Estrus cycle: influence on cardiac function following trauma-hemorrhage

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PREVIOUS STUDIES HAVE SHOWN that cardiac function is depressed following trauma-hemorrhage. Since cardiac function is depressed in males but not in proestrus (PE) females following trauma-hemorrhage (T-H), we examined whether different estrus cycles influence cardiac function in female rats under those conditions. We hypothesized that females in the PE cycle only will have normal cardiac function following T-H and resuscitation. Sham operation or T-H was performed in five groups of rats (250–275 g) including PE, estrus (E), metestrus (ME), diestrus (DE), and ovariectomized (OVX) females (n = 6–7 per group). Cardiac function was determined 2 h after T-H, following which cardiomyocytes were isolated and nuclei extracted. Cardiomyocyte IL-6 and NF-κB expressions were measured using Western blotting. Moreover, plasma IL-6, estradiol, and progesterone levels were measured using ELISA or EIA kits. Results (1-way ANOVA) indicated that following T-H, cardiac function was depressed in DE, E, ME, and OVX groups but maintained in the PE group; 2) the PE group had the highest plasma estrogen level; 3) plasma IL-6 levels increased significantly in DE, E, ME, and OVX groups, but the increase was attenuated in the PE group; 4) cardiomyocyte IL-6 protein level increased significantly in DE, E, ME and OVX groups after T-H, but the increase was attenuated in the PE group; and 5) cardiomyocyte NF-κB expression increased significantly but was attenuated in the PE group. These data collectively suggest that the estrus cycle plays an important role in cardiac function following TH. The salutary effect seen in PE following TH is likely due to a decrease in NF-κB-dependent cardiac IL-6 pathway.

Female reproductive cycle; ovariectomy; estrogen; trauma-hemorrhagic shock; interleukin-6; nuclear factor-κB

It has been shown that high estrogen levels in female rats in the proestrus cycle maintain cardiac function following trauma-hemorrhage (16). However, the estrogen levels vary in different estrus cycles, and thus, depending on the stage of estrus cycle, females may exhibit variations in their responses to trauma-hemorrhage. Studies also have shown a correlation between the sustained elevation in IL-6 levels and poor outcome following hypoxia (26, 36, 37). It also has been demonstrated that IL-6 gene expression that is induced by hypoxia is mainly through the activation of NF-κB in cardiomyocytes (24). Furthermore, the transcription factors NF-IL-6 and NF-κB are known to synergistically activate the transcription of inflammatory cytokines, chemokines, and adhesion molecules (27). More recently, we (40) found an increase in cardiac IL-6 protein levels and also increased cardiomyocyte IL-6 gene expression following trauma-hemorrhage. In this study we investigated whether different estrus cycles have an influence on cardiac function following trauma-hemorrhage and whether cardiac function under those conditions is related to cardiac IL-6 production. Thus, we hypothesized that downregulation of cardiac IL-6 might be one of the mechanisms by which proestrus females have protective effects on cardiac function following trauma-hemorrhage. Since NF-κB is involved in IL-6 regulation, we also examined whether the different female reproductive cycles have any effects on NF-κB/IκB-α.

MATERIALS AND METHODS

Experimental protocol. The aim of the present study was to determine whether different estrus cycles influence cardiac function following trauma-hemorrhage in female rats and to clarify the relationship between sex hormones and cardiac function following trauma-hemorrhage. Sham operation or trauma-hemorrhage was performed in five groups of female rats: proestrus (PE), estrus (E), metestrus (ME), diestrus (DE), and ovariectomized (OVX). Cardiac function was determined 2 h after trauma-hemorrhage, cardiomyocytes were isolated, and nuclei were then extracted. Plasma sex hormones including estradiol and progesterone were measured. Systemic IL-6 levels also were measured. Moreover, cardiomyocyte IL-6 and NF-κB expression levels were determined by Western blot analysis.

