ENDOGENOUS ESTROGEN MEDIATES A HIGHER THRESHOLD FOR ENDOTOXIN-INDUCED MYOCARDIAL PROTECTION IN FEMALEs

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Running Title: Estrogen Mediated Myocardial Protection Threshold

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ABSTRACT

Introduction: 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 endotoxin-induced protection in females.

Methods: Adult proestrus and ovariectomized (OVX) female rats were preconditioned with intraperitoneal injections of 125 (PC+125) or 500 (PC+500) mcg/kg Salmonella typhimurium lipopolysaccharide (ETX) or normal saline (PC-). 24 hours later injury dose ETX (500 mcg/kg) was injected. After six hours, myocardial function was measured via Langendorff. p38 MAPK and JNK activation as well as TNF, IL-1, and IL-6 expression were evaluated.

Results: ETX injury significantly decreased LVDP in PC- groups versus controls. PC+500 protected against ETX injury resulting in normal cardiac function. PC+125 protected OVX but not proestrus females which had diminished myocardial function. Activated JNK and TNF increased in PC- animals, but were diminished in PC+500. Importantly, activated JNK and TNF increased in PC+125 proestrus females, whereas PC+125 OVX females displayed decreases in these molecules. There were no differences in p38 MAPK activation or expression of IL-1 or IL-6.

Conclusions: These results demonstrate that proestrus females require a higher stimulus (PC+500) to achieve myocardial protection against ETX injury. Removal of endogenous estrogen (OVX) lowered the preconditioning threshold (PC+125) resulting in protection after lesser injury. Additionally, myocardial JNK and TNF expression was decreased in OVX PC+125 females, which correlated with myocardial function differences.
Therefore, we conclude that endogenous estrogen mediates a higher threshold for endotoxin tolerance in female myocardium.

Key Words: Tolerance, Gender, Acute Injury
INTRODUCTION

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 forms of preconditioning (18, 22, 29, 48) noting that delayed protection can persist up to 72 hours 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 one month 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 to 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 (I/R) had better functional recovery and decreased expression of inflammatory mediators than males (47). Additionally, studies
performed by Chaudry et al (31, 38, 51, 53) have demonstrated that gender differences affect pro-inflammatory 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 mitogen activated protein kinase (MAPK) and c-Jun n-terminal kinase (JNK) are increased by acute injury. Both enzymes can lead to increased expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF), 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 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). Based on this background we hypothesized that: 1) both normal and ovariectomized females can be preconditioned; 2) endogenous estrogen mediates the higher endotoxin-induced myocardial protection threshold in females; and, 3) preconditioning modulates activation of p38 MAPK and JNK, as well as the expression of the pro-inflammatory cytokines TNF, IL-1, and IL-6 after acute injury.

MATERIALS AND METHODS

Animal Use and Care. Adult proestrous and ovariectomized (OVX) female Sprague-Dawley rats (weight: 250 to 300 g, Harlan Inc., 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 one week prior to 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 micrograms/kg *Salmonella typhimurium* lipopolysaccharide (ETX) or 0.4 ml normal saline (NS). After 24 hours, another IP injection of 500 micrograms/kg ETX was given. One or six hours later, the animals were anesthetized; their hearts rapidly excised and myocardial function evaluated using the Langendorff perfusion model. Normal females were ensured to be in the proestrus state by performing daily vaginal swabs. Ovariectomy was performed four weeks prior to 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/group) were formed. Non-preconditioned (PC-) rats received an IP NS injection. Preconditioned (PC+ 500) rats received 500 micrograms/kg ETX as the preconditioning stimulus. Preconditioning threshold animals (PC+125) received a 125 micrograms/kg ETX preconditioning stimulus. After 24 hours, 500 micrograms/kg ETX injury dose was administered IP to all groups, followed one or six hours later by assessment of cardiac function (figure 1). Endotoxin 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 sodium pentobarbital (Nembutal, 150 milligrams/kg) mixed with 500 Units sodium heparin (Fisher Scientific, Inc., Fair Lawn, NJ). A median sternotomy was performed and the heart rapidly removed and placed in a 4°C bath of Krebs-Henseleit (KH) 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 KH solution at 37°C, and bubbled with 95% O₂-5% CO₂ (Medipure) to achieve a PO₂ of 450-460 mm Hg, PCO₂ 39-41 mm Hg, and pH 7.39-7.41. Total ischemic time was less than 45 seconds. The perfusion buffer was continuously filtered through a 0.45 micron filter to remove particulates. A pulmonary arteriotomy and left atrial resection were performed prior to 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 (LVEDP) of 8 mm Hg (range 6-10 mm Hg) 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 (approximately 350 beats per minute) throughout perfusion. After an equilibration period of 10 minutes, myocardial function was evaluated for 20 minutes by continuous assessment of LVDP using a computerized PowerLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA), an Apple iMac (Apple Computer Inc., Cupertino, CA), and collecting pulmonary effluent at 0, 10, and 20 minutes as a measure of coronary flow (CF). The rate of cardiac contractility (+dP/dt)
and relaxation (-dP/dt) were also calculated (Chart v4.2, PowerLab ADInstruments, Milford, MA). After 20 minutes, the heart was removed from the apparatus, sectioned and snap frozen in liquid nitrogen.

