ajpregu.00833.2005.—Chronic endogenous testosterone exposure adversely affects proinflammatory and proapoptotic signaling after ischemia/reperfusion; however, it remains unknown whether a single acute testosterone exposure is equally detrimental. We hypothesized that acute exogenous testosterone infusion before ischemia would worsen myocardial functional recovery, increase the activation of MAPKs and caspase-3, and increase myocardial proinflammatory cytokine production. To study this, isolated-perfused rat hearts (Langendorff) from adult females and castrated males were subjected to 25-min ischemia and 40-min reperfusion with and without acute testosterone infusion (17β-hydroxy-4-androstenone, 10 ng·ml⁻¹·min⁻¹) before ischemia. Myocardial contractile function was continuously recorded. After ischemia/reperfusion, hearts were assessed for levels of testosterone (ELISA), expression of proinflammatory cytokines (ELISA), and activation of MAPKs and caspase-3 (Western blot analysis). Data were analyzed with two-way ANOVA or Student’s t-test; P < 0.05 was statistically significant. All indices of posts ischemic functional recovery were decreased with acute exogenous testosterone compared with the untreated groups. Acute testosterone infusion increased activation of MAPKs and caspase-3 following ischemia/reperfusion. However, there were no significant differences in the myocardial proinflammatory cytokine production after brief testosterone infusion. A single acute exposure to exogenous testosterone before ischemia worsens myocardial functional recovery and increases activation of MAPKs and caspase-3. These findings confirm the deleterious effects of testosterone on myocardium, elucidate the nongenomic mechanistic pathways of testosterone, and may have important clinical implications for patients who have acute exposure to exogenous testosterone.

ISCHEMIC HEART DISEASE is the leading cause of death for both men and women. Restoration of blood flow to ischemic myocardium results in the ischemia-reperfusion (I/R) injury (27). Gender differences have been noted in I/R (4, 16, 17, 28, 29, 43, 44), with several studies implicating the sex hormone estrogen in the cardioprotection found in females (5, 18, 30, 32, 41). In contrast, testosterone has received little attention. Currently, the majority of evidence points toward the detrimental effects of testosterone on myocardium, possibly via adverse effects on lipoproteins, thrombosis, and cardiac hypertrophy (26, 37, 38, 50). Indeed, we have previously demonstrated that chronic endogenous testosterone had a deleterious effect in the normal isolated heart subjected to I/R (45); testosterone depletion (castration) or subcutaneous testosterone blockade 4 wk before I/R improved cardiac functional recovery and decreased myocardial cytokine production, inflammatory signaling, and expression of apoptotic-related proteins (45). However, this indirect measure of endogenous testosterone via depletion and blockade may have allowed for possible adaptation in the hormonal milieu, such as estrogen and growth factor regulation (9), to occur over the 4 wk before I/R. The latter study of chronic exposure also did not address the acute effects of testosterone.

It is widely recognized that steroid hormones bind to intracellular receptors and modulate transcription and protein synthesis, triggering genomic events responsible for physiological effects (6). However, very rapid effects of steroids have also been revealed that are clearly incompatible with the genomic model (33, 47). Recent investigations suggest that testosterone can exert rapid, nongenomic effects (7, 29, 46). No study has focused on the nongenomic effects of acute testosterone exposure on myocardium after I/R. Because of these discrepancies between genomic and nongenomic effects of steroids and androgens, it is important to elucidate the mechanistic pathways of testosterone in myocardium subjected to I/R.

Therefore, on the basis of the negative effect of chronic endogenous testosterone, we hypothesized that exogenous acute testosterone infusion (ATI) before ischemia may directly exert deleterious effects on myocardium not exposed to chronic testosterone (females and castrated males). The purposes of this study were to investigate the effect of acute direct exogenous testosterone on postischemic: 1) myocardial function, 2) proinflammatory cytokine production and MAPK activation, and 3) pro- and antiapoptotic signaling.

MATERIALS AND METHODS

Animals. Normal female and male (280–300 g, 9–10 wk) Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for 2 wk before the experiments. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 85-23, revised 1996).