Trauma-hemorrhagic shock model and experimental groups. Trauma-hemorrhagic shock (34) was induced in PE, E, ME, DE, and OVX (2 wk before trauma-hemorrhage was induced) female adult (225–275 g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as described previously by our group, with some modifications (34, 35). In brief, all experimental rats were fasted overnight but allowed water ad libitum before the experiment. After determination of the estrus cycle, both the right and left femoral arteries and right
vein were cannulated with PE-50 tubing under anesthesia with isoflurane inhalation. The tubing in the right femoral artery was connected to a blood pressure analyzer (BPA; Digi-Med, Louisville, KY) for measuring and monitoring mean arterial pressure (MAP) and heart rate (HR); the tubing in the left femoral artery was used for blood withdrawal; and the tubing in the right femoral vein was used for fluid resuscitation. The total circulating blood volume was calculated according to our previous studies (i.e., total circulating blood volume = body weight × 6%). MAP was monitored continuously before the onset of hemorrhage until the end of resuscitation. We allowed ~10–15 min after cannulation for the animal to awaken completely from anesthesia. The animals were bled rapidly under conscious conditions to reach MAP of 35–40 mmHg within 10 min, and MAP was maintained at that level by further withdrawing blood until maximum bleed out (MBO) occurred. The amount of blood volume withdrawn to reach MBO was ~60% of the total circulating blood volume. The rats were then maintained at the MAP of 40 mmHg with infusions of small volumes of Ringer lactate for 45 min, followed by fluid resuscitation with Ringer lactate (4× MBO volume) over 1 h. The total time for trauma-hemorrhage model preparation was ~2.5–3 h. The animals were then returned to cages and allowed food and water ad libitum.

In the OVX group, ovariectomy was performed 2 wk before trauma-hemorrhage. Ovariectomy was performed by placing the animal in a prone position. A 1-cm-long incision was made on both sides of the waist under isoflurane inhalation, and ovaries on both sides were removed, blood vessels secured, and incisions closed. The experiments described were performed in adherence to the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals. This project was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Estrus cycle determination. Estrus cycles were determined in early morning on the experimental day by vaginal smear. Three types of cells are found in vaginal smears: polymorphonuclear (PMN), nucleated epithelial, and cornified epithelial cells (ECs). Primarily nucleated ECs with a few cornified ECs were detected in proestrus; primarily cornified ECs with a few nucleated ECs were found in estrus, primarily nucleated ECs were found in metestrus, and very few cells of all types were found in diestrus (21, 33).

Cardiac function determination. At 2 h after trauma-hemorrhage or sham operation, cardiac output (CO) was determined using the indocyanine green dilution technique (40). MAP and HR were documented using the BPA. Left ventricular performance parameters such as the maximal rate of pressure increase (dP/dtmax) and decrease (−dP/dtmax) were also measured with a heart performance analyzer (HPA; Digi-Med). Stroke volume (SV), total peripheral resistance (TPR), systemic O2 delivery, and O2 consumption were calculated according to standard equations as previously described in our studies (40).

Measurement of plasma levels of sex hormones. At the onset of trauma-hemorrhage, 2 ml of blood were collected in EDTA-coated tubes via the right carotid arterial catheter in various groups. Plasma was separated immediately by centrifugation, and samples were stored at −70°C until assayed for sex hormones. Plasma levels of estradiol and progesterone were determined by EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

Systemic IL-6 measurement. Blood samples were collected in EDTA-coated tubes at 2 h after trauma-hemorrhage or sham operation following the measurement of cardiac function in various groups. Plasma was immediately separated by centrifugation, and samples were stored at −70°C until assayed for systemic IL-6. Systemic IL-6 levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Left ventricular cardiomyocytes isolation. After measurement of cardiac function, cardiomyocytes from ventricle were isolated immediately as described previously by our group, with modifications (28). Briefly, the heart was quickly removed from the chest and perfused in a retrograde manner via the aorta at 37°C and at a constant rate (12 mg·min⁻¹·g tissue⁻¹) for 5 min with a calcium-free Krebs buffer containing (in mM) 118 NaCl, 4.7 KCl, 25 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, 10 HEPEs, and 11 glucose, gassed with 95%O2-5%CO2. After the wash-perfusion, the calcium-free Krebs buffer was replaced by an enzymatic digestion buffer containing collagenase type II (Worthington, Lakewood, NJ), 0.1% fat-free BSA, 100 μM CaCl2, and 10 mM taurine and perfused at 37°C at a constant rate (5 mg·min⁻¹·g tissue⁻¹) for 9 min. When the heart became swollen and hard, the left ventricle was removed and cut into small chunks (~1× 1 mm³) and further digested with the incubation buffer containing the enzymatic digesting buffer and 2% fat-free BSA in a shaker (60–70 rpm) water bath at 37°C for 10 min. The supernatant containing the dispersed cardiomyocytes was filtered through a 300-μl filter into a 50-ml sterilized tube and gently centrifuged at 500 rpm for 1 min. The upper portion of the supernatant was discarded, and ~30 ml BSA-free buffer (calcium-free Krebs buffer + 50 μM CaCl2) was added and centrifuged at 480 rpm for 1 min. The upper portion of the supernatant was discarded again, and a 10-ml cell suspension was carefully layered onto high BSA (4%) medium and centrifuged (480 rpm, 1 min). The number of cardiomyocytes was then counted by suspending cardiomyocytes in 0.02% Trypan blue under a light microscope. All the buffers were filtered (0.2-μM filter) and equilibrated with 95%O2-5%CO2 for at least 20 min before use. To reduce bacterial and viral contamination, the perfusion setup was washed each day with 70% alcohol and then washed with sterilized distilled water before perfusion.

