Mechanism of salutary effects of estradiol on organ function after trauma-hemorrhage: upregulation of heme oxygenase

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IT IS WELL ESTABLISHED THAT shock decreases organ perfusion and depresses organ as well as immune functions (2, 9, 12, 32, 38, 42). There is also a growing body of evidence that heme degradation products may counteract the deleterious consequences of hypoxia and/or ischemia-reperfusion injury. Because heme oxygenase (HO)-1 induction after adverse circulatory conditions is known to be protective, and because females in the proestrous cycle (with high estrogen) have better hepatic function and less hepatic damage than males after trauma-hemorrhage, we hypothesized that estrogen administration in males after trauma-hemorrhage will upregulate HO activity and protect the organs against dysfunction and injury. To test this hypothesis, male Sprague-Dawley rats underwent 5-cm laparotomy and hemorrhagic shock (35–40 mmHg for 93 ± 2 min), followed by resuscitation with four times the shed blood volume in the form of Ringer lactate. 17β-Estradiol and/or the specific HO enzyme inhibitor chromium mesoporphyrin (CrMP) were administered at the end of resuscitation, and the animals were killed 24 h thereafter. Trauma-hemorrhage reduced cardiac output, myocardial contractility, and serum albumin levels. Portal pressure and serum alanine aminotransferase levels were markedly increased under those conditions. These parameters were significantly improved in the 17β-estradiol-treated rats. Estradiol treatment also induced increased HO-1 mRNA expression, HO-1 protein levels, and HO enzymatic activity in cardiac and hepatic tissue compared with vehicle-treated trauma-hemorrhage rats. Administration of the HO inhibitor CrMP prevented the estradiol-induced attenuation of shock-induced organ dysfunction and damage. Thus the salutary effects of estradiol administration on organ function after trauma-hemorrhage are mediated in part via upregulation of HO-1 expression and activity.

hemorrhagic shock; heme oxygenase-1; chromium mesoporphyrin; liver; heart

MATERIALS AND METHODS

Animals. Adult male (275–325 g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in this study. All experiments were performed in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Experimental procedures. Rats were fasted overnight before the experiment but allowed water ad libitum. Trauma-hemorrhage was induced as described in detail previously (13, 19). Briefly, the rats were anesthetized by isoflurane (Attane; Minrad, Bethlehem, PA) inhalation before the induction of soft-tissue trauma (i.e., 5-cm midline laparotomy). The abdomen was then closed in layers, and catheters were placed in both femoral arteries and the right femoral vein [polyethylene (PE-50) tubing; Becton-Dickinson, Sparks, MD]. The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to minimize postoperative pain. The rats were then allowed to awaken, after which they were rapidly bled to a mean arterial pressure (MAP) of 35–40 mmHg within 10 min. The time at which the animals could no longer maintain a MAP of 35–40 mmHg without fluid infusion was defined as maximum bleed-out volume. The rats were maintained at this MAP until 40% of the shed blood volume was returned in the form of Ringer lactate.

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animals were then resuscitated with four times the volume of shed blood with Ringer lactate over 60 min. After resuscitation, the catheters were removed, the vessels were ligated, and skin incisions were closed with sutures. Sham-operated animals underwent the same groin dissection, which included ligation of the femoral artery, and catheters were placed in the femoral vein to administer agents; however, neither trauma-hemorrhage nor resuscitation was carried out. The animals were returned to their cages and were allowed food and water ad libitum until death, 24 h after the end of resuscitation. In the treatment groups, estradiol (1 mg/kg body wt iv; Sigma, St. Louis, MO) and/or Cr(III) mesoporphyrin-IX chloride (CrMP, 2.5 mg/kg body wt ip; Frontier Scientific, Logan, UT) were administered at the end of the resuscitation.

**Determination of cardiac contractility and cardiac output.** At 24 h after hemorrhage or sham operation, the animals were anesthetized with pentobarbital sodium. A tip-reduced PE-50 catheter was placed into the right carotid artery to measure MAP. The catheter was then advanced into the left ventricle. Left ventricular contractility parameters such as the maximal rate of left ventricular pressure increase (+dP/dt max) and decrease (−dP/dt max) were determined (13, 19). Cardiac output was determined by using an indocyanine green (ICG) dilution technique (13, 19). Briefly, the right jugular vein was isolated and cannulated with PE-50 tubing under isoflurane anesthesia. A 2.4-Fr fiber-optic catheter (Hospex Fiberoptics, Chestnut Hill, MA) was inserted into the right carotid artery and placed at the level of the aortic arch for continuous measurement of ICG concentration with an in vivo hemoreflectometer (Schwarzer-Picker International, Munich, Germany). The area under the curve was determined, and cardiac output was calculated according to the principle of dye dilution.

