Role of endogenous testosterone in myocardial proinflammatory and proapoptotic signaling after acute ischemia-reperfusion

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Wang, Meijing, Ben M. Tsai, Ajay Kher, Lauren B. Baker, G. Mathenge Wairiuko, and Daniel R. Meldrum. Role of endogenous testosterone in myocardial proinflammatory and proapoptotic signaling after acute ischemia-reperfusion. Am J Physiol Heart Circ Physiol 288: H221–H226, 2005. First published September 16, 2004; doi:10.1152/ajpheart.00784.2004.—Myocardial ischemia is the leading cause of death in both men and women; however, very little information exists regarding the effect of testosterone on the response of myocardium to acute ischemic injury. We hypothesized that testosterone may exert deleterious effects on myocardial inflammatory cytokine production, p38 MAPK activation, apoptotic signaling, and myocardial functional recovery after acute ischemia-reperfusion (I/R). To study this, isolated, perfused rat hearts (Langendorff) from adult males, castrated males, and males treated with a testosterone receptor blocker (flutamide) were subjected to 25 min of ischemia followed by 40 min of reperfusion. Myocardial contractile function (left ventricular developed pressure, left ventricular end-diastolic pressure, positive and negative first derivative of pressure) was continuously recorded. After reperfusion, hearts were analyzed for expression of tissue TNF-α, IL-1β, and IL-6 (ELISA) and activation of p38 MAPK, caspase-1, caspase-3, caspase-11, and Bcl-2 (Western blot). All indices of postischemic myocardial functional recovery were significantly higher in castrated males or flutamide-treated males compared with untreated males. After I/R, castrated male and flutamide-treated male hearts had decreased TNF-α, IL-1β, and IL-6; decreased activated p38 MAPK; decreased caspase-1, caspase-3, and caspase-11; and increased Bcl-2 expression compared with untreated males. These results show that blocking the testosterone receptor (flutamide) or depleting testosterone (castration) in normal males improves myocardial function after I/R. These effects may be attributed to the proinflammatory and/or the proapoptotic properties of endogenous testosterone. Further understanding may allow therapeutic manipulation of sex hormone signaling mechanisms in the treatment of acute I/R myocardial infarction; sex hormones; inflammation; caspase cascades

Recent studies suggest that there may be sex differences in the myocardial response to acute ischemia-reperfusion (I/R) injury. These differences may be attributable to the effects of the sex hormones estrogen and testosterone. Surprisingly, little information exists regarding the effect of testosterone on myocardial injury, and the few studies on the effects of testosterone have produced conflicting results. On one hand, testosterone may have a beneficial effect on myocardial function. For example, it has been demonstrated that testosterone reduces myocardial ischemia in patients with coronary artery disease (39), and low plasma testosterone levels have been correlated with several risk factors for myocardial infarction (26, 27).

Conversely, some studies indicated that testosterone had fewer antioxidant effects in heart muscle and worsened cardiac dysfunction in mice with myocardial infarction compared with estrogen (2). Moreover, exogenous androgen supplementation increased apoptosis in adult rat ventricular myocytes (43). Most of these studies were performed in chronically diseased myocardium. Indeed, population studies indicate that men have higher mortality before reaching the hospital after a myocardial infarction (34). Therefore, it is important to elucidate the role of endogenous testosterone in myocardium subjected to acute I/R.

Myocardial infarction may play a crucial role in I/R-induced myocardial dysfunction and the associated loss of cardiomyocytes. The myocardium itself generates inflammatory cytokines, such as TNF-α and IL-1β, in response to acute I/R, and these locally produced inflammatory mediators contribute to posts ischemic myocardial functional depression as well as cardiomyocyte apoptosis. One of the signaling enzymes involved in both inflammatory cytokine production and myocyte apoptosis is p38 MAPK. I/R injury results in activation of myocardial p38 MAPK (6, 18, 31), whereas p38 MAPK inhibition leads to improved myocardial function after I/R (17, 23). However, it is unknown whether testosterone affects myocardial inflammatory cytokine production, p38 MAPK activation, apoptotic signaling, and myocardial recovery after acute ischemic injury. On the basis of the results of several recent papers in the trauma literature (29, 30), we hypothesized that testosterone may exert deleterious effects on these processes. The purposes of this study were to investigate the effect of testosterone depletion (castration) or testosterone receptor blockade (flutamide) on posts ischemic 1) myocardial function, 2) proinflammatory cytokine (TNF-α, IL-1β, and IL-6) production and p38 MAPK activation, and 3) pro- and antiapoptotic signaling.

Materials and Methods

Animals. Normal male Sprague-Dawley rats (280–300 g, 9–10 wk; 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 Institutional 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 Pub. No. 85–23, revised 1985).

