p38 MAPK-dependent eNOS upregulation is critical for 17β-estradiol-mediated cardioprotection following trauma-hemorrhage

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Kan WH, Hsu JT, Ba ZF, Schwacha MG, Chen JG, Choudhry MA, Bland KI, Chaudry IH. p38 MAPK-dependent eNOS upregulation is critical for 17β-estradiol-mediated cardioprotection following trauma-hemorrhage. Am J Physiol Heart Circ Physiol 294: H2627–H2636, 2008. First published April 11, 2008; doi:10.1152/ajpheart.91444.2007.—Studies have shown that p38 MAPK and nitric oxide (NO) synthase (eNOS), play key roles under physiological and pathophysiological conditions. Although administration of 17β-estradiol (E2) protects cardiovascular injury from trauma-hemorrhage, the mechanism by which E2 produces those effects remains unknown. Our objective was to determine whether the E2-mediated activation of myocardial p38 MAPK and subsequent eNOS expression/phosphorylation would protect the heart following trauma-hemorrhage. To study this, male Sprague-Dawley rats underwent soft-tissue trauma (midline laparotomy) and hemorrhagic shock (mean blood pressure 35–40 mmHg for 90 min), followed by fluid resuscitation. Animals were pretreated with specific p38 MAPK inhibitor SB-203580 (SB; 2 mg/kg), and nonselective NO synthase inhibitor Nω-nitro-l-arginine methyl ester (l-NAME; 30 mg/kg) 30 min before vehicle (cycloedextrin) or E2 (100 µg/kg) treatment, followed by resuscitation, and were killed 2 h thereafter. Cardiovascular performance and other parameters were measured. E2 administration following trauma-hemorrhage increased cardiac p38 MAPK activity, eNOS expression and phosphorylation at Ser1177, and nitrate/nitrite levels in plasma and heart tissues; these were associated with normalized cardiac performance, which was reversed by SB administration. In addition, E2 also prevented trauma-hemorrhage-induced increase in cytokines (IL-6 and TNF-α), chemokines (macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant-1), and ICAM-1, which was reversed by l-NAME administration. Administration of E2 following trauma-hemorrhage attenuated cardiac tissue injury markers, myeloperoxidase activity, and nitrotyrosine level, which were reversed by treatment with SB and l-NAME. The salutary effects of E2 on cardiac functions and tissue protection following trauma-hemorrhage are mediated, in part, through activation of p38 MAPK and subsequent eNOS expression and phosphorylation.

nitric oxide synthase; cytokine; chemokine; myeloperoxidase

PREVIOUS STUDIES HAVE SHOWN that prolonged depression of organ and immune functions occurs in male rats following trauma-hemorrhage, despite fluid resuscitation (17, 19, 20). However, treatment with 17β-estradiol (E2) following trauma-hemorrhage restored the depressed cardiac functions (18, 19). Although several mechanisms responsible for the salutary effects of E2 have been proposed (18, 19), it remains not entirely clear how E2 protects heart from trauma-hemorrhage-induced injury and depression. E2 is a key regulator and functions in a wide array of tissues, including the cardiovascular system (18–20, 43). There is compelling evidence that E2 activates distinct signaling pathways, such as protein kinase A, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) (19, 44). MAPK is a large family of serine/threonine kinases, which is activated by a variety of extracellular stimuli (2, 19, 25). The MAPK superfamily consists of three well-characterized subfamilies: extracellular signal-regulated kinase, p38 MAPK, and c-Jun NH2-terminal kinases. Studies have shown that MAPK plays an important role in sepsis and ischemic injury (19, 25, 35). Furthermore, activation of p38 MAPK has been suggested to be critical for cardioprotection during ischemia and reperfusion (15, 19, 22, 40). Nakano et al. found that using a p38 MAPK activator before ischemia resulted in cardioprotection (36). Pretreatment with SB-203580 (SB), a potent inhibitor of p38 MAPK, potentiated the deleterious effect of ischemic injury on the heart (36). Thus it appears that p38 MAPK plays a critical role in the regulation of ischemic and sepsis-induced myocardial injury.