**c-Jun N-terminal Kinase (JNK).** Western blot analysis was performed on cardiac tissue to measure total JNK and JNK activation (phosphor-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 hour. Then membranes were incubated with JNK antibody or phosphor-JNK (Thr 183/Tyr 185) antibody (Cell Signaling Technology) overnight at 4°C. The membrane was then washed 3 times for 5 minutes in TBS-T, incubated with HRP-conjugated secondary antibody for 1 hour, and again washed 3 times for 5 minutes in TBS-T. The membranes were developed using supersignal west pico stable peroxide solution (Pierce).

**Myocardial p38 Mitogen Activated Protein Kinase (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 hour. Then membranes were incubated with p38 MAPK antibody or phosphor-p38 MAPK (Thr 180/Tyr 182) antibody (Cell Signaling Technology) overnight at 4°C. The membrane was then washed 3 times for 5 minutes in TBS-T, incubated with HRP-conjugated secondary antibody for 1 hour, and again washed 3 times for 5 minutes in TBS-T. The membranes were developed using supersignal west pico stable peroxide solution (Pierce).
Myocardial TNF alpha, IL-1, and IL-6. Myocardial homogenate TNF-α, IL-1, and IL-6 content were determined by enzyme linked immunosorbent assay (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 Chemical Co., St. Louis, MO.

Presentation of Data and Statistical Analysis. All reported values are mean ± SEM. Differences at the 95% confidence level were considered significant. Data was compared using Student t-test analysis as well as ANOVA with Tukey post-test analysis (GraphPadPrism 4.0, Camino Real, San Diego, CA).

RESULTS

Myocardial Function

LVDP. One hour groups showed no decrease in left ventricular developed pressure (data not shown). Considerable differences however were seen in the six hour groups. Both normal and OVX non-preconditioned (PC-) groups experienced a profound decrement in LVDP compared to shams (p<0.0001 and p<0.004 respectively) (figure 2). In contrast, both normal and OVX groups preconditioned with 500 micrograms/kg ETX (PC+500) were protected from the second ETX insult resulting in preserved myocardial function (p>0.05 vs. sham). Interestingly, OVX rats preconditioned with 125 micrograms/kg ETX (OVX PC+125) maintained near normal LVDP compared to sham
animals (p>0.05) while the normal females preconditioned with 125 micrograms/kg were not protected and had a substantial decrease in LVDP compared to sham (p<0.001).

dP/dt. Positive and negative dP/dt are measures of myocardial contractility and relaxation, respectively. Both six hour groups of PC- animals had considerably less compliance compared to shams (figure 3A and 3B). Both normal and OVX PC+500 groups maintained near normal compliance after preconditioning, while 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 shams. There was no appreciable difference in + or – dP/dt for any of the one hour 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+125, OVX PC+500, and normal PC+500 groups compared to PC- groups indicating protection via as a result of preconditioning (figure 4). Importantly, normal PC+125 females had increased JNK activation similar to both normal and OVX PC- groups correlating with the differences seen in myocardial function.

p38 MAPK Activation

The amount of activated and total p38 MAPK was not different among any of the groups at the time point measured (figure 5).