Cardiomyocyte nuclear extraction. Isolated cardiomyocytes (~1.5 × 10⁶) were rapidly suspended in 1 ml of hypotonic buffer including (in mM) 10 HEPEs (pH 7.9), 1.5 MgCl2, 10 KCl, 0.5 DTT, 0.1 PMSF, and 10 μg/ml aprotinin. The cardiomyocytes were centrifuged at 1,500 rpm for 5 min at 4°C, and the supernatant was discarded. The packed cardiomyocytes were resuspended in hypotonic buffer and allowed to swell for 10 min on ice. The cardiomyocyte integrity was then broken using a 26.5 needle with six repeats of up-and-down pushing. The cardiomyocyte nuclei were then collected by centrifuging at 4,000 rpm for 15 min at 4°C. Finally, the packed nuclei pellet was resuspended in an equal volume of high-salt buffer (calcium-free Krebs buffer containing (in mM) 20 HEPEs (pH 7.9), 1.5 MgCl2, 0.6 KCl, 0.2 EDTA, 0.5 DTT, 0.1 PMSF, 25% glycerol, and 10 μg/ml aprotinin for 60 min with continuous gentle mixing to extract the nuclei. The extracted cardiomyocyte nuclei were collected by centrifuging at 14,000 rpm for 15 min at 4°C. The extracted cardiomyocyte nuclei were stored at −70°C until used.

Western blot analysis of cardiomyocyte IL-6, estrogen receptor (ER)-α, ER-β, NF-κB, and IκBα-phospho-IκBα expression. Approximately 1.5–2.0 × 10⁶ cardiomyocytes were homogenized in 0.5–1 ml of lysis buffer containing (in mM) 20 HEPEs (pH 7.9), 1.5 MgCl2, 20 KCl, 0.2 EDTA, 2 NaNO3, 10 NaN, 0.2 PMSF, 20% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cardiomyocyte lysate was centrifuged at 10,000 g for 10 min at 4°C, and the protein concentration of supernatant was measured (Bio-Rad Laboratories, Hercules, CA). The lysates were analyzed on SDS-PAGE (4–20%), and the proteins were transferred to nitrocellulose membranes. For IL-6, ERs, and IκBα-α, 40 μg of lysate per lane were loaded, whereas for NF-κB, 20 μg of cardiomyocyte nuclear extract per lane were loaded. After transfer, the membranes were immuno-oblotted with anti IL-6 (BioSource International, Camarillo, CA), ER-α/ER-β, NF-κB, and IκBα-phospho-IκBα antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, followed by the addition of horseradish peroxidase-conjugated secondary antibody. After the final wash, membranes were probed using enhanced chemiluminescence dye (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and autoradiographed. The density of the bands was determined using densitometric analysis. In addition, the membranes were re-probed using glyceraldehyde-3-phosphate dehydrogenase (Abcam,
Cambridge, MA) or histone H1 (Upstate, Charlottesville, VA) as loading control.

Statistical analysis. Data are presented as means ± SE. One-way analysis of variance and Tukey’s test were employed for comparison among different groups of animals. The differences are considered significant at \( P \leq 0.05 \).

RESULTS

Alterations in cardiac function and systemic hemodynamic parameters following trauma-hemorrhage and resuscitation. Table 1 shows cardiac functions in sham animals in various stages of estrus cycle, because the plasma sex hormone levels are different in various estrus cycles and these levels may influence the basal cardiac function. However, when conducting the experiment, we found that there was no difference in the basal cardiac function from various estrus cycles, including OVX after sham operation. Since the results in Table 1 do not show differences in various parameters in different estrus cycles, we have pooled “sham group” values, and those combined sham values are included in the subsequent data presentations where comparisons are made between sham and trauma-hemorrhage in various stages of estrus cycle.