Endothelial nitric oxide (NO) synthase (eNOS) is the primary physiological source of NO in the vascular system and is expressed in both endothelial cells and cardiomyocytes. It is thus possible that E2 plays a critical role in E2-mediated cardiac protection (14, 19). In cardiomyocytes, eNOS is associated with caveolin, which serves to inhibit eNOS. Cell stimulation with Ca2+ mobilizing agonists promotes calmodulin binding to eNOS and caveolin disassociation from it, rendering eNOS activity. Several isoforms of caveolin are expressed in myocytes (11), and it is likely that caveolin-1 may modulate the catalytic activity of cardiac eNOS, which, in turn, regulates NO production. Although E2 enhances eNOS expression and bioactivity, it is not known whether E2 modulates eNOS directly or indirectly via p38 MAPK. We hypothesized that the beneficial effects of E2 following trauma-hemorrhage are mediated via a p38 MAPK-dependent pathway through regulation of cardiac eNOS expression and phosphorylation.

MATERIALS AND METHODS

Rat trauma-hemorrhagic model. A nonheparinized model of trauma-hemorrhage was used, as described previously (18). Briefly, adult male Sprague-Dawley rats (275–325 g, Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane (Attane; Minrad, Bethlehem, PA) before soft tissue trauma was induced via a midline laparotomy. The

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abdominal incision was then closed, and polyethylene catheters (PE-50, Becton Dickinson, Sparks, MD) were placed in both femoral arteries and the right femoral vein. The wounds were bathed with 1% lidocaine throughout the surgical procedure. The rats were then placed into a Plexiglas box (21 × 9 × 5 cm) in a prone position and allowed to awaken, after which they were rapidly bled to a mean blood pressure (MBP) of 35–40 mmHg within 10 min. Hypotension was maintained until the animals could no longer keep a MBP of 35–40 mmHg, unless additional fluid in the form of Ringer lactate (RL) was administered. This time was defined as maximum bleed-out, and the amount of withdrawn blood was noted. Following this, the rats were maintained until the animals could no longer keep a MBP of 35–40 mmHg until the animals could no longer keep a MBP of 35–40 mmHg, unless additional fluid in the form of Ringer lactate (RL) was administered. This time was defined as maximum bleed-out, and the amount of withdrawn blood was noted. Following this, the rats were maintained at MBP of 35–40 mmHg until 40% of the maximum bleed-out volume was returned in the form of RL (~90 min from the onset of bleeding). The animals were then resuscitated with four times the volume of the shed blood over 60 min with RL. Sham-operated animals underwent the same soft tissue trauma, which included the ligation of the right femoral artery and vein, but neither hemorrhage nor resuscitation was carried out.

Animals were pretreated with p38 MAPK inhibitor SB (2 mg/kg body wt, San Diego, CA) and NO synthase (NOS) inhibitor Nω-nitro-ω-arginine methyl ester (l-NAMe, 30 mg/kg iv) 30 min before treatment with vehicle (cyclohextrin) or E2 (100 µg/kg BW) and resuscitation. Following resuscitation, the catheters were removed, the vessels were ligated, and the skin incisions closed. The animals were killed 2 h after the end of resuscitation or sham operation, and the samples of blood and tissues were collected for analysis.

All animal experiments were performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, 1996) and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Determination of cardiac performance. Two hours after trauma-hemorrhage and resuscitation or sham operation, the animals were reanesthetized with isoflurane and catheterized via the left femoral vein. Under continuous general anesthesia with pentobarbital sodium (25–30 mg/kg iv), a PE-50 catheter was placed into the right carotid artery and connected to a blood pressure analyzer (DigiMed, Louis-