TNF, IL-1, IL-6

TNF expression was decreased in the OVX PC+125 and OVX PC+500 animals compared to 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 to PC- rats at this time point (Figure 6A). Expression of IL-1 was higher in normal PC+125 animals; however no difference in expression was noted between animals in any other groups. Finally, IL-6 production resulting from endotoxic injury was not different between any of the animals at this selected time point (figure 6B and 6C).

**DISCUSSION**

These results clearly show that 1) both normal and OVX females can be preconditioned; 2) endogenous estrogen mediates the higher endotoxin-induced myocardial protection threshold in females; 3) decreased myocardial function correlates with increased active JNK (p-JNK) and TNF expression; 4) preconditioning modulates the activation of JNK in endotoxin-induced myocardial protection; and, 5) the decrease in p-JNK and expression of TNF in OVX PC+125, OVX PC+500, 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 and colleagues found that cardiac performance, after ETX administration, diminished significantly between 4 and 6 hours following exposure. In addition to demonstrating the myocardial depressive effect of ETX, their results also showed that myocardial function was preserved after preconditioning with endotoxin (29). Our data is consistent with this report, indicating that both normal and OVX females had similarly decreased cardiac performance 6 hours 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), which noted that OVX females were unable to be preconditioned and had resultant decreased myocardial function. This divergence in results may be due to differences between acute and delayed preconditioning as Song’s group evaluated early processes while our results evaluate 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+125 females were not preconditioned with the lower ETX injury, indicating they are relatively protected from small acute insults. In contrast, estrogen depleted OVX PC+125 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 and associates (6) found that estrogen depleted OVX females had larger areas of myocardial damage and resultant decreased function after injury than those animals receiving estrogen. Finally, our lab has demonstrated that normal females experience improved myocardial function and an attenuated inflammatory response after ischemic injury compared to males (47). Clearly endogenous estrogen, as demonstrated through in
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 or not endogenous estrogen was the mediator 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 nuclear factor kappa B (NFkB) and pro-inflammatory cytokines such as TNF (13, 25). The results of this study showed preconditioned animals expressed lower amounts of p-JNK. These findings are consistent with work performed by Sato et al (39) who found that preconditioning attenuated the increased expression of JNK after I/R injury. In an examination by Asai and colleagues (2), they noted that normal females had lower cytokine levels in response to ETX challenge compared to 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. In order to evaluate the effect of preconditioning on cytokine expression we measured TNF, IL-1β, and IL-6.

The time sequence of cytokine expression was described by Maass and associates (17) after burn. They noted significantly increased in levels TNF-α at one hour, without changes in the expression of IL-1β or IL-6. Harken’s group (27) also described this finding noting that TNF expression occurred 1-2 hours after ETX injury, while
myocardial dysfunction did not occur until 4-6 hours after this insult. Findings from our evaluations were consistent with the above studies. We found that, in the one hour groups, OVX PC+125, OVX PC+500, and PC+500 animals expressed less TNF, while the PC+125 females at one hour released TNF in amounts similar to that of non-preconditioned animals. Further, the amounts of IL-1β and IL-6 were not different among any of the groups after one hour incubation with ETX, with the exception of PC+125 females which expressed elevated IL-1β at this time point. Work performed by our group has (49) provided evidence that IL-1β and IL-6 expression 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.
ACKNOWLEDGMENT

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REFERENCES


Figure Legend

**Figure 1.** Injection time line and groups with their respective injection schedule. At time equal to zero and 24 hours, rats were given an intraperitoneal (IP) injection of either normal saline (NS) or endotoxin (ETX) per the injection schedule shown. At time equal to 25 or 30 hours, hearts were harvested and myocardial function assessed.

**Figure 2.** Left ventricular developed pressure (LVDP) normal and OVX females, 6 hours. PC- groups showed decreased LVDP compared to sham. Normal PC+125 females also were not protected which resulted in impaired myocardial function. Importantly, OVX PC+125, OVX PC+500 and PC+500 normal females were protected and retained near normal LVDP after preconditioning compared to sham. * p<0.05 vs. sham, † p<0.05 vs. PC+500, # p<0.05 vs. OVX sham.

