Endogenous estrogen mediates a higher threshold for endotoxin-induced myocardial protection in females

Jeffrey M. Pitcher, Meijing Wang, Ben M. Tsai, Ajay Kher, Nicholas T. Nelson, and Daniel R. Meldrum. Endogenous estrogen mediates a higher threshold for endotoxin (ETX)-induced protection in females. Am J Physiol Regul Integr Comp Physiol 290: R27–R33, 2006. First published September 8, 2005; doi:10.1152/ajpregu.00452.2005.—Myocardial endotoxin tolerance may be induced in both males and females; however, it remains unknown whether there are mechanistic and threshold differences between the sexes. We hypothesized that endogenous estrogen mediates a higher threshold for ETX-induced protection in females. Adult proestrous and ovariectomized (OVX) female rats were preconditioned (PC) with intraperitoneal injections of 125 (PC125) or 500 (PC500) µg/kg Salmonella typhimurium LPS (ETX) or normal saline (PC–). Twenty-four hours later, injury dose ETX (500 µg/kg) was injected. After 6 h, myocardial function was measured via Langendorff. p38 MAPK and JNK activation, IL-1, and IL-6 expression were evaluated. ETX injury significantly decreased left ventricular developed pressure in PC– vs. controls. PC500 regimen protected against ETX injury, resulting in normal cardiac function. PC125 regimen protected OVX but not proestrous females, which had diminished myocardial function. Activated JNK and TNF-α increased in PC– but were diminished in PC500 animals. Importantly, activated JNK and TNF increased in PC125 proestrous females, whereas PC125 OVX females displayed decreases in these molecules. There were no differences in p38 MAPK activation or expression of IL-1 or IL-6. These results demonstrate that proestrous females require a higher stimulus (PC500) to achieve myocardial protection against ETX injury. Removal of endogenous estrogen (OVX) lowered the preconditioning threshold (PC125), resulting in protection after lesser injury. Additionally, myocardial JNK and TNF expression was decreased in OVX PC125 females, which correlated with myocardial function differences. Therefore, we conclude that endogenous estrogen mediates a higher threshold for ETX tolerance in female myocardium.

tolerance; gender; acute injury

PRECONDITIONING IS PROTECTION resulting from prior sublethal acute injury. This protective mechanism was initially described in 1986 by Murry et al. (32), who found that canine myocardium, subjected to a small ischemic injury, was protected from a second, larger insult, resulting in smaller areas of infarct and improved cardiac function. Since that time, studies have provided evidence of both acute (5, 8, 14, 44) and delayed (18, 22, 29, 48) forms of preconditioning, noting that delayed protection can persist up to 72 h after the preconditioning stimulus. Furthermore, other investigations have noted that, in addition to ischemia, other stressors such as endotoxemia (29, 30, 36), heat stress (26, 28, 41), and trauma/hemorrhage (21, 23, 33) are capable of inducing a preconditioned state. Finally, there is evidence that acute injury is capable of producing cross-tolerance in different organs distant from the initial insult, resulting in protection from additional injury (11, 14, 15, 52).

Another mechanism associated with protection from acute injury is related to gender. Studies have shown that females are relatively protected from acute injury (1, 7, 47, 50, 54). Clinical investigations have indicated that females fare better in the immediate aftermath of myocardial infarction than males (43, 45, 46), although 1-mo mortality is equivalent between the sexes. Females have also been noted to have lower rates of arrhythmias and congestive heart failure after ischemic events compared with males (10). In addition to clinical data, small animal studies have shown that females have improved cardiac function after acute injury. We have previously demonstrated that female rats experiencing ischemia-reperfusion had better functional recovery and decreased expression of inflammatory mediators than male rats (47). Additionally, studies performed by Chaudry and colleagues (31, 38, 51, 53) have demonstrated that gender differences affect proinflammatory cytokine expression and immune function after acute injury.

Investigations on acute injury have indicated that inflammation plays a central role in altering myocardial function (19, 20, 27). Activation of p38 MAPK and c-Jun NH2-terminal kinase (JNK) are increased by acute injury. Both enzymes can lead to increased expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, which negatively affect myocardial function (4, 17, 49, 55). Additionally, JNK activation has been implicated in apoptotic processes (16), thereby increasing injury and degrading heart function. Interestingly, evaluations of the enzymes p38 MAPK and JNK and their production of the inflammatory cytokines associated with acute injury suggest that the expression of these potentially deleterious products may be decreased by preconditioning (9, 12, 24, 35).