CO decreased by 26.2, 27.8, 35.9, and 51.1% in E, ME, DE, and OVX females compared with shams, respectively, at 2 h after trauma-hemorrhage (Fig. 1A). However, CO was normal in PE females and was significantly higher compared with that in E, ME, DE, and OVX groups at 2 h after trauma-hemorrhage (Fig. 1A). Moreover, SV decreased by 16.3, 17.0, 19.6, and 36.4% in E, ME, DE, and OVX females compared with shams, respectively, after trauma-hemorrhage (Fig. 1B). Furthermore, TPR was not significantly altered in PE, E, ME, and DE groups under those conditions (Fig. 1C). Similarly, +dP/dt increased by 28.6, 29.2, 44.1, and 54.2%, and −dP/dt decreased by 25.4, 36.3, 53.4, and 66.5%, in E, ME, DE, and OVX females, respectively, at 2 h after trauma-hemorrhage compared with shams (Fig. 1D and E). Both +dP/dt and −dP/dt were maintained in PE females at 2 h after trauma-hemorrhage (Fig. 1, D and E). There was a significant decrease in MAP in E, ME, DE, and OVX females and in HR in DE and OVX females after trauma-hemorrhage compared with sham; however, both MAP and HR were maintained in the PE group (Table 2). The hemoglobin and hematocrit results indicate that the trauma-hemorrhagic shock models were consistent in various groups (Table 2). Although 60% of total circulating blood was withdrawn and hemoglobin and hematocrit significantly decreased in all trauma-hemorrhage groups, systemic O2 delivery in PE significantly improved in the PE compared with DE and OVX groups. Moreover, the increase in circulating lactate was prevented in the PE trauma-hemorrhage group. This finding suggests that acidosis following trauma-hemorrhage was prevented and the metabolic situation was improved in this group (Table 2). In addition, the mortality rate in the present study was 10.0% (1 of 10), 18.2% (2 of 11), and 33.3% (4 of 12) in E, DE, and OVX females within 2 h after trauma-hemorrhage, respectively. In contrast, all PE and ME females were alive at 2 h after trauma-hemorrhage in this study.

Alterations in plasma levels of sex hormones. Plasma levels of estradiol were highest at the start of the experiment in the PE group (9.34 ± 0.16 pg/mg protein) and were significantly lower in E (5.34 ± 0.27 pg/mg protein), ME (3.29 ± 0.27 pg/mg protein), DE (2.32 ± 0.27 pg/mg protein), and OVX groups (0.59 ± 0.12 pg/mg protein) (Fig. 2A). Moreover, the highest plasma progesterone levels were found in the E and ME groups (Fig. 2B).

Alterations in systemic IL-6 levels. Plasma levels of IL-6 were significantly increased in the ME, DE, and OVX groups after trauma-hemorrhage compared with shams (Fig. 2A); however, plasma IL-6 levels were markedly lower in PE females after trauma-hemorrhage.

Alterations in cardiomyocyte IL-6 expression. A significant increase occurred in IL-6 protein level in the cardiomyocytes homogenates prepared from the DE and OVX groups after trauma-hemorrhage compared with the homogenates from sham-operated animals; however, the trauma-hemorrhage-me-

Table 1. Cardiac function, systemic \( \text{DO}_2 \) and \( \text{VO}_2 \), Hb, systemic hematocrit, and plasma lactate at 2 h after sham operation in different estrus cycles

