Mechanism of cardiac depression after trauma-hemorrhage: increased cardiomyocyte IL-6 and effect of sex steroids on IL-6 regulation and cardiac function

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Studies from our laboratory have shown that, in a nonheparinized, trauma-hemorrhagic shock model in rats, a prolonged depression of cardiovascular function occurs in males after trauma-hemorrhagic shock (T-H). Although a correlation between increased circulating IL-6 levels and poor outcome has been reported after T-H, it remains unknown whether T-H increases IL-6 levels locally in cardiomyocytes and whether there is a correlation between altered cardiac function and local IL-6 production after T-H. T-H was induced in normal, castrated (2 wk before T-H), and 17β-estradiol (E2)-treated (0.5 mg sc, 1 wk before T-H) adult male rats. At 2 h after T-H or sham operation, cardiac output, heart rate, mean arterial pressure, positive and negative first derivative of pressure (dP/dt), stroke volume, and total peripheral resistance were determined. Cardiomyocytes were isolated and divided into two parts: one was used for measurements of intracellular IL-6 levels using fluorescent activated cell sorting, and the other was used to isolate RNA to determine IL-6 gene expression by quantitative real-time PCR. In addition, cardiac IL-6 protein levels were measured in freshly isolated hearts by Western blotting. Cardiac output, stroke volume, +dP/dt, −dP/dt, and total peripheral resistance were markedly altered after T-H. These parameters, except −dP/dt, improved significantly in the castrated group; however, all these parameters were restored in E2-treated males. Cardiomyocyte IL-6 mRNA expression and intracellular IL-6 production increased after T-H. Cardiac IL-6 protein levels increased after T-H in freshly isolated heart. Castration and E2 treatment attenuated cardiomyocyte intracellular IL-6 levels and cardiac IL-6 protein levels after T-H; however, only E2 treatment attenuated cardiomyocyte IL-6 gene expression. Thus there is an inverse correlation between cardiomyocyte IL-6 levels and cardiac function after T-H. The salutary effects of E2 on cardiac function after T-H may be due in part to decreased IL-6 synthesis in cardiomyocytes.

estrogen; ribonucleic acid; quantitative real-time polymerase chain reaction; Western blot; cytokines; fluorescein-activated cell sorting; interleukin-6

STUDIES FROM OUR LABORATORY have shown that, in a nonheparinized, trauma-hemorrhagic shock model in rats, a prolonged depression of cardiovascular function is evident in males, despite fluid resuscitation (6, 41). However, decreasing the androgen levels by castration 2 wk before trauma-hemorrhage or administration of flutamide (a testosterone receptor antagonist) in normal males after trauma-hemorrhage significantly improved/restored cardiac function (6). Furthermore, treatment of males after trauma-hemorrhage with 17β-estradiol (E2) improved/restored the depressed cardiac function (19, 25, 34). Studies also showed that systemic IL-6 levels increased after trauma-hemorrhage, and sustained elevation in IL-6 levels correlated with poor outcome after trauma-hemorrhage and resuscitation (34, 40).

IL-6 is a 21-kDa cytokine that is produced by a variety of cells, including fibroblasts, endothelial cells, mononuclear phagocytes, neutrophils, hepatocytes, T and B lymphocytes (31, 45), macrophages (38), and myocytes (3). IL-6 receptor contains two chains: an α-chain (glycoprotein 80; CD 126), which is specific for IL-6, and a β-chain (glycoprotein 130), which is shared with all other members of the IL-6 family, including IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, and cardiotropin 1 (31). IL-6 is a multifunctional cytokine that affects various cell types. Studies have shown that hypoxia, a condition associated with trauma-hemorrhagic shock, induced the expression of IL-6 mRNA and increased IL-6 production in cardiomyocytes (45). Moreover, IL-6 has been reported to be involved in the pathogenesis of myocardial injury in ischemic heart disease (11) and congestive heart failure (12, 42). The clinical relevance of these effects of IL-6 are supported by a complementary study that reported elevated IL-6 levels in humans after cardiopulmonary bypass, which depressed human cardiac tissue in vitro (13). Studies have also demonstrated that IL-6 mRNA and protein are produced in the lungs, liver, and intestinal tracts of rats subjected to hemorrhagic shock (17, 18). Previous studies have also shown that Kupffer cells (KC) are the major producers of systemic IL-6 after trauma-hemorrhage, because depletion of KC by pretreatment of rats with gadolinium chloride abolished the increase in systemic IL-6 after trauma-hemorrhage (38, 46). Nonetheless, it remains unknown whether local cardiac IL-6 levels increase after trauma-hemorrhage and resuscitation and, if so, whether there is a correlation between cardiac IL-6 levels and cardiac function. We hypothesized that cardiac IL-6 production increases after trauma-hemorrhagic shock and that this increase in IL-6 locally in cardiomyocytes is responsible for producing the depression in cardiac function under those con-
tions. We also hypothesized that treatment with E2 in male rats will attenuate cardiac local IL-6 production and prevent the depression in cardiac functions after trauma-hemorrhage and resuscitation.