Previous investigations on acute injury and preconditioning have noted that both males and females benefit from this form of protection (34, 36, 40). Interestingly, studies performed by
our group have noted that females have a higher preconditioning injury threshold than males (36, 37). On the basis of this background, we hypothesized that 1) both normal and ovariectomized (OVX) females can be preconditioned, 2) endogenous estrogen mediates the higher endotoxin (ETX)-induced myocardial protection threshold in females, and 3) preconditioning modulates activation of p38 MAPK and JNK, as well as the expression of the proinflammatory cytokines TNF, IL-1, and IL-6, after acute injury.

MATERIALS AND METHODS

Animal Use and Care

Adult proestrus and OVX female Sprague-Dawley rats (weight: 250–300 g; Harlan, Indianapolis, IN) were given a diet of standard rat chow and allowed to eat and drink ad libitum, while acclimating in a quiet quarantine room for 1 wk before experimentation. 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 1985).

Experimental Protocol

Animals were given an intraperitoneal (IP) injection of 125 or 500 μg/kg *Salmonella typhimurium* LPS (ETX) or 0.4 ml of normal saline. After 24 h, another IP injection of 500 μg/kg ETX was given. Either 1 or 6 h later, the animals were anesthetized; their hearts were rapidly excised, and myocardial function was evaluated using the Langendorff perfusion model. Normal females were ensured to be in the proestrus stage by performing daily vaginal swabs. Ovariectomy was performed 4 wk before the initiation of these experiments. This time period allowed for complete depletion of endogenous estrogen as well as avoidance of the potentially confounding effects of acute injury associated with surgical ovariectomy.

Experimental Groups

The following experimental groups (n = 4–5 rats/group) were formed. Nonpreconditioned (PC−) rats received an IP normal saline injection. Preconditioned rats received 500 μg/kg ETX as the preconditioning stimulus (PC+500 group). Preconditioning threshold animals received a 125 μg/kg ETX preconditioning stimulus (PC+125 group). After 24 h, 500 μg/kg ETX injury dose was administered IP to all groups, followed 1 or 6 h later by assessment of cardiac function (Fig. 1). ETX solutions were prepared with sterile 0.9% saline.

Myocardial Function

Intrinsic cardiac contractility was determined by a modified isovolumetric Langendorff technique. Rats were anesthetized and heparinized with an IP injection of pentobarbital sodium (Nembutal; 150 mg/kg) mixed with 500 U sodium heparin (Fisher Scientific, Fair Lawn, NJ). A median sternotomy was performed, and the heart was rapidly removed and placed in a 4°C bath of Krebs-Henseleit solution (in mM: 11 dextrose, 119 NaCl, 1.2 CaCl2, 4.7 KCl, 20.8 NaHCO3, 1.18 KHPO4, 1.17 MgSO4). The aorta was cannulated and antegrade perfused in the isolated, isovolumetric Langendorff mode with Krebs-Henseleit solution at 37°C, and bubbled with 95% O2-5% CO2. The perfusion buffer was continuously filtered through a 0.45-μm filter to remove particulates. A pulmonary arteriotomy 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 (balloon volume) was held constant during the entire experiment to allow continuous recording of the left ventricular developed pressure (LVDP). The balloon was adjusted to a mean left ventricular end-diastolic pressure 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. After an equilibration period of 10 min, myocardial function was evaluated for 20 min by continuous assessment of LVDP using a computerized PowerLab 8 preamplifier/digitizer (ADInstruments, Milford, MA), an Apple iMac (Apple Computer, Cupertino, CA), and collecting pulmonary effluent at 0, 10, and 20 min as a measurement of coronary flow. The rates of cardiac contractility (positive dP/dt) and relaxation (negative dP/dt) were also calculated (Chart v4.2, PowerLab, ADInstruments). After 20 min, the heart was removed from the apparatus, sectioned, and snap frozen in liquid nitrogen.

JNK

Western blot analysis was performed on cardiac tissue to measure total JNK and JNK activation (phospho-JNK). The protein extracts (30 μg/lane) were electrophoresed on a 12% Tris·HCl gel from Bio-Rad and transferred to a nitrocellulose membrane, which was incubated in 5% dry milk for 1 h. Then membranes were incubated with JNK antibody or phospho-JNK (Thr 183/Tyr 185) antibody (Cell Signaling Technology) overnight at 4°C. The membrane was then washed three times for 5 min in TBS-Tween 20 (TBST), incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and again washed three times for 5 min in TBST. The membranes were developed using Supersignal West Pico stable peroxide solution (Pierce).