<table>
<thead>
<tr>
<th></th>
<th>PE</th>
<th>E</th>
<th>ME</th>
<th>DE</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO, ml/min−1·100 g body wt−1</td>
<td>41.1 ± 0.4</td>
<td>40.6 ± 0.4</td>
<td>40.2 ± 0.3</td>
<td>40.2 ± 0.3</td>
<td>40.0 ± 0.4</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>15338 ± 1160</td>
<td>14698 ± 600</td>
<td>15081 ± 1150</td>
<td>13983 ± 1045</td>
<td>14078 ± 988</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>9705 ± 591</td>
<td>9222 ± 564</td>
<td>9324 ± 120</td>
<td>9303 ± 443</td>
<td>9111 ± 814</td>
</tr>
<tr>
<td>SV, μl·beat−1·100 g tissue−1</td>
<td>100.3 ± 0.9</td>
<td>100.9 ± 0.7</td>
<td>99.7 ± 0.8</td>
<td>100.1 ± 0.3</td>
<td>99.7 ± 0.9</td>
</tr>
<tr>
<td>TPR, mmHg·ml−1·100 g body wt−1</td>
<td>2.98 ± 0.04</td>
<td>2.98 ± 0.03</td>
<td>3.01 ± 0.14</td>
<td>2.98 ± 0.12</td>
<td>3.01 ± 0.16</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>123 ± 1</td>
<td>121 ± 2</td>
<td>121 ± 6</td>
<td>120 ± 4</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>HR, beat/min</td>
<td>410 ± 57</td>
<td>403 ± 22</td>
<td>403 ± 24</td>
<td>402 ± 24</td>
<td>402 ± 24</td>
</tr>
<tr>
<td>( \text{Do}_2 ), ml/min−1·100 g body wt−1</td>
<td>7.7 ± 0.2</td>
<td>7.7 ± 0.1</td>
<td>7.7 ± 0.1</td>
<td>7.8 ± 0.2</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>( \text{Vo}_2 ), ml/min−1·100 g body wt−1</td>
<td>2.4 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.4</td>
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<tr>
<td>Hb, g/dl</td>
<td>14.4 ± 0.5</td>
<td>14.5 ± 0.2</td>
<td>14.9 ± 0.1</td>
<td>14.7 ± 0.4</td>
<td>14.7 ± 0.2</td>
</tr>
<tr>
<td>Hematocrit sys</td>
<td>44.0 ± 1.0</td>
<td>44.6 ± 0.4</td>
<td>45.0 ± 0.8</td>
<td>44.8 ± 1.8</td>
<td>44.6 ± 0.7</td>
</tr>
<tr>
<td>Lactate, mg/dl</td>
<td>8 ± 2</td>
<td>7 ± 1</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
<td>8 ± 1</td>
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</table>

Values are presented as means ± SE (n = 4/group) and were compared using 1-way ANOVA and Tukey’s test. PE, proestrous; E, estrus; ME, metestrus; DE, diestrous; OVX, ovariectomized. CO, cardiac output; +dP/dt, rate of pressure increase; −dP/dt, rate of pressure decrease; SV, stroke volume; TPR, total peripheral resistance; MAP, mean arterial pressure; HR, heart rate; \( \text{Do}_2 \), oxygen delivery; \( \text{Vo}_2 \), oxygen consumption; Hb, hemoglobin; hematocrit sys, systemic hematocrit.
dilated increase in IL-6 expression was markedly attenuated in PE females (Fig. 3, B and C).

**Alterations in cardiomyocyte ERs.** Cardiac ER-α expression decreased significantly in the DE and OVX groups after trauma-hemorrhage compared with shams (P < 0.05, Figs. 4, A and B); however, no difference in cardiomyocyte ER-α levels was observed in the PE group compared with shams (Fig. 4, A and B). Similarly, cardiac ER-β expression decreased significantly in the DE and OVX groups after trauma-hemorrhage (P < 0.05, Fig. 4, A and B); however, the levels in the PE group after trauma-hemorrhage were similar to those in shams (P < 0.05, Fig. 4, A and C).

**Alterations in cardiomyocyte IκB-α and NF-κB.** Similar levels of cardiomyocyte IκB-α expression were detected among different groups (Fig. 5, A and B); however, phospho-IκB-α expression was significantly increased in DE and OVX females after trauma-hemorrhage (P < 0.05, Fig. 5, A and C). Expression of cardiomyocyte phospho-IκB-α did not change in the PE group (Fig. 5, A and C) after trauma-hemorrhage. Cardiac NF-κB expression increased significantly following trauma-hemorrhage in the DE and OVX groups (P < 0.05, Fig. 5, A and C). Table 2.