MATERIALS AND METHODS

Trauma-hemorrhagic shock model. Trauma-hemorrhagic shock was induced in normal male (225–275 g) adult Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), castrated male rats (2 wk before trauma-hemorrhage), and E2-treated male rats (1 wk before trauma-hemorrhage), as described previously by us with modification (8, 19, 41). Briefly, all experimental rats were fasted overnight but allowed water ad libitum before the experiment. Under anesthesia with isoflurane inhalation, the right and left femoral arteries and right veins were cannulated with PE-50 tubing. The right femoral artery was connected to a blood pressure analyzer (DigiMed, Louisville, KY) for the measurement and monitoring of mean arterial pressure (MAP) and heart rate (HR). The right femoral vein was used for fluid resuscitation, and the left femoral artery was used for withdrawal of blood. The total circulating blood volume was calculated according to our previous studies (i.e., total circulating blood volume = body weight × 6%) (15). MAP was monitored continuously before the onset of hemorrhage, during hemorrhage, and until the end of resuscitation. The animals were bled rapidly to an MAP of 35–40 mmHg within 10 min and then maintained at that pressure by further withdrawal of small volumes of blood until maximum bleed out (MBO). The amount of blood withdrawn to reach MBO was ~60% of the total circulating blood volume. The rats were then maintained at that pressure by return of small volumes of Ringer lactate until 40% of the MBO was returned in the form of Ringer lactate. The animals were then resuscitated with Ringer lactate (4 times the MBO) 90 min after the onset of bleeding via a right femoral venous catheter over 1 h. In a group of males, castration was performed 2 wk before induction of trauma-hemorrhage. Briefly, a 1-cm-long midline incision was performed in the scrotum under isoflurane anesthesia, testes including epididymides in both sides were removed, the stumps were ligated securely, and the scrotum incision was closed. The animals were then returned to cages and allowed free access to food and water.

In E2-treated males, 0.5 mg of E2 was administered subcutaneously to each rat 1 wk before induction of trauma-hemorrhage through a tunnel made by a 10-gauge precision trochar under anesthesia with isoflurane inhalation. Animals were then returned to cages and allowed food and water ad libitum. The experiments were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. The Institutional Animal Care and Use Committee of The University of Alabama at Birmingham approved this project.

Determination of plasma levels of sex hormones. Blood samples (2 ml) were collected in EDTA-coated test tubes after the onset of hemorrhage in various trauma-hemorrhage groups and after the measurement of cardiac function via the femoral arterial catheter or the hemorrhage in various trauma-hemorrhage groups and after the measurement of cardiac function via the femoral arterial catheter or the hemorrhage in various trauma-hemorrhage groups and after the measurement of cardiac function via the femoral arterial catheter. They were permeabilized with 0.35 ml (for 10^6 cells) of Cytofix/Cytoperm (BD Biosciences, Palo Alto, CA) according to the manufacturer’s instruction. The number of cardiomyocytes was then counted by flow cytometric analysis using a spectrophotometer (Smart Spec 300, Bio-Rad). Cardiomyocyte RNA was stored at −80°C until used. Determination of cardiac function. At 2 h after trauma-hemorrhage or sham operation, the animals were anesthetized again with pentobarbital sodium (50 mg/kg ip). Cardiac output (CO) was determined using the indocyanine green (ICG) dilution technique (15). A 2.4-Fr fiber-optic catheter (Hospex Fiberoptics, Chestnut Hill, MA) was inserted into the right carotid artery and advanced to the level of the aortic arch for continuous measurement of ICG concentration by using an in vivo hemoremeter (Schwarzer-Picker International, Munich, Germany). A silicone rubber catheter was inserted into the right jugular vein to reach the level of the right atrium. ICG (50 µl, 1 mg/ml) was injected via the right jugular vein catheter. The concentration of ICG was recorded by using a computer-assisted data acquisition program (Asyst), a fast-growing, international research and development company headquartered in Rochester, New York. After the measurement of CO, the right carotid artery was cannulated with PE-50 tubing and connected with a blood pressure analyzer (DigiMed, Louisville, KY). After MAP and HR were recorded, this PE-50 tubing was advanced into the left ventricle and connected to a heart performance analyzer (DigiMed) to monitor and record positive and negative first derivatives of pressure (+dP/dt and −dP/dt), CO, stroke volume (SV), total peripheral resistance (TPR), systemic O2 delivery, and O2 consumption were calculated according to standard equations.