Experimental groups. Rats were divided into four experimental groups: normal females (n = 9), females with acute testosterone infusion (ATI) before ischemia (n = 5), castrated males (n = 8), and castrated males with ATI before ischemia (n = 6). Acute testosterone...
infusion (17β-hydroxy-4-androstenone) was administered at physiological levels, 10 ng/ml−1 min−1 for 5 min (Eppendorf Centrifuge 5417R, Westbury, NY). Male rats (100–125 g, 5–6 wk) received bilateral castration and were allowed at least 4 wk of recovery. That normal females were in the proestrus stage was ensured by the performance of daily vaginal swabs. Isolated rat hearts in all groups were subjected to the same I/R protocol: 15-min equilibration period, 25 min of global ischemia (37°C), and 40 min of reperfusion.

**Isolated heart preparation (Langendorff).** Rats were anesthetized (pentobarbital sodium, 60 mg/kg ip) and heparinized (500 units ip), and then hearts were rapidly excised via median sternotomy and placed in 4°C Krebs-Henseleit (KH) solution. The aorta was cannulated, and the heart was retrograde perfused in the isolated, isovolumetric Langendorff mode (70 mmHg) with KH solution (in mM: 11 dextrose, 110 NaCl, 1.2 CaCl2, 4.7 KCl, 20.8 NaHCO3, 1.18 KHPO4, 1.17 MgSO4) at 37°C. The KH solution was bubbled with 95% O2-5% CO2 (Medipure) to achieve a PO2 of 450–460 mmHg, PCO2 39–41 mmHg, and pH 7.39 to 7.41. Total ischemic time was less than 45 s. The perfusion buffer was continuously filtered through a 0.45-μm filter to remove particulates. A pulmonary arteriometry and left atrial resection were performed before insertion of a water-filled latex balloon through the left atrium into the left ventricle. The preload volume (volume of buffer) was held constant during the entire experiment to allow for continuous recording of the left ventricular developed pressure (LVDP). The balloon was adjusted to a mean left ventricular end-diastolic pressure (LVEDP) of 8 mmHg (range 6–10 mmHg) during the initial equilibration. Pacing wires were fixed to the right atrium and left ventricle, and hearts were paced at 6 Hz, 3 V, 2 ms (~350 beats/min) throughout perfusion. A three-way stopcock above the aortic root was used to create global ischemia during which the heart was placed in a 37°C degassed organ bath. Coronary flow was measured by collecting pulmonary artery effluent. Data were continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments, Milford, MA) and an Apple G4 PowerPC computer (Apple Computer, Cupertino, CA). The maximal positive and negative value of the first derivative of pressure (dP/dt) and the slope of the staircase were calculated using PowerLab software. After reperfusion, the heart was removed from the apparatus, immediately sectioned, and snap frozen in liquid nitrogen.

**Myocardial proinflammatory cytokine expression, and testosterone expression.** Heart tissue was homogenized in cold buffer containing (in mM) 20 Tris (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 NaVO4, 1 PMSF, plus 1 μg/ml leupeptin and 1% Triton X-100, and centrifuged at 12,000 rpm for 5 min (Eppendorf Centrifuge 5417R, Westbury, NY). Myocardial TNF-α, IL-1β, IL-6, and testosterone in the cardiac tissue were determined by ELISA using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

**Western blot analysis.** Western blot analysis was performed to measure MAPK and apoptosis-related proteins. Heart tissue was homogenized in cold buffer containing (in mM) 20 Tris (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 NaVO4, 1 PMSF, plus 1 μg/ml leupeptin and 1% Triton X-100, and centrifuged at 12,000 rpm for 5 min (Eppendorf Centrifuge 5417R). The protein extracts (30 μg/lane) were subjected to electrophoresis on a 12% Tris-HCl gel (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane that was stained by Naphthol Blue-Black to confirm equal protein loading. The membranes were incubated in 5% dry milk for 1 h and then incubated with the following primary antibodies: p38 MAPK antibody, phosphor-p38 MAPK (Thr180/Tyr182) antibody, SAPK/JNK antibody, phosphor-SAPK/JNK (Thr183/Tyr185) antibody, p44/p42 MAPK antibody, phosphor-p44/p42 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA), caspase-3 (H-277) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (Ab-4) antibody, and GAPDH antibody (Oncogene Research Products, San Diego, CA). Subsequently, the membranes were incubated with hors eradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody. Detection was performed using supersignal west pico stable peroxide solution (Pierce, Rockford, IL). Films were scanned using an Epson Perfection 3200 Scanner (Epson America, Long Beach, CA), and band density was analyzed using ImageJ software (National Institutes of Health). Spots of dirty background were digitally erased.