**Table 2. Alterations in MAP, HR, systemic DO$_2$ and VO$_2$, Hb, systemic hematocrit, and plasma lactate at 2 h after trauma-hemorrhage and resuscitation**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>PE-TH</th>
<th>E-TH</th>
<th>ME-TH</th>
<th>DE-TH</th>
<th>OVX-TH</th>
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<tr>
<td>MAP, mmHg</td>
<td>121±4</td>
<td>100±2</td>
<td>81±6*</td>
<td>88±5*</td>
<td>76±3*</td>
<td>73±8†</td>
</tr>
<tr>
<td>HR, beat/min</td>
<td>403±5</td>
<td>360±12</td>
<td>357±12</td>
<td>365±16</td>
<td>322±8*</td>
<td>316±22*</td>
</tr>
<tr>
<td>DO$_2$, ml/min/100 g body wt$^{-1}$</td>
<td>7.6±0.1</td>
<td>2.9±0.1*</td>
<td>2.4±0.1*</td>
<td>2.5±0.1*</td>
<td>2.0±0.1*</td>
<td>1.7±0.1†</td>
</tr>
<tr>
<td>VO$_2$, ml/min/100 g body wt$^{-1}$</td>
<td>2.4±0.3</td>
<td>1.5±0.1*</td>
<td>1.2±0.1*</td>
<td>1.0±0.1*</td>
<td>1.0±0.1*</td>
<td>1.0±0.1*</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>14.2±0.3</td>
<td>5.4±0.3*</td>
<td>5.6±0.2*</td>
<td>5.8±0.2*</td>
<td>5.7±0.1*</td>
<td>6.2±0.3*</td>
</tr>
<tr>
<td>Hematocrit$_{sys}$</td>
<td>43±1</td>
<td>17±1*</td>
<td>18±1*</td>
<td>18±1*</td>
<td>18±1*</td>
<td>19±1*</td>
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<td>Lactate, mg/dl</td>
<td>7±1</td>
<td>10±1</td>
<td>18±2§</td>
<td>21±4*§</td>
<td>35±5*†</td>
<td>38±3*</td>
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</tbody>
</table>

Values are presented as means ± SE (n = 6–7/group) and were compared using 1-way ANOVA and Tukey’s test. *P < 0.05 vs. sham. †P < 0.05 vs. PE-TH. ‡P < 0.05 vs. DE-TH. §P < 0.05 vs. OVX-TH. T-H, trauma-hemorrhage.
trauma-hemorrhage in the E, ME, DE, and OVX groups ($P < 0.05$); however, there was no significant difference in NF-κB in the PE group following trauma-hemorrhage compared with the sham group ($P < 0.05$, Fig. 6, A and B).

**DISCUSSION**

Our results indicate that cardiac function was significantly depressed in E, ME, DE, and OVX female rats as demonstrated by significantly decreased CO, SV, and $\frac{dP}{dt_{\text{max}}}$ following trauma-hemorrhage and resuscitation. Females in the PE cycle, however, maintained all of the above cardiac functional parameters following trauma-hemorrhage. The maintenance of cardiac function following trauma-hemorrhage in PE females was associated with the highest levels of estradiol, whereas all others had significantly lower plasma levels of estradiol. Although estradiol levels were higher in E and ME cycles compared with OVX females, it appears that those levels were not sufficient to prevent cardiac depression following trauma-hemorrhage. Moreover, both cardiomyocyte ER-α and ER-β expression levels were significantly decreased in DE and OVX females following trauma-hemorrhage; however, they were maintained at levels similar to those in shams in the PE group following trauma-hemorrhage. Furthermore, these levels were significantly higher compared with those in DE and OVX females following trauma-hemorrhage. It should also be noted that cardiomyocyte and plasma IL-6 levels were significantly increased in E, ME, DE, and OVX groups at 2 h after trauma-hemorrhage, whereas they were significantly attenuated in the PE group under those conditions. Although high levels of progesterone were found in E and ME females, it appears that progesterone did not have sufficient protective effects on cardiac function following trauma-hemorrhage. This conclusion may appear at variance from previous findings by our group (20), which showed that administration of progesterone (25 mg/kg body wt) in OVX rats following trauma-hemorrhage ameliorated the proinflammatory response and prevented hepatic injury under those conditions. However, it should be noted that the highest level of progesterone observed in the plasma of mice was $\sim 30$ pg/mg protein, whereas 25 mg/kg progesterone was administered in the previous study (20). Thus far higher levels of progesterone are needed for progesterone to have salutary effects following trauma-hemorrhage.

**Fig. 2.** Alterations in plasma estradiol (A) and progesterone (B) levels in PE, E, ME, DE, and OVX females. There were 6–7 animals in each group. Data are expressed as means ± SE and were compared using 1-way ANOVA and Tukey’s test. $\# P < 0.05$ vs. PE. $+ P < 0.05$ vs. OVX. $\ast P < 0.05$ vs. DE.