Isolation of cardiomyocytes. Ventricular myocytes were isolated immediately after the measurement of cardiac function. 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 ml·min⁻¹·g tissue⁻¹) for 5 min with a Ca²⁺-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 perfusion, Ca²⁺-free Krebs buffer was replaced by enzymatic digesting buffer containing Ca²⁺-free Krebs buffer, collagenase type II (Worthington, Lakewood, NJ), 0.1% fat-free BSA (NSA), and 0 mM Ca²⁺. The heart was then cannulated with PE-50 tubing. The right femoral artery was connected to a blood pressure analyzer (DigiMed, Louisville, KY). After MAP and HR were recorded, this PE-50 tubing was advanced into the left ventricle and connected to a heart performance analyzer (DigiMed) to monitor and record positive and negative first derivatives of pressure (+dP/dt and −dP/dt), CO, stroke volume (SV), total peripheral resistance (TPR), systemic O₂ delivery, and O₂ consumption were calculated according to standard equations.

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CALIBUR (Becton Dickinson, San Jose, CA) to determine cardiomyocyte levels of intracellular and extracellular IL-6 after sham operation or trauma-hemorrhage, and the percentage of gated IL-6-positive cardiomyocyte population was recorded.

Western blotting for measurement of cardiac IL-6 protein levels. Freshly isolated hearts from each animal (~0.2 g) were homogenized in 1 ml of lysis buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 20 mM KCl, 20% glycerol, 0.2 mM EDTA, 2 mM Na₃VO₄, 10 mM NaF, 1% Triton X-100, 0.2 mM PMSF, 10 mg/ml aprotonin, and 10 mg/ml leupeptin. Tissue lysates were centrifuged at 10,000 g for 10 min, and the supernatants were stored at −80°C until use. Tissue lysate protein concentrations were assayed (Bio-Rad Laboratories, Hercules, CA), and lysates (30 μg per lane) were analyzed by SDS-12% polyacrylamide gel and transferred to nitrocellulose paper. An equivalent amount of protein from various samples was loaded on the gel. The membranes were immunoblotted with anti-rat IL-6 antibody (Biosource) followed by addition of horseradish peroxidase-conjugated secondary antibody. After the final wash, membranes were probed using enhanced chemiluminescence dye (ECL, Amershams Pharmacia Biotech, Piscataway, NJ) and autoradiographed. The intensity of the bands was determined using densitometric analysis (47). In addition, the membranes were reprobed using mouse monoclonal antiactin antibody (Santa Cruz Biototechnology, Santa Cruz, CA).

Determination of cardiomyocyte IL-6 mRNA expression by quantitative real-time PCR. Cardiomyocyte IL-6 gene expression was determined by quantitative real-time PCR (QT-PCR). IL-6 primers were designed using the ABI Prism Primer Express Program (Applied Biosystems, Foster City, CA), and amplification of cDNA was performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). To perform QT-PCR, all the isolated cardiomyocyte RNA was reverse transcribed into cDNA by addition of TaqMan Gold RT-PCR reagent (Applied Biosystems) and use of an Eppendorf Master Cycler Gradient RT-PCR instrument (Eppendorf, Westbury, NY). A relative quantification (ΔΔCt, where Ct is cycle threshold) method was chosen for QT-PCR. 18S was used for internal control, and cDNA from heart tissue from a rat subjected to 2 h of trauma-hemorrhage was used to calibrate the instrument. A 384-well plate was used for QT-PCR. To each well, a 3-μl aliquot of cDNA, 5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μl of 20X IL-6 or TNF-α primer and probe (Applied Biosystems), and 1.5 μl of water were added (total volume = 10 μl). All the samples were measured in triplicate. Samples were amplified for 1 cycle at 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Fluorescence was measured at the end of each cycle and after 40 cycles of reaction; a profile of fluorescence vs. cycle number was obtained. An arbitrary threshold of fluorescence was set within the exponential phase of amplification (reference normalized = 0.1). The cycle at which the amplification of the product exceeded this threshold was determined and designated cycle threshold (Ct). The Ct for the gene of interest ranged from 23 to 31 depending on the gene amplified, whereas the Ct for 18S was between 14 and 16 cycles. The expression of each gene within each sample was normalized against 18S and expressed relative to a calibrator sample using the formula 2^-(ΔΔCt), as described elsewhere (K. Livak, PE-ABI, Sequence Detector User Bulletin 2). ΔΔCt can be defined as follows: [Ct gene of interest (unknown sample) – Ct18S (unknown sample)] – [Ct gene of interest (calibrator sample) – Ct18S (calibrator sample)]. The calibrator sample was designated the most highly expressed time point for each gene of interest and, therefore, has an expression of 1. In view of this, all other data are expressed as the fold expression of this value. Before it was used as an internal control, expression of 18S was verified to be constant across all sampling times. To choose and decide the calibrator sample, preliminary experiments were performed to ensure that amplification efficiencies for the target genes and 18S were equivalent (see PE-ABI, Sequence Detector User Bulletin 2).