**Presentation of data and statistical analysis.** All reported values are means ± SE (n = 5–9/group). Data were compared using two-way ANOVA with the post hoc Bonferroni test or Student’s t-test (female control vs. female with ATI and castrated male control vs. castrated male with ATI). A two-tailed P < 0.05 was considered statistically significant. Representative gels are shown with all lanes/samples from the same gel for each respective figure.

**RESULTS**

**Testosterone levels following I/R.** Testosterone levels following I/R assessed via ELISA were significantly greater in females (170.9 ± 21.2 pg/mg) and castrated males (118.6 ± 9.7 pg/mg) with ATI compared with females and castrated males without ATI (38.3 ± 9.7 and 54.2 ± 16.9 pg/mg, respectively (Fig. 1). There were no significant differences between females with ATI and castrated males with ATI.

**Myocardial function.** I/R resulted in markedly decreased LVDP in all groups. LVDP (mmHg) decreased from 96.1 ± 3.7 to 68.6 ± 2.5 in females, 97.5 ± 5.0 to 26.8 ± 7.9 in females with ATI, 102.0 ± 6.1 to 68.8 ± 7.4 in castrated males, and 120.3 ± 8.6 to 9.1 ± 1.9 in castrated males with ATI. Postischemic recovery of LVDP (expressed as a percentage of preischemic function) was significantly higher in females (71.7 ± 3.3%) and castrated males (64.5 ± 6.0%) than in females and castrated males with ATI (26.1 ± 7.5%, 7.1 ± 1.3%), respectively (Fig. 2A).

LVDP was elevated in response to I/R as shown in Fig. 2B. Normal females and castrated males without intervention demonstrated lower LVDP at each time point following I/R than females and castrated males with ATI.

**Maximum positive and negative dP/dt were impaired at the start of reperfusion.** Female hearts and castrated male hearts exposed to ATI demonstrated more depression of +dP/dt and
elevation of $-dP/dt$ compared with castrated males and normal females (Fig. 2, C and D).

Myocardial MAPK signaling pathway following I/R. The myocardial activation of phosphorylated p38 (active), non-phosphorylated p38 (total) MAPK, phosphorylated SAPK/JNK, nonphosphorylated SAPK/JNK, phosphorylated p44/42 MAPK, and nonphosphorylated p44/42 MAPK were assessed by Western blot analysis (Fig. 3). The phosphorylated forms of p38 MAPK and JNK were increased in females and castrated males with ATI compared with females and castrated males without intervention. Total p38 MAPK, total JNK, active p44/42 MAPK, and total p44/42 MAPK were equivalent in females, females with ATI, castrated males, and castrated males with ATI following I/R.

Myocardial inflammatory response to I/R. Myocardial production of TNF-$\alpha$, IL-1$\beta$, and IL-6 was measured via ELISA. Compared with females (TNF-$\alpha$: 150.4 $\pm$ 13.1 pg/mg, IL-1$\beta$: 85.2 $\pm$ 5.0 pg/mg, and IL-6: 714.8 $\pm$ 49.2 pg/mg protein), and castrated males (TNF-$\alpha$: 138.1 $\pm$ 12.6 pg/mg, IL-1$\beta$: 47.1 $\pm$ 9.2 pg/mg, and IL-6: 586.7 $\pm$ 39.1 pg/mg protein), females with ATI (TNF-$\alpha$: 144.1 $\pm$ 7.5 pg/mg, IL-1$\beta$: 84.3 $\pm$ 5.8 pg/mg, and IL-6: 655.7 $\pm$ 41.8 pg/mg protein) and castrated males with ATI (TNF-$\alpha$: 149.0 $\pm$ 15.9 pg/mg, IL-1$\beta$: 74.42 $\pm$ 7.2 pg/mg, and IL-6: 658.6 $\pm$ 60.2 pg/mg protein) had equivalent myocardial TNF-$\alpha$, IL-1$\beta$, and IL-6 levels following I/R injury as shown in Fig. 4.