**Fig. 3.** Plasma (A) and cardiomyocyte IL-6 protein levels (B and C) in sham, PE-TH, E-TH, ME-TH, DE-TH, and OVX-TH females. Representative blot is shown in B. Blots were analyzed densitometrically; densitometric values are pooled from 4 animals in each group and are shown in C. Data are expressed as means ± SE and were compared using 1-way ANOVA and Tukey’s test. $\ast P < 0.05$ vs. sham. $\# P < 0.05$ vs. PE-TH. $+ P < 0.05$ vs. OVX-TH.
Our results collectively demonstrate that ovarian and gonadal sex steroids are associated with the sexual dimorphic response to trauma-hemorrhage. Previous studies by our group have shown that immunological functions are markedly depressed in males following trauma-hemorrhage but not in PE females under those conditions (1, 42). The reason for the diverse immunological effects between males and PE females appears to be due to high estradiol levels that are observed in the proestrus state (1, 16). Support for this notion comes from studies showing that OVX females responded to trauma-hemorrhage in a manner even worse than that of males. Furthermore, administration of estradiol in OVX females and even in males normalized the depressed cardiovascular and immunological responses following trauma-hemorrhage (16, 26, 40). These studies therefore suggest that the maintenance or depression of cardiac function is dependent on the estrogen levels prevailing at the time of trauma-hemorrhage. Support for this notion comes from the present study, which indicates that the PE females, which have the highest estrogen levels, maintained cardiac function following trauma-hemorrhage. Although E and ME females showed some improvement in CO, SV, and \( \frac{d\text{P}}{dt} \) compared with OVX females following trauma-hemorrhage, all of the measured parameters were not restored to normal levels. The slight improvement in the above parameters may be due to the relatively higher estrogen levels in E and ME females compared with OVX females. It therefore appears that the protective effects of estrogen on cardiac function following trauma-hemorrhage are likely due to the estrogen levels present at the time of injury.

It also appears that not only low levels of estradiol but also the levels of male sex steroids are responsible for the depression of cardiac function following trauma-hemorrhage. In this

Fig. 4. Cardiomyocyte estrogen receptor (ER)-\( \alpha \) and ER-\( \beta \) expression in sham, PE-TH, E-TH, ME-TH, DE-TH, and OVX-TH females. Cardiomyocyte lysates prepared from various groups were analyzed on SDS-PAGE and immunoblotted using antibodies to ER-\( \alpha \) and ER-\( \beta \). A representative blot is shown in A. Blots were analyzed densitometrically; densitometric values are pooled from 4 animals in each group and are shown in B (ER-\( \alpha \)) and C (ER-\( \beta \)). For equal protein loading, the membranes were reprobed with GAPDH for control. Data are expressed as means \( \pm \) SE and were compared using 1-way ANOVA and Tukey’s test. *\( P < 0.05 \) vs. sham. #\( P < 0.05 \) vs. PE-TH. +\( P < 0.05 \) vs. OVX-TH.

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Fig. 5. Cardiomyocyte I\( \kappa \)B-\( \alpha \)/phospho (P)-I\( \kappa \)B-\( \alpha \) expression levels in sham, PE-TH, E-TH, ME-TH, DE-TH, and OVX-TH females. Cardiomyocyte lysates prepared from various groups were analyzed on SDS-PAGE and immunoblotted using antibodies to I\( \kappa \)B-\( \alpha \) and P-I\( \kappa \)B-\( \alpha \). A representative blot is shown in A. Blots were analyzed densitometrically; densitometric values are pooled from 4 animals in each group and are shown in B (total I\( \kappa \)B-\( \alpha \)) and C (P-I\( \kappa \)B-\( \alpha \)). For equal protein loading, the membranes were reprobed with GAPDH for control. Data are expressed as means \( \pm \) SE and were compared using 1-way ANOVA and Tukey’s test. *\( P < 0.05 \) vs. sham. #\( P < 0.05 \) vs. PE-TH. +\( P < 0.05 \) vs. OVX-TH. ^\( P < 0.05 \) vs. DE-TH.
beneficial effects of estradiol on cardiac function following trauma-hemorrhage appear to be due in part to the decreased IL-6 synthesis in cardiomyocytes (40). Although our previous studies suggest that Kupffer cells are the primary source of circulatory IL-6 levels, we found that cardiomyocytes also can synthesize IL-6 and that the local production of IL-6 plays a key role in regulating cardiac function following adverse circulatory conditions (40). In the present study, plasma IL-6 levels were increased at 2 h after trauma-hemorrhage and resuscitation, and the elevated plasma and cardiac IL-6 levels in E, ME, DE, and OVX females were associated with adverse effects on cardiac function. However, in PE females the level of IL-6 following trauma-hemorrhage was not significantly different from that observed in sham animals. Since the levels of estrogen are highest in the PE state, it is likely that the elevated level of estrogen in PE females downregulates cardiomyocyte IL-6 production and thus protects cardiac functions following trauma-hemorrhage. To establish the relationship between cardiac IL-6 and cardiac function following trauma-hemorrhage, we administered goat anti-rat-IL-6 monoclonal antibody at the middle of resuscitation after trauma-hemorrhage to neutralize IL-6 and measured the effect of this treatment on cardiac function following trauma-hemorrhage. Our results suggested that treatment of animals with anti-IL-6 antibodies downregulated cardiac IL-6 and improved cardiac function (39).