Statistical analysis. Values are means ± SE. One-way ANOVA and Tukey’s test were employed for comparison among different groups of animals. The differences were considered significant at P ≤ 0.05.

RESULTS

Alteration in plasma levels of sex hormones. The male group has the lowest estradiol and the highest androgen (testosterone and DHT) levels in plasma (Fig. 1). Castration did not increase plasma levels of estradiol; however, it significantly decreased (by 98.5%) plasma androgen, including testosterone and DHT levels. E₂ treatment significantly increased (by 46-fold) plasma levels of estradiol and decreased (by 90.1%) plasma levels of androgen.

Effects of E₂ on cardiac function and systemic hemodynamic parameters after trauma-hemorrhage and resuscitation. CO decreased by 41% (P < 0.05; Fig. 2A) and SV decreased by 42% (P < 0.05; Fig. 2B) compared with sham-operated males, respectively, 2 h after trauma-hemorrhage and resuscitation (Fig. 2). In contrast, TPR increased by 38% (P < 0.05) under these conditions (Fig. 2C). Castration and E₂ treatment, however, increased CO by 33% and 46%, respectively (P < 0.05; Fig. 2A). Moreover, castration and E₂ treatment maintained SV and TPR at levels similar to those of sham-operated animals 2 h after trauma-hemorrhage and resuscitation (Fig. 2, B and C). In addition, +dP/dt decreased by 40% 2 h after trauma-hemorrhage and resuscitation compared with sham-operated males (P < 0.05). Castration and E₂ treatment, however, maintained +dP/dt (Fig. 3A). Similarly, −dP/dt decreased by 54% (P < 0.05) 2 h after trauma-hemorrhage. Castration did not increase −dP/dt; however, E₂ treatment maintained −dP/dt and significantly increased it compared with males subjected to trauma-hemorrhage as well as castrated males subjected to trauma-hemorrhage (Fig. 3B). There was no significant difference in MAP and HR among groups (Table 1). The mortality rate, however, was 22% (2 of 9 rats) 2 h after trauma-
hemorrhage and resuscitation in male rats. In contrast, all castrated and E2-treated males were alive 2 h after trauma-hemorrhage and resuscitation in the present study.

Effects of E2 on cardiomyocyte intracellular IL-6 levels after trauma-hemorrhage. The representative histograms of percentage of IL-6-positive population in gated cardiomyocytes from sham animals and males subjected to trauma-hemorrhage with and without castration or E2 treatment are shown in Fig. 4. The percentage of IL-6-positive cardiomyocyte population was <10% in all normal, castrated, and E2-treated sham-operated males; however, the percentage of IL-6-positive cardiomyocyte population increased from 9.6% to 53.4% 2 h after trauma-hemorrhage and resuscitation in males (P < 0.05; Fig. 4A). Although the percentage of IL-6-positive cardiomyocyte population was increased from 8% to 23.3% and from 7.5% to 16.5% in castrated and E2-treated males, respectively, 2 h after trauma-hemorrhage and resuscitation, this shift was markedly reduced compared with males subjected to trauma-hemorrhage (P < 0.05; Fig. 4, B and C). Additional experiments were performed to confirm these observations, and the results from these experiments (Fig. 5) suggest a significant increase in IL-6-positive cardiomyocyte population 2 h after trauma-hemorrhage. However, the shift in IL-6-positive cardiomyocyte population was significantly decreased in castrated as well as in E2-treated male animals subjected to trauma-hemorrhage (Fig. 5). Furthermore, our results suggest that the shift in IL-6-positive population in animals subjected to trauma-hemorrhage is due to an increase in intracellular IL-6 production, rather than extracellular IL-6. This suggestion is based on the fact that <1% of cardiomyocytes from sham animals and animals subjected to trauma-hemorrhage (0.33% and 0.91%, respectively, 2 h after trauma-hemorrhage; E2-T-H, preestrogen (E2)-treated male animals subjected to trauma-hemorrhage (Fig. 5).