Myocardial p38 MAPK

Western blot analysis was performed on cardiac tissue to measure total p38 MAPK and activated p38 MAPK. The protein extracts (30 μg/lane) were electrophoresed on a 12% Tris·HCl gel from Bio-Rad and transferred to a nitrocellulose membrane, which was incubated in 5% dry milk for 1 h. Then membranes were incubated with p38 MAPK antibody or phospho-p38 MAPK (Thr 180/Tyr 182) antibody (Cell Signaling Technology) overnight at 4°C. The membrane was then washed three times for 5 min in TBST, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and again washed 3 times for 5 min in TBST. The membranes were developed using Supersignal West Pico stable peroxide solution (Pierce).
Myocardial TNF-α, IL-1, and IL-6

Myocardial homogenate TNF-α, IL-1, and IL-6 contents were determined by ELISA (Genzyme, Cambridge, MA). ELISA was performed by adding 100 μl of each sample (equal protein and tested in duplicate) to wells in a 96-well plate of a commercially available ELISA kit. ELISA was performed according to the manufacturer’s instructions. Final cytokine results were expressed as picograms per milligram protein.

Chemicals and Reagents

Unless otherwise specified, all chemicals and reagents were obtained from Sigma (St. Louis, MO).

Presentation of Data and Statistical Analysis

All reported values are means ± SE. Differences at the 95% confidence level were considered significant. Data were compared using Student’s t-test analysis as well as ANOVA with Tukey’s posttest analysis (GraphPadPrism 4.0, Camino Real, San Diego, CA).

RESULTS

Myocardial Function

LVDP. One-hour groups showed no decrease in LVDP (data not shown). Considerable differences, however, were seen in the 6-h groups. Both normal and OVX PC− groups experienced a profound decrement in LVDP compared with sham rats (P < 0.0001 and P < 0.004, respectively) (Fig. 2). In contrast, both normal and OVX PC+500 groups were protected from the second ETX insult, resulting in preserved myocardial function (P > 0.05 vs. sham). Interestingly, OVX PC+125 rats maintained near normal LVDP compared with sham animals (P > 0.05), whereas the normal PC+500 females were not protected and had a substantial decrease in LVDP compared with sham (P < 0.001).

\[ \frac{dP}{dt} \]
Positive and negative dP/dt are measures of myocardial contractility and relaxation, respectively. Both 6-h groups of PC− animals had considerably less compliance compared with sham animals (Fig. 3). Both normal and OVX PC+500 groups maintained near normal compliance after preconditioning, whereas PC+125 females were not protected, resulting in noticeably reduced contractility and relaxation. In contrast, the PC+125 OVX females retained compliance similar to that of sham animals. There was no appreciable difference in positive dP/dt or negative dP/dt for any of the 1-h groups (data not shown). No difference was found between any of the groups in coronary flow rates (data not shown).
JNK Activation

Active JNK expression was decreased in OVX PC/H11001125, OVX PC/H11001500, and normal PC/H11001500 groups compared with PC/H11002 groups, indicating protection as a result of preconditioning (Fig. 4). Importantly, normal PC/H11001125 females had increased JNK activation similar to both normal and OVX PC/H11002 groups, correlating with the differences seen in myocardial function.

p38 MAPK Activation

The amounts of activated and total p38 MAPK were not different among any of the groups at the time point measured (Fig. 5).

TENF-α, IL-1, and IL-6

TNF-α expression was decreased in the OVX PC+125 and OVX PC+500 animals compared with the OVX PC− rats. In normal females, TNF expression was elevated in the PC− and PC+125 groups. PC+500 animals exhibited decreased TNF production, although not significantly, compared with PC− rats at this time point (Fig. 6A). Expression of IL-1 was higher in normal PC+125 animals; however, no difference in expression was noted between animals in any other group. Finally, IL-6 production resulting from endotoxic injury was not different between any of the animals at this selected time point (Fig. 6, B and C).

DISCUSSION

These results clearly show that 1) both normal and OVX females can be preconditioned; 2) endogenous estrogen mediates the higher ETX-induced myocardial protection threshold in females; 3) decreased myocardial function correlates with increased active JNK (phosphor-JNK) and TNF expression; 4) preconditioning modulates the activation of JNK in ETX-induced myocardial protection; and 5) the decrease in phosphor-JNK and expression of TNF in OVX PC/H11001125, OVX PC/H11001500, and normal PC+500 females emphasizes that the higher preconditioning injury threshold is mediated by endogenous estrogen in females.