### Table 1. Effects of 17β-estradiol on cardiac function following trauma-hemorrhage

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<th>Sham</th>
<th>Trauma-Hemorrhage</th>
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<tr>
<td>Mean BP</td>
<td>Vehicle</td>
<td>E2</td>
</tr>
<tr>
<td>mmHg</td>
<td>122±6</td>
<td>124±8</td>
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<tr>
<td>+dP/dtmax, mmHg/s</td>
<td>12,925±12,146</td>
<td>13,102±1,095</td>
</tr>
<tr>
<td>−dP/dtmax, mmHg/s</td>
<td>9,552±1,008</td>
<td>9,687±553</td>
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Values are means ± SE of 6 animals in each group. Mean blood pressure (BP), maximal rate of left ventricular pressure increase (+dP/dtmax), and maximal rate of left ventricular pressure decrease (−dP/dtmax) values at 2 h after resuscitation (sham) or trauma-hemorrhage are given. Rats were treated with either vehicle (cyclohextrin) or 17β-estradiol (E2). The results were analyzed by one-way ANOVA and Tukey’s test. *P < 0.05 vs. sham or trauma-hemorrhage plus E2, †P < 0.05 vs. sham.

Institutional Animal Care and Use Committee of the University of California, Berkeley, CA), and GAPDH (Abcam, Cambridge, MA; 1:25,000) overnight at 4°C. After washing with Tris-buffered saline-Tween 20 three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody for 1 h at room temperature. The detection of bound antibodies was performed by enhanced chemiluminescence (Amersham, Piscataway, NJ). Signals were quantified using ChemiImager 5500 imaging software (Alpha Innotech, San Leandro, CA), and the densitometric values (6 rats/group) were obtained.

Determination of heart cytokines, chemokines, and ICAM-1 levels. The heart tissue cytokines IL-6 and TNF-α and chemokine cytokine-induced neutrophil chemoattractant (CINC)-1 and adhesion molecule ICAM-1 levels were determined using ELISA kits (R&D, Minneapolis, MN), according to the manufacturer’s instructions. The chemokine macrophage inflammatory protein (MIP)-2 was measured using Rat MIP-2 CytoSet kit (BioSource Cytokines and Singaling, Invitrogen). Briefly, ~100 mg of snap-frozen heart tissue were homogenized in 1 ml of lysis buffer containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail. The lysates were clarified at 14,000 g for 20 min at 4°C. The protein concentration of supernatant was determined, and the same supernatant was assayed for cytokines, chemokines, and ICAM-1 levels.

Measurement of heart myeloperoxidase activity. Heart tissue injury is characterized by increased neutrophil accumulation. Myeloperoxidase (MPO) is a well-accepted indicator of neutrophil tissue infiltration. MPO activity in tissues was determined as described previously (19). Briefly, heart tissues (100 mg) were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in 50 mmol/l phosphate buffer, pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were centrifuged at 14,000 rpm at 4°C, and the supernatants were stored at −80°C. Protein concentration was determined (BioRad, Hercules, CA). The samples were incubated with a substrate o-dianisidine dihydrochloride. The reaction was carried out in a 96-well plate by adding 290 µl of 50 mmol/l phosphate buffer, 3 µl substrate solution (containing 20 mg/ml o-dianisidine dihydrochloride), and 3 µl

Western blot analysis. Approximately 0.1 g of frozen heart tissue from each rat was homogenized in 1 ml of lysis buffer containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma, St. Louis, MO) and centrifuged at 12,000 g for 20 min at 4°C. For measuring nitrate/nitrite, supernatant and plasma (250 µl) were further centrifuged by using a 30-kDa molecular mass cut-off filter (Fisher Scientific, Pittsburgh, PA) at 12,000 g for 45 min. The flow of cut-off filters was analyzed according to the manufacturer’s instructions.

Table 1. Effects of 17β-estradiol on cardiac function following trauma-hemorrhage
H₂O₂ (20 mmol/l). Sample (10 µl) was added to each well to start the reaction. Light absorbance at 460 nm was determined, and MPO activity was calculated using a standard curve obtained from human MPO (Sigma).