Table 1. Alterations in MAP, HR, systemic O2 delivery, O2 consumption, and systemic Hct 2 h after trauma-hemorrhage and resuscitation

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Male T-H</th>
<th>Cast-T-H</th>
<th>E2-T-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>127±3</td>
<td>102±4</td>
<td>104±2</td>
<td>107±5</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>389±18</td>
<td>394±10</td>
<td>351±10</td>
<td>338±12</td>
</tr>
<tr>
<td>DO2, ml/min·100 g body wt^-1</td>
<td>7.4±0.2</td>
<td>1.8±0.1*</td>
<td>2.4±0.1*</td>
<td>2.4±0.1*</td>
</tr>
<tr>
<td>VO2, ml/min·100 g body wt^-1</td>
<td>2.2±0.3</td>
<td>0.8±0.1*</td>
<td>0.9±0.1*</td>
<td>1.0±0.2*</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>13.6±0.3</td>
<td>5.4±0.1*</td>
<td>5.5±0.1*</td>
<td>5.1±0.1*</td>
</tr>
<tr>
<td>Hct</td>
<td>41.9±1.0</td>
<td>17.0±0.2*</td>
<td>17.5±0.3*</td>
<td>16.2±0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5). T-H, trauma-hemorrhage; cast-T-H, precastrated male trauma-hemorrhage; E2-T-H, preestrogen (E2)-treated male trauma-hemorrhage; MAP, mean arterial pressure; HR, heart rate; DO2, O2 delivery; VO2, O2 consumption; Hct, systemic hematocrit. Data were compared by 1-way ANOVA and Tukey’s test. *P < 0.05 vs. sham.
were positive for extracellular IL-6 when cardiomyocytes were stained in the absence of permeabilization (Fig. 6).

**Cardiac IL-6 protein levels in freshly isolated hearts.** There was a significant increase in cardiac IL-6 protein in homogenates prepared from freshly isolated heart 2 h after trauma-hemorrhage compared with homogenates from sham-operated male animals (Fig. 7). However, the trauma-hemorrhage-mediated increase in IL-6 protein was significantly attenuated in castrated rats. Similarly, E2 treatment also prevented the increase of cardiac IL-6 protein level after trauma-hemorrhage (Fig. 7).

**Effects of E2 on cardiomyocyte intracellular IL-6 gene expression after trauma-hemorrhage and resuscitation.** The relative quantification of cardiomyocyte IL-6 mRNA significantly increased 2 h after trauma-hemorrhage and resuscitation in males as well as in castrated males subjected to trauma-hemorrhage (Fig. 8). However, E2 treatment attenuated cardiomyocyte IL-6 gene expression after trauma-hemorrhage (Fig. 8).

**DISCUSSION**

The results of this study indicate that cardiac function was significantly depressed 2 h after trauma-hemorrhage and resuscitation in male rats, as demonstrated by the significant decrease of CO, SV, and left ventricular performance, as measured by +dP/dt max and −dP/dt max and the significantly increased TPR. These results are similar to our previous findings (19, 34, 39, 41). E2 treatment (0.5 mg/300 g body wt, 1 wk

**Fig. 5.** Alterations in cardiomyocyte intracellular IL-6 levels in sham-operated males and males subjected to trauma-hemorrhage (A), castrated sham-operated males and males subjected to trauma-hemorrhage (B), and E2-treated sham-operated males and males subjected to trauma-hemorrhage (C). Curve with arrow represents negative control. M1 gate was established at a point containing 95% of negative control. M2 begins at the end of M1 and excludes most negative events. Thus numbers above M2 represent positive events falling within the M2 region. PE, phycoerythrin.

**Fig. 6.** Comparison of cardiomyocyte extracellular IL-6 levels in sham-operated animals (A) and males subjected to trauma-hemorrhage (B). Representative results are shown. Curve with arrow represents negative control. M1 gate was established at a point containing ≥95% of negative control. M2 begins at the end of M1 and excludes most negative events. Thus numbers above the M2 region represent positive events falling within the M2 region.
before onset of trauma-hemorrhage), however, significantly improved all the above-measured cardiac parameters, which include a restored +dP/dt_{max} and −dP/dt_{max}, SV, TPR, and improved CO 2 h after trauma-hemorrhage and resuscitation. Although castration partially improved cardiac function, which included CO, SV, TPR, and +dP/dt_{max}, it did not improve −dP/dt_{max}. Our previous studies showed that plasma levels of IL-6 increased significantly after trauma-hemorrhage and resuscitation, and the elevated plasma IL-6 levels were associated with adverse effects on organ function (34, 40). Previous studies suggested that KC represent the major source of inflammatory cytokine release after adverse circulatory conditions, because reduction of KC by administration of gadolinium chloride before trauma-hemorrhage markedly reduced IL-6 release after trauma-hemorrhage (38). We found a significant increase in IL-6 protein levels in freshly isolated heart tissue 2 h after trauma-hemorrhage and resuscitation. In addition, cardiomyocyte IL-6 (mRNA) expression and intracellular IL-6 production dramatically increased 2 h after trauma-hemorrhage and resuscitation. Our studies further suggested that castration and E2 treatment attenuated the increases in IL-6 protein levels in heart as well as cardiomyocyte intracellular IL-6 levels after trauma-hemorrhage. However, the increase in IL-6 mRNA was attenuated only in E2-treated animals subjected to trauma-hemorrhage and not in castrated animals. These results suggest that removal of male hormones via castration may regulate cardiomyocyte IL-6 production via a mechanism that is different from that used by E2. Further studies are needed to delineate the differences in castration- and E2-mediated cardiomyocyte IL-6 regulation after trauma-hemorrhage. In the present study, a correlation was observed between the increased cardiomyocyte IL-6 mRNA expression, cardiomyocyte intracellular IL-6 production, and cardiac dysfunction. These findings therefore suggest that the local production of IL-6 plays a key role in regulating cardiac function after adverse circulatory conditions. In this regard, our previous studies showed that plasma IL-6 levels were significantly elevated at 24 h in vehicle-treated animals subjected to trauma-hemorrhage, whereas E2 administration during resuscitation down-regulated plasma IL-6 to levels that were not significantly different from those in sham-operated males (40). Although studies showed KC to be the primary source of circulatory IL-6 release (38), the results presented here suggest that cardiomyocytes can also synthesize IL-6. The findings that E2 treatment prevented IL-6 production locally in cardiomyocytes and improved cardiac function support the notion of a close relationship between cardiac function and cardiomyocyte IL-6 levels. Furthermore, there was a significant correlation between IL-6 and