**Figure 3A.** Normal and OVX female cardiac contractility (+dP/dt) 6 hour groups. Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). ETX injury resulted in decreased myocardial contractility in PC- animals but was preserved in PC+500 rats. Cardiac contractility was near normal in PC+125 males, but diminished in PC+125 females. Results are shown as mean ± SEM, * p<0.05 vs. sham, † p<0.05 vs. PC+500, # p<0.05 vs. OVX sham.

**Figure 3B.** Normal and OVX female cardiac relaxation (-dP/dt) 6 hour groups. Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). ETX injury resulted in decreased myocardial relaxation in PC- animals but...
was preserved in PC+500 rats. Cardiac relaxation was near normal in PC+125 males, but impaired in PC+125 females. Results are shown as mean ± SEM, * p<0.05 vs. sham, † p<0.05 vs. PC+500, # p<0.05 vs. OVX sham.

**Figure 4.** Normal and OVX female activated myocardial c-Jun n-terminal kinase (JNK) 1 hour groups. A. Shown are representative immunoblots of total JNK in the top row with active (p-) JNK in the bottom row. B. Densitometry data of p-JNK (% of total JNK). Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). ETX injury resulted in increased JNK activation in both normal and OVX PC- groups as well as normal PC+125 females. In contrast, OVX PC+125 as well as both normal PC+500 and OVX PC+500 animals exhibited decreased amount of activated JNK after acute ETX injury. Results are shown as mean ± SEM, * p<0.05 vs. OVX PC-, † p<0.05 vs. PC-, # p<0.05 vs. PC+125.

**Figure 5.** Normal and OVX female activated myocardial p38 mitogen activated protein kinase (MAPK) 1 hour groups. A. Shown are representative immunoblots of total p38 MAPK in the top row with active (p-) p38 in the bottom row. B. Densitometry data of p-p38 (% of total p38). Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). ETX injury resulted in no appreciable difference between the groups in expression of activated p38 MAPK. Results are shown as mean ± SEM.

**Figure 6A.** Normal and OVX female cardiac TNF expression 1 hour groups. Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after
preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). ETX injury resulted in greater TNF expression in PC+125 rats similar to shams, while OVX PC+125, OVX PC+500, and normal PC+500 animals were protected and had decreased levels of TNF production. Results are shown as mean ± SEM. * p<0.05 vs. OVX PC-, † p<0.05 vs. PC+125

**Figure 6B.** Normal and OVX female cardiac IL-1 expression 1 hour groups. Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). Expression of IL-1 was elevated in normal PC+125 rats compared to normal PC- animals. There was no other disparity seen between any of the groups in IL-1 expression after ETX injury at this time point. Results are shown as mean ± SEM. * p<0.05 vs. PC-.

**Figure 6C.** Normal and OVX female cardiac IL-6 expression 1 hour groups. Rats were subjected to endotoxin (ETX) injury without preconditioning (PC-) or after preconditioning with 125 micrograms/kg ETX (PC+125) or 500 micrograms/kg ETX (PC+500). ETX injury resulted in no difference among any of the groups in expression of IL-6 at the selected time point. Results are shown as mean ± SEM.
Figure 1

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Figure 2

![Bar chart showing LVDP mmHg for different groups: Sham, PC-, PC+125, PC+500. The chart compares Normal Female and Ovariectomized Female groups, with statistical significance indicated by asterisks (*) and hash symbols (#).]
Figure 3A
Figure 3B
Figure 4

A

B

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* p-JNK (% of JNK)
Figure 5

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B

- **Normal Female**
- **OVX Female**

![Bar chart showing p-p38 (% of p38) for PC-, PC+125, and PC+500 in Normal Female and OVX Female categories.](image-url)
Figure 6A

![Graph showing TNF pg/mg protein levels for Normal Female and OVX Female groups in PC-, PC+125, and PC+500 conditions.](image-url)
Figure 6B
Figure 6C

![Bar chart showing IL-6 levels in Normal Female and OVX Female groups with PC-500, PC+125, and PC+500 treatments.]