Measurement of heart nitrotyrosine level. Formation of nitrotyrosine in the heart was evaluated as a cardiac injury marker using a commercially available nitrotyrosine ELISA kit (Cell Science, Canton, MA). Heart tissue (100 mg wet wt) was homogenized in 1 ml of lysis buffer (pH 7.4) containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM MgCl₂, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1M NaF, 10% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma) on ice and centrifuged at 12,000 g for 20 min at 4°C. Nitrotyrosine content of the resulting supernatant (100 µl) was determined by spectrophotometry, according to the manufacturers’ instructions.

Statistical analysis. Data are presented as means ± SE (n = 6 rats/group). The Western blot analyses were performed with at least four animals per group. Statistical differences among groups were determined by one-way ANOVA, followed by Tukey’s test. Differences were considered significant if P < 0.05.

RESULTS

Effects of E₂ on cardiac performance. A significant decrease in MBP and ±dP/dt_max following trauma-hemorrhage was observed in the vehicle-treated group compared with shams (Table 1). Administration of E₂ after trauma-hemorrhage significantly ameliorated the trauma-hemorrhage-induced decrease in MBP; however, the values remained lower than those in the sham-treated animals. E₂ administration following trauma-hemorrhage, however, normalized ±dP/dt_max and markedly improved −dP/dt_max (Table 1).

p38 MAPK activation in the heart. Trauma-hemorrhage induced a significant decrease in the phosphorylation of cardiac p38 MAPK compared with shams at 2 h after the end of resuscitation; this was normalized by E₂ administration (Fig. 1). Coadministration of p38 MAPK inhibitor SB prevented the E₂-mediated cardiac p38 MAPK activation (Fig. 1). There was no difference in p38 MAPK phosphorylation in

Fig. 1. Expression of total p38 mitogen-activated protein kinase (p38 MAPK) and phosphorylated (p-p38) MAPK in the heart following sham operation (sham) or trauma-hemorrhage. Blots obtained from several experiments were analyzed using densitometry; densitometric values were pooled from 6 animals in each group and are presented as means ± SE. The results were compared by one-way ANOVA and Tukey’s test. *P < 0.05 vs. sham or trauma-hemorrhage plus 17β-estradiol (E₂). SB, SB-203580, p38 MAPK inhibitor.

Fig. 2. Nitrate/nitrite levels in plasma (A) and heart (B) following sham operation or trauma-hemorrhage. Rats were treated with either vehicle, SB, E₂, E₂ + SB, N⁵-nitro-L-arginine methyl ester (L-NAME; L), or E₂ + L. Data are means ± SE of 6 animals in each group. The results were compared by one-way ANOVA and Tukey’s test. *P < 0.05 vs. sham or trauma-hemorrhage + L. #P < 0.05 vs. all other groups.
sham rats treated with E2, vehicle, or SB. Furthermore, the difference was not evident in the total cardiac p38 MAPK protein expression between trauma-hemorrhage and sham-treated animals.

Effects of inhibition of p38 MAPK or NOS on nitrate/nitrite levels in the heart and plasma. Since the blood was diluted after resuscitation, we used protein content of the heart tissue and plasma to quantitate the concentration of nitrate/nitrite in samples. The levels of nitrate/nitrite in the plasma and heart increased significantly after trauma-hemorrhage (Fig. 2). E2 treatment further increased the content of nitrate/nitrite in the heart and plasma. SB treatment significantly decreased the levels of nitrate/nitrite; but the values were still significantly higher than in the shams. SB in the absence of E2 did not further alter cardiac and plasma nitrate/nitrite levels compared with trauma-hemorrhage. The content of cardiac and plasma nitrate/nitrite was not different among sham animals treated with vehicle, E2, SB, or L-NAME.