**Measurement of portal pressure and bile flow.** At 24 h after the end of resuscitation or sham operation, a laparotomy was performed, after which the intestines were covered with wet gauze to minimize evaporative fluid loss during the measurements (36). The portal vein was identified and exposed. The common bile duct was cannulated with a PE-10 catheter, and the bile flow was measured in preweighed tubes for 10 min. A PE-10 catheter, filled with saline, was inserted into the portal vein without compromising the flow. The catheter was connected to a Digi-Med Low Pressure Analyzer to monitor portal blood pressure.

**Measurement of hepatic injury and function.** Blood samples were obtained and placed in microcentrifuge tubes at 24 h after the end of resuscitation or sham operation. After centrifugation, plasma and serum samples were separated, immediately frozen, and stored at −80°C until being assayed. Hepatic injury was determined by measuring alanine aminotransferase (ALT) with a commercially available colorimetric reaction kit according to the manufacturer’s instructions (Sigma). To evaluate hepatic function, serum albumin level was measured by a colorimetric reaction kit (Tina-quant, Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Serum samples were homogenized in a buffer containing (in mM) 50 NaF, 10 Na4P2O7, 2.5 Na3VO4, and 1 PMSF, with 0.1% 2-mercaptoethanol and protease inhibitor cocktail (1:200 dilution; Sigma). Homogenates were centrifuged at 14,000 g for 30 min at 4°C. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). Protein al althoughs were mixed with 4× lithium dodecyl sulfate sample buffer and were electrophoresed on 4–12% SDS-polyacrylamide gels (Invitrogen) and transferred electrophoretically onto nitrocellulose transfer membranes (Invitrogen). Membranes were incubated with anti-HO-1 (1:10,000) or anti-HO-2 (1:2,000) antibody overnight at 4°C and then washed with Tris-buffered saline-Tween 20 buffer. Membranes were later incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:10,000) for 1 h at room temperature and washed with Tris-buffered saline-Tween 20. Blots were immersed for 5 min in SuperSignal West Pico detection reagent (Pierce Biotechnologies, Rockford, IL) and then exposed to film. Signals were quantified with ChemiImager 5500 imaging software (Alpha Innotech, San Leandro, CA).

**Determination of HO activity.** We determined HO activity in liver and heart homogenates by measuring the amount of bilirubin formed in a two-step enzymatic reaction, as described previously (36). Tissue samples were homogenized in 10 mM phosphate-buffered saline (pH 7.2) containing protease inhibitors (Complete EDTA-free: Roche, Basel, Switzerland). According to the manufacturer, Complete EDTA-free is a protease inhibitor cocktail containing chymotrypsin, thermolysin, papain, pronase, pancreatic extract, and trypsin. The homogenates were centrifuged at 10,000 g for 15 min at 4°C. The supernatant was further centrifuged at 105,000 g for 1 h at 4°C. The resulting pellet was resuspended in phosphate-buffered saline with protease inhibitors. Protein content was determined with a protein assay kit (Bio-Rad Laboratories). A solution mixture was prepared containing 0.5 ml of microsomal protein (6–9 mg/ml), 200 μl of a 105,000 g centrifuged supernatant fraction (10 mg/ml of protein) as a source for biliverdin reductase, a reduced NADP (NADPH)-generating system (in mM: 0.8 β-NADPH, 0.8 glucose-6-phosphate, and 2 magnesium chloride, with 1.5 μU of glucose-6-phosphate dehydrogenase), and 20 μl of 2.5 mM heme solution. The final volume was adjusted to 1.5 ml with 0.1 M phosphate-buffered saline. Samples were prepared on ice and then incubated in the dark with agitation for 60 min at 37°C. Bilirubin content was measured spectrophotometrically at 460 and 530 nm; HO activity was expressed as nanomoles of bilirubin per 60 min per milligram of protein.

**Statistical analysis.** Data are presented as means ± SE. Statistical differences among groups were determined by one-way ANOVA followed by Fisher’s least significant difference test as a post hoc test. Differences were considered significant if P < 0.05.