Myocardial caspase cascades following I/R. The expression of apoptosis-related and inflammation-related caspases in I/R-injured myocardium was assessed by Western blot analysis. Caspase-3 cleavage/activation products were increased in females with ATI compared with females without intervention as shown in Fig. 5A. Caspase-3 cleavage/activation products were similar in castrated males with and without ATI. Myocardial expression of the antiapoptosis protein Bcl-2 was significantly lower in castrated males with ATI relative to castrated males without ATI as shown in Fig. 5B. Bcl-2 was not significantly different in females with ATI and without ATI.

DISCUSSION

The results of this study clearly demonstrate that after I/R, in hearts devoid of chronic testosterone exposure, a single dose of exogenous testosterone 1) depresses myocardial functional recovery, 2) increases activation of p38 MAPK and JNK, 3) increases expression of apoptotic protein caspase 3, and 4) decreases expression of antiapoptotic protein Bcl-2.

Testosterone may play an important role in the contractile dysfunction induced by I/R injury for several reasons. Functional androgen receptors are present in isolated cardiac myocytes (24, 25), and the heart can accumulate testosterone.

Fig. 2. Changes in myocardial function following I/R in normal females, females with ATI, castrated males, and castrated males with ATI. A: left ventricular developed pressure (LVD; %equilibration). End reperfusion LVD %equilibration was significantly higher in females and castrated males than in females and castrated males with ATI, respectively. B: left ventricular end diastolic pressure. C: first derivative of pressure $(+dP/dt)$ maximum; D: $-dP/dt$. Results are means $\pm$ SE. *$P < 0.05$ females with ATI vs. normal females, †$P < 0.05$ castrated males with ATI vs. castrated males at the corresponding time points.
one at higher concentrations than other androgen target organs (21). Acute exogenous testosterone produces a hypertrophic response in the heart by acting directly on androgen receptors in cardiac muscle cells (24, 34). This cardiac hypertrophy may result in an increase in ventricular stiffness (42), which reduces myocardial contractility. Indeed, the results of this study confirm the acute deleterious effects of testosterone on myocardial functional recovery after I/R; ATI caused statistically significant depression of %recovery \( \frac{\Delta P}{\Delta t} \) (a measure of compliance) in both females and castrated males. However, it remains unclear through which mechanisms testosterone exerts its myocardial depression following I/R.

Although testosterone traditionally mediates its effects via nuclear transcription, recent investigations indicate that testosterone can also exert rapid effects (12, 14, 46). In particular, recent investigations have elucidated that the androgen receptor is able to activate the MAPK family through a mechanism independent of their transcriptional activity (20, 31). Two members of the MAPK family, p38 MAPK and JNK, have been identified as signaling enzymes of myocardial inflammation (8, 15). I/R injury results in activation of myocardial p38 MAPK and JNK (11, 23, 39), whereas p38 MAPK and JNK inhibition leads to improved myocardial function following I/R (22, 36). Gender differences exist in the activation of p38 MAPK (3). This study demonstrates that exogenous ATI in females and castrated males decreased myocardial contractility and increased both activated p38 MAPK and activated JNK following I/R. Therefore, it is possible that acute exogenous testosterone adversely affects myocardial function via androgen receptor activation of nontranscriptional mechanistic pathways, such as MAPK inflammatory signaling.