Effects of inhibition of p38 MAPK or NOS on E2-mediated improvement in cardiac performance. To evaluate whether the cardioprotective effects of E2 were mediated via p38 MAPK/eNOS, trauma-hemorrhage rats were treated with SB, E2 + SB, L-NAME, or E2 + L-NAME. Coadministration of SB prevented the E2-induced improvement of cardiac performance following trauma-hemorrhage (Fig. 3). SB in the absence of E2 did not further alter cardiovascular function after trauma-hemorrhage. Coadministration of L-NAME did not affect the E2-mediated improvement of heart performance. There was no significant difference in MBP and \( \pm \frac{dP}{dt_{\text{max}}} \) in sham animals treated with SB, L-NAME alone, or E2 compared with vehicle-treated shams.

Cardiac eNOS expression and phosphorylation. Trauma-hemorrhage produced a marked decrease in both cardiac eNOS protein expression and phosphorylation compared with shams (Fig. 4). Administration of E2 following trauma-hemorrhage significantly increased cardiac eNOS expression (Fig. 4A) and phosphorylation (Ser1177) (Fig. 4B). To investigate whether the E2-mediated increase in eNOS expression and phosphorylation following trauma-hemorrhage was via p38 MAPK, a group of animals was administered SB alone or E2 plus SB. The E2-mediated increase in eNOS expression and phosphorylation was abolished by coadministration of SB with E2. No significant difference in cardiac eNOS expression or phosphorylation was observed between trauma-hemorrhage and trauma-hemorrhage treated with SB. There was also no difference in eNOS expression or phosphorylation among the sham animals treated with vehicle, E2, or SB. These data strongly suggest that E2-induced eNOS activation is mediated by activation of p38 MAPK pathway in the heart following trauma-hemorrhage.

Cardiac caveolin-1 and calmodulin expression. Cardiac caveolin-1, a negative regulator of eNOS, was significantly increased by E2 treatment following trauma-hemorrhage. The levels of calmodulin in the tissue and plasma following trauma-hemorrhage was via p38 MAPK, a group of animals was administered SB alone or E2 plus SB. The E2-mediated increase in eNOS expression and phosphorylation was abolished by coadministration of SB with E2. No significant difference in cardiac eNOS expression or phosphorylation was observed between trauma-hemorrhage and trauma-hemorrhage treated with SB. There was also no difference in eNOS expression or phosphorylation among the sham animals treated with vehicle, E2, or SB. These data strongly suggest that E2-induced eNOS activation is mediated by activation of p38 MAPK pathway in the heart following trauma-hemorrhage.
following trauma-hemorrhage compared with shams, which was, however, decreased by E2 administration (Fig. 5A). Trauma-hemorrhage induced a decrease in cardiac calmodulin, which positively regulates eNOS phosphorylation compared with shams (Fig. 5B). Administration of E2 prevented the trauma-hemorrhage-induced decrease in the cardiac calmodulin-1 protein level. To determine the role of p38 MAPK in the E2-induced decrease in cardiac caveolin-1 and increase in calmod-
ulin-1 protein expression following trauma-hemorrhage, rats were treated with E2 along with SB following trauma-hemorrhage. The results show that coadministration of E2 with SB inhibited E2-mediated decrease in cardiac caveolin-1 (Fig. 5A) and increase in calmodulin-1 (Fig. 5B) protein expression. No significant difference in cardiac caveolin-1 and calmodulin levels was observed between trauma-hemorrhage and trauma-hemorrhage treated with SB. The protein expression of cardiac caveolin-1 and calmodulin-1 was not different among sham animals treated with vehicle, E2, or SB. These data suggest that eNOS activation is coupled to the changes in caveolin-1 and calmodulin expression.

Effects of NOS inhibition of cardiac cytokines, chemokines, and ICAM-1 levels. To understand whether eNOS activation induced by E2 treatment following trauma-hemorrhage was protective in the heart, a NOS inhibitor, L-NAME, was administered along with E2. There was no significant difference in IL-6 (Fig. 6A), TNF-α (Fig. 6B), MIP-2 (Fig. 7A), CINC-1 (Fig. 7B), and ICAM-1 (Fig. 7C) levels in sham-treated groups. Following trauma-hemorrhage, the protein expression of cytokines, chemokines, and adhesion molecules was significantly increased in the heart. Treatment with E2 normalized the levels of these mediators toward those of sham animals. Inhibition of NOS with L-NAME abolished the salutary effects of E2 on these proinflammatory mediators.