Experimental groups. Rats were divided into three experimental groups: normal males (n = 12), castrated males (n = 8); castration occurred when the animals weighed 100 g, and males treated with the testosterone receptor blocker (TRB) flutamide (n = 9). Male rats
(100–125 g, 5–6 wk) received bilateral castration and were allowed at least 4 wk of recovery. Subcutaneous implantation of 21-day-release pellets containing 10 mg of flutamide was performed in normal male rats (animals were utilized at the end of the 21-day blockade). 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 U ip), and hearts were rapidly excised via median sternotomy and placed in 4°C Krebs-Henseleit solution. The aorta was cannulated, and the heart was perfused (70 mmHg) with oxygenated (95% O2-5% CO2) Krebs-Henseleit solution (37°C). 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 with a PowerLab 8 preamplifier/digitizer (AD Instruments, Milford, MA) and an Apple G4 PowerPC computer (Apple Computer, Cupertino, CA). The maximal positive and negative values of the first derivative of pressure (+dP/dt and −dP/dt) were calculated with PowerLab software.

Myocardial TNF-α, IL-1, and IL-6. 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 Na3VO4, and 1 PMSF with 1 g/ml leupeptin and 1% Triton X-100 and centrifuged at 12,000 rpm for 5 min. Myocardial TNF-α, IL-1β, and IL-6 in the cardiac tissue were determined by ELISA with a commercially available ELISA kit (BD Biosciences, San Diego, CA, and R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

Western blotting. Western blot analysis was performed to measure p38 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 Na3VO4, and 1 PMSF with 1 μg/ml leupeptin and 1% Triton X-100 and centrifuged at 12,000 rpm for 5 min. The protein extracts (30 μg/lane) were subjected to electrophoresis on a 12% Tris-HCl gel from Bio-Rad and transferred to a nitrocellulose membrane, which was stained with 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 (Cell Signaling Technology, Beverly, MA); caspase-3 (H-277) antibody, caspase-1 p20 (G-19) antibody, caspase-11 (M-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA); Bcl-2 (Ab-4) antibody (Oncogene Research Products, San Diego, CA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse IgG secondary antibody and detection with SuperSignal West Pico stable peroxide solution (Pierce, Rockford, IL). Films were scanned with an Epson Perfection 3200 Scanner (Epson America, Long Beach, CA), and band density was analyzed with

Fig. 1. Changes in myocardial function after ischemia and reperfusion (I/R) in normal age-matched males (n = 12), castrated males (n = 8), and males treated with testosterone receptor blocker (TRB, flutamide; n = 9). A: left ventricular developed pressure (LVDP). B: left ventricular end-diastolic pressure (LVEDP). C: maximum positive first derivative of pressure (+dP/dt). D: −dP/dt. Results are means ± SE. *P < 0.01 vs. normal male at the corresponding time points.
Presentation of data and statistical analysis. All reported values are means ± SE (n = 8–12/group). Data were compared with two-way ANOVA with post hoc Bonferroni test or Student’s t-test. A two-tailed probability value of <0.05 was considered statistically significant. Representative gels are shown in Figs. 2, 4, and 5 with all lanes/samples from the same gel.

RESULTS

Myocardial function. I/R resulted in markedly decreased left ventricular developed pressure (LVDP) in all groups. LVDP (mmHg) decreased from 97.1 ± 3.52 to 52.1 ± 5.04 in normal males, from 98.7 ± 6.48 to 69.3 ± 7.13 in castrated males, and from 114.6 ± 4.83 to 80.8 ± 4.89 in males treated with TRB (Fig. 1A). Postischemic recovery of LVDP (expressed as % of preischemic function) was significantly higher in castrated males (68.9 ± 4.70%) and males treated with TRB (71.4 ± 4.38%) than in normal males (51.5 ± 5.03%).

Left ventricular end-diastolic pressure (LVEDP) was elevated in response to I/R as shown in Fig. 1B. Castrated males and TRB-treated males demonstrated lower LVEDP than normal males at each time point after I/R.

Maximum +dP/dt and −dP/dt were impaired at the start of reperfusion. Normal male hearts demonstrated more depression of +dP/dt and increase of −dP/dt compared with castrated males and TRB-treated males (Fig. 1, C and D).

Myocardial p38 MAPK signaling pathway after I/R. The myocardial activation of phosphorylated p38 (active) and non-phosphorylated p38 (total) MAPK was assessed by Western blot. Total p38 MAPK was equivalent in normal males, castrated males, and males treated with TRB after I/R (Fig. 2). However, the phosphorylated forms of p38 MAPK were decreased in castrated males and males treated with TRB compared with normal males.