Fig. 7. IL-6 protein levels in freshly isolated hearts from sham-operated animals, males subjected to trauma-hemorrhage, males subjected to castration and trauma-hemorrhage, and E2-treated males subjected to trauma-hemorrhage. A: representative blot. For equal protein loading, membranes were reprobed for actin using mouse monoclonal antibody. Intensity of bands was determined using densitometry. B: ratio of IL-6 to actin-integrated density values. Values are means ± SE of 3 animals in each group. Data were compared by 1-way ANOVA and Tukey’s test. *P < 0.05 vs. sham; #P < 0.05 vs. T-H.

Fig. 8. Quantitative real-time PCR of cardiomyocyte IL-6 gene expression in sham-operated males, males subjected to trauma-hemorrhage, males subjected to castration and trauma-hemorrhage, and E2-treated males subjected to trauma-hemorrhage. IL-6 gene expression is expressed as a fold change of relative quantification (RQ) to calibrator (Calib) sample that has an expression set at 1. A: raw data. B: graphic analysis. There were 4 animals in each group 2 h after sham operation or trauma-hemorrhage. Values are means ± SE. Data were compared by 1-way ANOVA and Tukey’s test. *P < 0.05 vs. sham; #P < 0.05 vs. E2-T-H.
hepatocellular function ($V_{\text{max}}$ of ICG clearance) (40). Thus downregulation of this proinflammatory cytokine may be responsible for restoring the depressed cardiac function after trauma-hemorrhage and resuscitation.

Another aspect to consider is whether isolation of cardiomyocytes might introduce artifacts and negatively influence the results. Although we cannot entirely rule out this possibility, we do not believe our results to be isolation artifacts. The reason for this conclusion is that, in the measurement of cardiomyocyte intracellular IL-6 levels by fluorescein-activated cell-sorting analysis, the population of positively gated IL-6 cardiomyocytes was <10% in sham animals, and this population increased significantly after trauma-hemorrhage. Furthermore, cardiomyocyte extracellular IL-6 level was <1%. These results collectively provide strong evidence that the results after trauma-hemorrhage are not isolation artifacts. It could also be argued that the IL-6 results obtained using isolated myocytes should be confirmed with those obtained from the left ventricle. However, the left ventricle contains at least three different types of cells: cardiomyocytes, fibroblasts, and endothelial cells. Thus measurement of IL-6 in the left ventricle would potentially provide the combined results of IL-6 from at least three different cell types, instead of only myocytes. Because the focus of this study was on cardiac contractile function and because cardiomyocytes are responsible for contractile function, our studies examined IL-6 levels only in isolated cardiomyocytes, and not in other cells such as fibroblasts or endothelial cells.

Because we examined only the short-term hemodynamic effects of E2 treatment after trauma-hemorrhage, it could be argued that the effects may be short lived and, thus, may not translate into improvements in long-term survival. Although we have not measured survival rates in this study, our previous results showed that if an agent had a salutary effect early after trauma-hemorrhage and resuscitation, the beneficial effects were sustained for longer periods of time and they also resulted in improved survival rates of animals (33). The present results also showed that the mortality rate was 22% in male rats 2 h after trauma-hemorrhage and resuscitation; however, all castrated and E2-treated males were alive 2 h after trauma-hemorrhage and resuscitation. Furthermore, previous studies showed that the mortality rate was 30% in the vehicle-treated hemorhaged group and 11% in the E2-treated group (33). In view of this information, it would appear that the observed salutary effects of E2 do not appear to be short term.