Effects of inhibition of p38 and NOS on cardiac MPO activity and nitrotyrosine levels. To determine whether inhibition of p38 MAPK and NOS was associated with the elevation of cardiac tissue injury following trauma-hemorrhage, the cardiac injury markers, MPO activity and cardiac nitrotyrosine protein, were measured. As shown in Fig. 8, differences in heart tissue MPO content (A) and nitrotyrosine protein (B) were not noted among sham animals treated with vehicle, E2, SB, and L-NAME. In contrast, trauma-hemorrhage resulted in markedly increased MPO levels and nitrotyrosine formation compared with shams. Treatment with E2 attenuated the increase in cardiac injury markers in the hemorrhaged animals, which was reversed by pretreatment with SB or L-NAME.

DISSCUSSION

Understanding the pathophysiology of trauma-hemorrhage and how E2 mitigates subsequent cardiac depression is important. In the present study, we report mechanistic details of how E2 protects against cardiac dysfunction and tissue injury from trauma-hemorrhage through p38 MAPK-dependent eNOS regulation. We found that cardiac performance, eNOS expression/phosphorylation, and calmodulin were markedly decreased following trauma-hemorrhage. Consequently, nitrate/nitrite levels, cytokines/chemokines levels in heart tissue, cardiac MPO activity and nitrotyrosine formation, as well as caveolin-1 expression were increased at 2 h after the end of resuscitation. Our findings also indicate that these alterations were accompanied by a decrease in p38 MAPK activation. The depressed cardiac functions following trauma-hemorrhage were restored by administration of E2 after trauma-hemorrhage and were accompanied with increased cardiac p38 MAPK activity, eNOS levels and its regulatory protein (calmodulin) expression, eNOS phosphorylation, nitrate/nitrite levels in plasma and heart, and a decrease in cardiac inflammation, MPO activity, nitrotyrosine levels, and caveolin-1 expression. The E2-mediated cardioprotection and the increased eNOS expression/phosphorylation, calmodulin expression, as well as the levels of nitrate/nitrite were abolished if the p38 MAPK-

![Fig. 6. Effects of nitric oxide synthase (NOS) inhibition with L on cardiac IL-6 (A) and TNF-α (B) production. Rats were treated with either vehicle, L, E2, or L + E2. Values are means ± SE of 6 animals in each group. The results were compared by one-way ANOVA and Tukey’s test. *P < 0.05 vs. sham or trauma-hemorrhage + E2.](http://ajpheart.physiology.org/10.1152/ajpheart.00557.2007)
specific inhibitor SB was used with E2. These findings, therefore, indicate that the beneficial effects of E2 on cardiac function following trauma-hemorrhage are mediated through the activation of p38 MAPK, subsequent eNOS expression/phosphorylation, and the changes in its regulatory protein expression. Several potential mechanisms have been proposed for the beneficial effects of E2 on organ function following trauma-hemorrhage (18–20, 43). Our present study utilizing the in vivo signal transduction mechanism for the first time demonstrates that E2-mediated cardioprotection is through the regulation of p38 MAPK and eNOS expression/phosphorylation following trauma-hemorrhage.

A major finding of the present study is that the trauma-hemorrhage induced a profound increase in neutrophil infiltration into the heart tissue (i.e., MPO activity). Neutrophil infiltration is a major hallmark of the inflammatory process and tissue injury. The extravasation of neutrophils into the heart tissue involves leukocyte rolling, followed by adherence and transendothelial migration. Adhesion molecules (ICAM-1, P-selectin) and chemokines play an important role in this process. A close correlation between MPO activity and nitrotyrosine formation under acute and chronic vascular inflammatory conditions has been previously demonstrated (4). In this regard, MPO-generated HOCl has been shown to affect eNOS activity and nitrotyrosine formation in ischemic vascular lesions. The present study confirms the formation of cardiac nitrotyrosine, which may induce cytotoxicity and provide a catalyst for further generation of deleterious oxygen species from neutrophils (45). Our study provides a potential mechanism by which estrogen contributes to the regulation of neutrophil infiltration and nitrotyrosine formation by showing that administration of E2 resulted in a decrease in MPO activity, nitrotyrosine levels, lower ICAM-1 expression, and production of chemokines in heart tissues following trauma-hemorrhage. These results are consistent with our laboratory’s previous studies (16, 20).