Ultimately, depression of myocardial function following I/R and loss of cardiomyocytes result from myocardial apoptosis. Apoptosis may be mediated by either the extrinsic death receptor signaling pathway or the intrinsic mitochondrial control pathway (19). Caspases play a crucial role in each of these pathways. The activation of caspase-3 has been observed in response to hypoxia or ischemia (35). Gender differences exist in the caspase cascades leading to apoptosis. Clinically, myocyte apoptosis in heart failure is increased in men compared with women (13). In isolated rat hearts, increased activation of proapoptotic caspase-3 after I/R was associated with chronic endogenous testosterone exposure (45). In vitro studies also found increased apoptosis in rat myocytes exposed to acute testosterone (48, 52). Similarly, in this study, we observed increased activation of caspase-3 in females with ATI compared with females without ATI following I/R. In addition, we also found a significant decrease in activation of Bcl-2, an antiapoptotic signaling enzyme, in castrated males with ATI compared with castrated males without ATI. Whether these effects are mediated by nongenomic or genomic effects remains uncertain. Nevertheless, elevated caspase-3 levels and decreased Bcl-2 levels in hearts subjected to endogenous and exogenous testosterone imply that the detrimental effect of testosterone on myocardium may be induced by activation of proapoptotic signaling caspases and the downregulation of antiapoptotic signaling enzymes.

We found no significant difference in the myocardial production of TNF-\( \alpha \), IL-1\( \beta \), and IL-6 in hearts with exogenous ATI compared with hearts without intervention. Other investigators have found divergent cytokine release when myocardia were exposed to estrogen and testosterone (1, 2, 10, 40, 49, 51). Previously, we found that endogenous testosterone exposure increased myocardial cytokine production and decreased posts ischemic cardiac function, suggesting that endogenous testosterone exerts a deleterious effect on myocardium via increased myocardial inflamma-
The discrepancy in myocardial cytokine production between chronic endogenous testosterone and acute exogenous testosterone exposure may reflect the innate difference between transcriptional effects (chronic testosterone) and acute nongenomic effects (acute testosterone). In this study, a single brief exposure to testosterone was administered immediately before ischemia; previously, rats were testosterone depleted (castrated) or testosterone was blocked for 4 wk before I/R (45). Perhaps the lack of time for adaptation or nuclear transcription in acute exogenous testosterone infusion precluded myocardial cytokine production. This discrepancy in cytokine production between chronic and acute testosterone exposure intimates that testosterone may exert its deleterious effects on myocardium via different genomic and nongenomic pathways depending on the temporal application.

There still remains a great deal of controversy over the role of gender and injury. This study confirms the deleterious effects of testosterone on myocardium after acute injury. This investigation also lends insight into the complex mechanistic genomic and nongenomic pathways of testosterone action. These findings may have important clinical implications for patients who have acute exposure to exogenous testosterone. This may also help explain the variation in clinical outcomes between males and females after myocardial infarction. Perhaps modification of testosterone-dependent mechanisms associated with I/R will alter the myocardial response to ischemia. Additional investigations into testosterone-dependent mechanisms and the resultant detrimental effects must be performed to gain a more complete understanding of gender and injury.

ACKNOWLEDGMENTS
This work was supported in part by National Institute of General Medical Science Grant R01-GM-070628 (to D. R. Meldrum), an American Heart Association Postdoctoral Fellowship (to M. Wang), the Clarian Values Fund (to D. R. Meldrum), the Showalter Trust (to D. R. Meldrum), and the Cryptic Masons Medical Research Foundation (to D. R. Meldrum and M. Wang).

Fig. 4. Effect of acute testosterone on myocardial TNF-α, IL-1β, and IL-6 production (in pg/mg) after I/R. Cardiac TNF-α (A), IL-6 (B) and IL-1β (C) production was not significantly different between females (F I/R + ATI) and castrated males (CM I/R + ATI) with ATI compared with those (F I/R, CM I/R) without ATI. Results are means ± SE.

Fig. 5. Myocardial expression of proapoptotic and antiapoptotic enzymes in females (F I/R), females with ATI (F I/R + ATI), castrated males (CM I/R), and castrated males with ATI (CM I/R + ATI) after I/R. Shown are representative immunoblots. A: active caspase-3 levels were increased in female hearts with ATI compared with normal females. Castrated males with and without ATI had no significant difference. All samples were on the same membrane. B: Bcl-2 expression on immunoblot was decreased in castrated males with ATI compared with castrated males without intervention. No difference in Bcl-2 expression was observed in females with and without ATI. All samples were on the same membrane.
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