Proinflammatory cytokines are considered the key mediators of cellular damage in immune as well as inflammatory responses. Recent studies showed that proinflammatory cytokines also play a role in the myocardium (44). TNF-α was the first cytokine that was suggested to be associated with development of congestive heart failure (19, 26, 27, 34). More recently, clinical and basic studies suggested that IL-6 may also be involved in development of congestive heart failure (12, 24, 35). The interest in IL-6 has been prompted by the fact that IL-6, like TNF-α, is elevated in patients with congestive heart failure and is cardiodepressive (12, 24, 43). Furthermore, IL-6 and other proinflammatory cytokines are not only in the pathogenesis of myocardial injury in myocarditis (29) and ischemic heart diseases (32). Ancye et al. (3) showed that cardiomyocytes and fibroblasts are able to spontaneously produce IL-6. Moreover, studies have shown that hypoxia induces the expression of IL-6 mRNA and increases production of IL-6 in cultures of cardiomyocytes (45). The increased IL-6 production at all culture periods appears to be the consequence of fibroblast proliferation and glycoprotein 130 stimulation (3). Although increased IL-6 mRNA expression and production have been noted in congestive heart failure and myocarditis, studies have not been conducted to determine whether expression and production of IL-6 in cardiomyocytes are increased after trauma-hemorrhage and, if so, whether the increase is associated with depressed cardiac function under these conditions.

Little is known regarding the molecular mechanisms that regulate myocardial expression of proinflammatory cytokines. Studies have suggested that adenosine, a breakdown product of ATP, inhibits the release of TNF-α and IL-6 by human monocytes through activation of the A2 receptor (7). In isolated cardiomyocytes, adenosine inhibits the expression of TNF-α through activation of the A2 receptor but induces robust expression of IL-6 through activation of the A1 and, possibly, A1 receptor (20). Thus the mechanism by which E2 inhibits cardiomyocyte IL-6 production does not appear to be due to the A2 or A1 receptor, because IL-6 production was decreased in those cardiomyocytes. Deshpande et al. (10) demonstrated that E2 attenuates cytokine production by inhibiting activation of the transcription factor NF-κB in murine macrophages. In this regard, our recent studies showed that E2 also inhibits KC IL-6 release, whereas DHT enhanced its production (4, 46). Thus it appears that E2 is capable of downregulating not only KC, but also cardiomyocyte, IL-6 production.

Previous studies from our laboratory showed that cardiovascular and immunologic functions are markedly depressed in males after trauma-hemorrhage but not in proestrus females under those conditions (4, 19). The reason for the diverse cardiovascular and immunologic effects between males and females appears to be high estradiol levels in proestrus (4, 19). Support for this notion also comes from the studies in which the response of ovariectomized females to trauma-hemorrhage was similar to that of males (4, 19, 34). Furthermore, administration of E2 in ovariectomized animals and even in males normalized the depressed cardiovascular and immunologic responses after trauma-hemorrhage (4, 19, 34). In addition, our preliminary findings suggest that levels of cardiomyocyte intracellular IL-6 as well as IL-6 mRNA expression are low in proestrus females with high levels of estrogen. These females do not exhibit cardiac depression 2 h after trauma-hemorrhage (unpublished data). Together, these studies therefore suggest that the local production of IL-6 plays a key role in regulating cardiac function after trauma-hemorrhage.

The precise mechanism of the beneficial effects of E2 and castration on cardiac function after trauma-hemorrhage and resuscitation remains unknown. The present results indicate significant differences in plasma sex hormone levels in normal, castrated, and E2-treated males. The highest plasma levels of androgen (testosterone and DHT) as well as the lowest levels of estradiol were found in normal males. In contrast, the highest estradiol levels were found in E2-treated males. Although the lowest levels of plasma androgens were found in castrated males, plasma levels of estradiol were also low in that group. Because the ovaries are the predominant source of estradiol production in females, the testes and peripheral aromatization of testosterone and DHT account for the low levels...
of estradiol in males. It appears that not only low circulating levels of estrogen but also high levels of male sex steroids, which include testosterone and DHT, are responsible for depression of organ functions after hemorrhagic shock. In this regard, estradiol has been shown to produce various beneficial effects after circulatory stress (1), and studies have shown that a high level of estrogen in proestrus females and in E2-treated male rats as well as mice produces salutary effects on cardiovascular and immunologic function after trauma-hemorrhage (1, 2, 4, 19, 25, 34).