NO is regulated by the amount of NOS protein in cardiac tissue and by its activity or phosphorylation. Our results demonstrate that the expression of cardiac eNOS proteins was downregulated following trauma-hemorrhage and normalized after E2 treatment. In agreement with our findings, others have shown that E2 facilitates the expression of eNOS and activity in endothelial cells. These effects are E2-specific because they are inhibited by estrogen receptor blockade (3). Nonetheless, the present data are the first to demonstrate the enhancement of eNOS expression by E2 in the rat heart in the trauma-hemorrhage model. In addition, inhibition of NOS, including eNOS, with L-NAME in vivo following trauma-hemorrhage reversed the attenuation of cardiac inflammatory response (IL-6, TNF-α, ...
MIP-2, and CINC-1) and heart tissue injury mediated by E2. From these results, it is feasible to suggest that the role of eNOS activation induced by E2 during trauma-hemorrhage is to act as an early protective trigger. It is most likely that this protective action involves modulation of cytokines (IL-6, TNF-α), chemokines (MIP-2, CINC-1), and the adhesive proteins (ICAM-1) expressed at the interface between the endothelium and neutrophils (26, 34, 38).

The precise molecular mechanism by which E2 regulates eNOS activity remains unknown. In this regard, two amino acids are particularly important in regulating eNOS activity: a serine residue in the reductase domain (Ser1177) and a threonine residue (Thr495) located within the calmodulin-binding domain (9). The finding that eNOS can be phosphorylated on serine, threonine, and tyrosine residues supports the important role of phosphorylation in regulating eNOS activity (14). For instance, in unstimulated cultured endothelial cells, Ser1177 is not phosphorylated, but is rapidly phosphorylated after the application of estrogen (30) and other stimuli (12, 15). Notably, eNOS activity is under a dynamic regulation, and several agents have been implicated in modulating eNOS function in arterioles from estradiol-depleted rats, and only the simultaneous upregulation of eNOS and downregulation of caveolin-1 has been associated with the normalization of activity (41). Our results suggest that interaction of E2 with cardiac eNOS, which has been directly correlated with the favorable effects on cardioprotection, involves not only an upregulation of eNOS protein expression, but also the phosphorylation of eNOS and its regulatory proteins, calmodulin and caveolin.

Most of the kinases, such as MAPKs and extracellular signal-regulated kinase 1/2, as well as the cyclic nucleotide-dependent kinases PKA and PKG, phosphorylate eNOS on tyrosine or threonine residues physically associated with the enzyme, either directly or via binding to an adaptor protein (6). However, whether these kinases play any role in affecting cardiac function or tissue injury following trauma-hemorrhage remains to be established. Although study has indicated that, following heart ischemia-reperfusion, there is differential association of p38 MAPK with different type of caveolin-induced survival or death signal (11), our study demonstrates for the first time that p38 MAPK inhibition attenuates the E2-induced decrease in caveolin-1 expression; however, the precise mechanism responsible for p38 MAPK regulation of caveolin-1 remains to be established. The roles of p38 MAPK in heart tissue injury and the recovery of postischemic organ function are complex and controversial. Some studies suggest that p38 MAPK activation is beneficial (2, 19, 22, 29). Others
indicated its activation is detrimental, and that its inhibition during reperfusion is protective (10, 24, 31, 33, 39). There are four isoforms of p38 MAPK: α, β, γ, and δ. Both p38-α and p38-β, which are found in the heart, are activated by several stimuli. The p38-α activation has been associated with death signaling, and p38-β is related to cell survival. E2 can act to modulate the balance between the two isoforms (p38-α and p38-β) favoring cell survival during stress (27). In this regard, additional findings demonstrate that the acute activation of p38 MAPK induced by E2 is protective and reduces dysfunction following trauma-hemorrhage or ischemia-reperfusion (19, 28). Our data demonstrate that estradiol activated p38 MAPK in the heart, and blocking p38 MAPK activation partially inhibited the tissue protection by E2. In addition to p38, our laboratory’s previous studies have shown the role of other signaling molecules, such as PI3K/Akt, cGMP-dependent kinase (PKG), peroxisome proliferators-activator receptor coactivator-1α, heat shock proteins, and heme oxygenase-1, in estrogen-mediated protective effects following trauma-hemorrhage (18–20, 43). However, whether they are linear or are regulated independently by E2 remains unclear at present. Thus more studies are needed to identify the relationship between p38 and other pathways, such as PI3K and heat shock proteins.