Studies have shown that ETX exposure leads to depressed myocardial function (19). Meng et al. (29) found that cardiac performance, after ETX administration, diminished significantly between 4 and 6 h after exposure. In addition to demonstrating the myocardial depressive effect of ETX, their results also showed that myocardial function was preserved after preconditioning with ETX. Our data are consistent with this report, indicating that both normal and OVX females had similarly decreased cardiac performance 6 h after ETX administration. Interestingly, our results show that both normal and OVX females can be protected when ETX is used as the preconditioning stimulus. This is in contrast to an investigation by Song et al. (42) that noted that OVX females were unable to be preconditioned and had resultant decreased myocardial function.
function. This divergence in results may be due to differences between acute and delayed preconditioning, as Song’s group evaluated early processes, whereas our results evaluated delayed preconditioning. Furthermore, the injury applied may not have been sufficient to surpass the injury threshold and therefore did not induce a preconditioned state.

In work previously performed by our group, we determined that females have a higher preconditioning injury threshold than males (36). This study clearly demonstrates that the higher injury threshold in females is mediated by endogenous estrogen. Our results show that normal PC/H11001 females were not preconditioned with the lower ETX injury, indicating that they are relatively protected from small acute insults. In contrast, estrogen-depleted OVX PC/H11001 females developed preconditioned protection, showing that the smaller acute injury was capable of inducing preconditioning mechanisms, again emphasizing endogenous estrogen’s role in mediating the higher injury threshold. The protective effects of estrogen have also been described by Mizushima et al. (31) who noted that males given estrogen had improved myocardial function and less inflammatory cytokine expression after trauma/hemorrhage than controls. Furthermore, a study by Cavasin et al. (6) found that estrogen-depleted OVX females had larger areas of myocardial damage and resultant decreased function after injury than those animals that received estrogen. Finally, our laboratory (47) demonstrated that normal females experience improved myocardial function and an attenuated inflammatory response after ischemic injury compared with males. Clearly, endogenous estrogen, as demonstrated by the results of this study, exerts powerful protective effects. Other investigations, as previously noted, have provided evidence that exogenous estrogen also provides protection from acute injury. Because the stresses associated with estrogen administration and determination of estrogen levels after exogenous administration may influence the preconditioned states, this study aimed only to determine whether endogenous estrogen was the mediator of the higher preconditioning threshold in females.

One of the mechanisms by which acute injury produces myocardial dysfunction is through a profound inflammatory response (3, 20, 27). Acute injury results in signal transduction through kinases such as JNK, leading to increased expression of NF-κB and proinflammatory cytokines such as TNF (13, 25). The results of this study showed that preconditioned animals expressed lower amounts of phosphor-JNK. These findings are consistent with work performed by Sato et al. (39), who found that preconditioning attenuated the increased expression of JNK after ischemia-reperfusion injury. Asai et al. (2) noted that normal females had lower cytokine levels in response to ETX challenge compared with males. This diminished cytokine release by normal females may therefore be insufficient to induce the preconditioning mechanisms, resulting in lack of protection from a second insult. To evaluate the
effects of preconditioning on cytokine expression, we measured TNF, IL-1β, and IL-6.

The time sequence of cytokine expression was described by Maass et al. (17) after burn. They noted significantly increased levels of TNF-α at 1 h, without changes in the expression of IL-1β or IL-6. Harken and colleagues (27) also described this finding, noting that TNF expression occurred 1–2 h after ETX injury, whereas myocardial dysfunction did not occur until 4–6 h after this insult. Findings from our evaluations were consistent with the above studies. We found that, in the 1-h groups, OVX PC +125, OVX PC +500, and PC +500 animals expressed less TNF, whereas the PC +125 females at 1 h released TNF in amounts similar to those of PC– animals. Furthermore, the amounts of IL-1β and IL-6 were not different among any of the groups after 1 h of incubation with ETX, with the exception of PC +125 females, which expressed elevated IL-1β at this time point. Work performed by our group (49) has provided evidence that IL-1β and IL-6 expressions are downstream of TNF release. These findings suggest that differences in IL-1β and IL-6 expression may occur at a time point later than was measured in our study.

These results clearly show that endogenous estrogen plays an important role in mediating the higher preconditioning injury threshold in females. This highlights the influence of gender in the injury response and may begin to explain the many discrepant clinical findings regarding gender and myocardial injury.

GRANTS

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REFERENCES

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