Our previous studies showed that estrogen’s salutary effects on organ functions were evident even 24 h after trauma-hemorrhage and resuscitation (34). The present study demonstrates that the salutary effects of estrogen on cardiovascular function are evident soon, i.e., 2 h, after trauma-hemorrhage and resuscitation. The actions of estrogen have been well characterized and can be divided into rapid nongenomic and slower genomic effects (30). The immediate effects of estrogen are mediated via constitutive nitric oxide (NO) synthase (cNOS) (9, 22), including endothelial NO production, presumably by increasing the expression or activity of the isoform of cNOS (9). The reduced release of endothelium-derived NO under various adverse circulatory conditions is most likely due to the decreased activity of endothelial cNOS (26). In this regard, our previous studies showed that vascular endothelial cell function is depressed early after the onset of trauma-hemorrhagic shock, and administration of L-arginine (the substrate for cNOS) restored the depressed CO (5). Thus it is possible that the beneficial effects of estrogen on cardiovascular function after trauma-hemorrhage are due to the upregulation of cNOS. In this regard, studies have indicated that activation of cNOS after severe shock is beneficial and results in an attenuated inflammatory response (14, 16). The ability of E2 to diminish leukocyte adherence and migration could be another potential mechanism for the improved organ functions in animals with higher estradiol levels (37). Several studies showed that the failure of microcirculatory perfusion after shock was attributed to the enhanced leukocyte-endothelial interaction under those conditions (23). Moreover, it has been shown that estradiol attenuates cytokine production by inhibiting the transcription factor NF-κB and inducing expression of the 90-kDa heat shock protein (HSP)-90 in various tissues (10).

Our previous studies also showed that induction of HSPs was associated with protective effects under adverse circulatory conditions (33). Thus the normalized organ function after trauma-hemorrhage in E2-treated males may be also due to induction of HSP-90. Although the precise mechanism of beneficial effects of estrogen remains to be clarified, one or more of the above mechanisms could be responsible for the protective effects of E2 observed in the males after trauma-hemorrhage and resuscitation.

Studies have shown that testosterone increases the levels of vascular smooth muscle thromboxane A2 receptors in the aorta (28). Moreover, testosterone treatment enhances the vasoconstrictor response of thromboxane A2 in the coronary circulation (21, 28). Furthermore, it has been shown that testosterone treatment inhibits prostacyclin synthesis in rat aorta smooth cells in culture (36). It is therefore possible that castration, i.e., depletion of testosterone, inhibits the vasoconstrictive actions of thromboxane A2 or enhances release of the vasodilator prostacyclin in the coronary circulation. In addition, it is possible that depletion of androgen by castration enhances the specific cellular effects of other sex hormones such as estradiol in males. Although castration partially improved cardiac function 2 h after trauma-hemorrhage and resuscitation, it did not improve the depressed −dP/dt max, nor did it attenuate cardiomyocyte IL-6 gene expression. The low levels of plasma estradiol may account for the lack of improvement in these parameters. Yu et al. (47) showed that JAK2/STAT3, not ERK1/2, mediates IL-6-induced activation of inducible NO synthase and decrease in contractility of adult ventricular myocytes. Because we did not measure JAK2/STAT3 or ERK1/2, we fully recognize that additional studies are needed to determine the precise mechanism of the protective effects of castration as well as estrogen on cardiac function after trauma-hemorrhage and resuscitation. Furthermore, although our present study indicates an inverse correlation between IL-6 and cardiac function, a causal relation between these two parameters cannot be established until additional studies using anti-IL-6 antibody are carried out.

In summary, our results suggest that the depressed cardiac functions after trauma-hemorrhage may be due to upregulation of cardiomyocyte IL-6 production. Support for this suggestion comes from our study showing that treatment with E2 in male rats prevented the increase in cardiomyocyte IL-6 production and the depression of cardiac function after trauma-hemorrhage and resuscitation. The results also indicate that castration only partially improved cardiac function after trauma-hemorrhage and resuscitation. Furthermore, administration of estrogen in males attenuated cardiomyocyte IL-6 mRNA expression as well as cardiomyocyte intracellular IL-6 production; however, castration attenuated only cardiomyocyte intracellular IL-6 production. In addition, cardiac IL-6 protein levels increased significantly after trauma-hemorrhage. Nonetheless, castration and E2 treatment prevented the increase in cardiac IL-6 protein levels. These results, taken together, suggest that there is a close correlation between cardiac function and regional IL-6 synthesis after trauma-hemorrhage and resuscitation and that the beneficial effects of E2 on cardiac function after trauma-hemorrhage may be due in part to decreased IL-6 synthesis in the cardiomyocytes.

GRANTS
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REFERENCES
EFFECT OF 17β-ESTRADIOL AFTER TRAUMA-HEMORRHAGE