There are some conflicting results concerning the effects of SB on p38 MAPK phosphorylation. Some studies show that SB does not inhibit the phosphorylation, but rather inhibits the downstream activities of p38 MAPK (42), whereas other studies show the inhibitory effect of this agent on p38 MAPK phosphorylation (5, 19). We confirmed that the dose of SB used in this study blocked E2-induced cardiac p38 MAPK phosphorylation following trauma-hemorrhage.

The present study utilized measurement of cardiac function and the increase in the phosphorylation of p38 MAPK, eNOS expression, and phosphorylation at a single time point, i.e., 2 h after trauma-hemorrhage. In view of this, it remains unclear whether similar effects of estradiol are maintained for periods longer than 2 h after trauma-hemorrhage. Our previous studies, however, have shown that, if improvement in organ functions by any pharmacological agent occurs early after treatment, those salutary effects are sustained for prolonged intervals, and they also improved the survival of animals (8). Thus, although a time point other than 2 h was not examined in this study, based on our previous studies, it would appear that the salutary effects of E2 would be evident, even if one examined the effects at another time point following trauma-hemorrhage and resuscitation.

Although L-NAME did not affect the E2-mediated cardiac performance after trauma-hemorrhage, it did prevent the E2-mediated attenuation of cardiac injury markers (MPO activity, nitrotyrosine), cytokine and chemokine levels, under such conditions. These findings suggest that, early after trauma-hemorrhage (2 h), the detriment in cardiac function is not linked to neutrophil-mediated tissue damage. Nonetheless, the increase in eNOS phosphorylation induced by E2 treatment, which reduced neutrophil infiltration, may be important to the recovery of cardiac function at later times postinjury, which warrants investigation.

It can also be argued that we should have examined cardiac output in this study. In this regard, our previous studies have shown that the cardiac output was significantly decreased following trauma-hemorrhage and was restored to normal after estradiol administration. Additionally, the +dP/dmax and −dP/dmax do reflect the value of cardiac output in sham and trauma-hemorrhage rats. Furthermore, if we had used the radioactive microsphere technique to determine cardiac output, the heart tissues could not have been used for Western blot analysis or nitrate/nitrite determination. In view of these limitations, we did not determine cardiac output in this study.

In summary, the present results indicate that E2 administration following trauma-hemorrhage upregulates p38 MAPK activation, eNOS expression, and eNOS phosphorylation in the heart; restores cardiac function; and attenuates cardiac injury. These findings suggest that the salutary effects of E2 are mediated via a p38 MAPK-dependent pathway through increased expression/phosphorylation of eNOS and the changes of eNOS regulatory protein caveolin-1 and calmodulin. Nonetheless, since E2 can mediate its effects in multiple ways, we do not consider activation of p38 MAPK to be the exclusive action of E2 under such conditions. Nonetheless, the findings highlight the relationship between E2 and the p38 MAPK/eNOS pathway, and this may help in developing new therapeutic modalities for the treatment of trauma-hemorrhagic shock.

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GRANTS

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