Myocardial inflammatory response to I/R. Myocardial production of TNF-α, IL-1β, and IL-6 was measured to investigate whether endogenous testosterone affects the myocardial inflammatory response to I/R injury (Fig. 3). Compared with normal males (TNF-α 153.5 ± 6.78, IL-1β 37.7 ± 13.5, and IL-6 1,449 ± 143.3 pg/mg protein), castrated males (TNF-α 125.6 ± 8.70, IL-1β 14.5 ± 2.89, and IL-6 1,105 ± 81.96 pg/mg protein) and males treated with TRB (TNF-α 103.9 ± 6.84, IL-1β 9.51 ± 2.03, and IL-6 998.6 ± 124 pg/mg protein) had less myocardial TNF-α, IL-1β, and IL-6 after I/R injury.

Myocardial caspase cascades after I/R. The expression of apoptosis-related and inflammation-related caspases in I/R-injured myocardium was assessed by Western blotting (Figs. 4 and 5). Caspase-1, caspase-3, and caspase-11 cleavage/activation products were decreased in castrated males and flutamide-treated males compared with normal males. Conversely, myocardial expression of the antiapoptosis protein Bcl-2 was increased in castrated males and flutamide-treated males compared with normal males.

DISCUSSION

The main findings of the present study were that after I/R, compared with normal males, castrated males and males treated with testosterone receptor blockade (flutamide) 1) exhibited cardiac functional protection, 2) had decreased proinflammatory cytokine production (TNF-α, IL-1β, and IL-6) and active p38 MAPK and caspase-1, 3) had decreased expression of apoptosis-related proteins caspase-3 and caspase-11, and 4) had increased expression of antiapoptotic protein Bcl-2.

Sex differences exist in the response of myocardium to acute injury (3, 11, 32). Most studies have focused on the role of estrogen in cardiac protection, whereas few data exist regarding the effect of testosterone on cardiovascular disease. The role of testosterone in cardiac injury may be important because the heart can accumulate testosterone at higher concentrations than other androgen target organs (16), and functional andro-
I/R injury induces the local production of TNF-α and IL-1β, and IL-6 production and decreased myocardial p38 MAPK signaling. Stimuli such as I/R, oxidant stress, and hydrogen peroxide directly activate p38 MAPK (20). Activation of p38 MAPK is required for TNF-α and IL-1β production in cardiomyocytes (24, 31, 37). TNF-α production after I/R is dependent on NF-κB translocation, which may occur via p38 MAPK activation, and regulation of this process occurs pretranscriptionally (20). On the other hand, caspase-1 and caspase-11 are shown to function upstream of IL-1β maturation (36). Caspase-1 is recognized as the IL-1β-converting enzyme (ICE) for cleavage of IL-1β precursor to the mature form (7, 8). Precursor caspase-1 results in the generation of mature p10/p20, and p10/p20 form ICE (28). ICE is required for IL-1β activation in the postischemic heart (25). Furthermore, the activation of caspase-1 is dependent on caspase-11 (38). Caspase-11 is thought to activate downstream signals caspase-1 and caspase-3, and thus it may be important in both inflammation and apoptosis (13). It has been demonstrated that p38 MAPK mediates caspase-11 induction in microglia subjected to hypoxia and endotoxin exposure (12, 14). In the current study, we observed increased procaspase-1, but decreased caspase-1 p20 and caspase-11, in castrated males and TRB-treated males compared with untreated males.

Myocardial apoptosis is responsible for the loss of cardiomyocytes and depression of myocardial function after I/R. Myocyte apoptosis in heart failure is reduced in women compared with men (9). Apoptosis may be mediated by either the extrinsic death receptor signaling pathway or the intrinsic mitochondrial control pathway (15). Caspases play a crucial role in each of these pathways. The activation of the intrinsic apoptotic pathway (the release of cytochrome c and the act-
viation of caspase-9) during reperfusion, but not ischemia, has been observed in chick cardiomyocytes (35), and activation of caspase-8 and caspase-3 occurs in response to hypoxia or ischemia (22). However, no information exists regarding the influence of testosterone on myocardial proapoptotic signaling cascades after acute I/R. In this study, we observed decreased activation of proapoptotic signaling cascade (caspase-3, caspase-11) and increased antiapoptotic Bcl-2 in castrated males and TRB-treated males compared with untreated male hearts after I/R. Clinically, anabolic androgenic steroid abuse has been associated with myocardial ischemia and sudden cardiac death (5). Some studies have demonstrated that anabolic androgenic steroids induce injury and apoptosis in myocardial cells (41, 43) and skeletal muscle cells in culture (1). Our observations of decreased apoptotic signaling with testosterone depletion or testosterone receptor blockade are consistent with these studies.

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REFERENCES