The protective effects of estrogen in cardiovascular diseases are currently under intensive investigation because of the potential therapeutic hormone effects (7, 25). However, the molecular mechanism by which estrogen downregulates cardiac IL-6 production is still not fully understood. Studies have demonstrated that estradiol attenuates cytokine production by inhibiting the transcription factor (nuclear factor) NF-κB (18). NF-κB is a pleiotropic transcription factor implicated in the regulation of diverse biological phenomena, including the cellular responses to stress, hypoxia, ischemia (18), and hemorrhagic shock (13). Studies have shown that NF-κB is activated in various heart diseases such as myocarditis, congestive heart failure, dilated cardiomyopathy, and heart transplant rejection (18). In the heart, NF-κB is found to be activated following burn, ischemia-reperfusion, and hypoxia (23, 24, 28). Moreover, studies have demonstrated that induction of IL-6 by hypoxia, a condition associated with trauma-hemorrhage, is mediated by NF-κB and NF-IL-6 in cardiomyocytes (28) and that hypoxia induces IL-6 gene expression through NK-κB activation (3). NF-κB and IκB-α interact in an autoregulatory mechanism (24). Transcription factors NF-IL-6 and NF-κB are known to synergistically activate the transcription of inflammatory cytokines (23). Studies also have shown that hypoxia-induced transcriptional activation of IL-6, i.e., IL-6 gene expression, is induced mainly through the activation of NF-κB in cardiomyocytes (29). Studies also have shown that estrogen represses IL-6 gene expression through inhibition of the DNA-binding activities of the transcription factors NF-IL-6 and NF-κB by the ERs (31). The present findings suggest a relationship between the increased cardiac IL-6 and the cardiac NF-κB/IκB-α system following trauma-hemorrhage. Thus the normalized cardiac function following trauma-hemorrhage in PE females also may be due to inhibition of the NF-κB/IκB system. Females in the PE cycle had a significantly attenuated increase in cardiac NF-κB and improvement in cardiac func-
tion under those conditions. It also has been demonstrated that JAK2/STAT3, not ERK1/2, mediates IL-6-induced activation of inducible NO synthase and a decrease in contractility of adult ventricular myocytes (41). Studies also have shown that estrogen inhibits growth hormone (GH) signaling by suppressing GH-induced JAK2 phosphorylation, and the suppressors of cytokine signaling (SOCS) play a central mechanistic role. Moreover, STAT3 is a molecular participant in ER inhibition of the IL-6 signaling pathway (21). In addition, studies have indicated sexual dimorphism in the permeability response of coronary microvessels to adenosine (15). Nonetheless, the precise mechanisms of estrogen’s effect in the attenuation of IL-6 following trauma-hemorrhage remain to be clarified.

In summary, the present results suggest that cardiac function was significantly depressed at 2 h after trauma-hemorrhage and resuscitation in E, ME, DE, and OVX female rats; however, it was maintained in PE females under those conditions. Furthermore, cardiomyocyte IL-6 expression and plasma IL-6 levels was maintained in PE females under those conditions. Further- more, cardiomyocyte NF-κB activity and plasma IL-6 levels were not apparent in PE females. Moreover, the cardiomyocyte NF-κB/IκB-α system was activated in E, ME, DE, and OVX females following trauma-hemorrhage; however, it was inhibited in PE females. These data collectively suggest that the estrus cycle plays an important role in cardiac function following trauma-hemorrhage. The salutary effect seen in PE females following trauma-hemorrhage is likely due to a decrease in NF-κB-dependent cardiac IL-6 pathway.

GRANT

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REFERENCES