**RESULTS**

**Alterations in cardiac performance.** Cardiac output decreased significantly in vehicle-treated rats after trauma-hemorrhage. Administration of estradiol after trauma-hemorrhage increased cardiac output, and the values were similar to those in the sham-operated animals (Fig. 1A). Furthermore, +dP/dt max and −dP/dt max were significantly depressed at 24 h after trauma-hemorrhage. Treatment of rats with estradiol prevented the decrease in both +dP/dt max and −dP/dt max (Fig. 1, B and C). The restoration of heart performance with estradiol treat-
ment was prevented by concurrent administration of estradiol with the HO inhibitor CrMP.

Alterations in portal pressure and bile flow. Trauma-hemorrhage significantly increased portal pressure (Fig. 2A) and decreased bile production (Fig. 2B) in vehicle-treated rats. Estradiol treatment attenuated the increase in portal pressure and restored bile production to the levels in sham-operated rats. However, administration of CrMP along with estradiol prevented both the estradiol-induced restoration of portal pressure and the enhancement of bile production.

Alteration in levels of ALT and albumin. Trauma-hemorrhage induced a significant increase in serum ALT levels (Fig. 3A). Estradiol administration attenuated the trauma-hemorrhage-induced increase in ALT. However, administration of CrMP along with estradiol prevented both the estradiol-induced restoration of portal pressure and the enhancement of bile production. Estradiol administration after trauma-hemorrhage prevented the decrease in serum albumin levels. This estradiol-induced normalization of albumin synthesis was prevented by coadministration of CrMP. Hematocrit was reduced by ~50% after trauma-hemorrhage, irrespective of estradiol or CrMP treatment (Table 1), indicating that the differences in serum albu-
HO activity and expression. Trauma-hemorrhage induced a slight, but insignificant, increase in heart and liver HO activity compared with those in sham-operated animals. Administration of estradiol after trauma-hemorrhage induced a marked increase in HO enzyme activity in the heart (Fig. 4A) and the liver (Fig. 4B). This increase in HO activity was completely blocked by administration of the HO inhibitor CrMP along with estradiol. To determine which isoform of the HO enzyme was responsible for the observed increase in activity, HO-1 and HO-2 protein levels were determined by Western blot analysis. Estradiol administration increased HO-1 (Fig. 5A) but not HO-2 (Fig. 5B) expression in the heart. Similarly, estradiol increased HO-1 (Fig. 6A) but not HO-2 (Fig. 6B) levels in the liver. PCR analysis showed elevated HO-1 mRNA levels in the liver of estradiol-treated animals (Fig. 7B). A trend toward increased HO-1 mRNA levels in the heart was observed under such conditions, but it did not reach statistical significance (Fig. 7A).

Table 1. Hematocrit levels at 24 h after trauma-hemorrhage

<table>
<thead>
<tr>
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<th>Sham</th>
<th>T-H</th>
<th>T-H + E2</th>
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<tr>
<td>Vehicle</td>
<td>43±3</td>
<td>20±4*</td>
<td>20±2*</td>
</tr>
<tr>
<td>CrMP</td>
<td>42±3</td>
<td>21±4*</td>
<td>22±3*</td>
</tr>
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Values are means ± SE; n = 6 animals/group. Sham, sham-operated rats; T-H, trauma-hemorrhage rats; T-H + E2, trauma-hemorrhage rats receiving estradiol; CrMP, chromium mesoporphyrin. *P < 0.05 vs. Sham group.

Fig. 4. Effects of estradiol treatment and HO blockade on HO activity in heart (A) and liver (B) homogenates after trauma-hemorrhage. At the end of the resuscitation, estradiol (1 mg/kg iv) and/or the HO blocker CrMP (2.5 mg/kg ip) were administered. Rats were killed at 24 h after trauma-hemorrhage, and HO activity was estimated by measuring bilirubin production in heart and liver homogenates (nmol bilirubin·h⁻¹·mg protein⁻¹). Data are presented as means ± SE (6 animals/group). *P < 0.05 vs. Sham group; #P < 0.05 vs. T-H group; X P < 0.05 vs. T-H + E2 group.

DISCUSSION

There is a growing body of evidence that indicates that the inducible form of HO (HO-1) is upregulated after hemorrhagic shock and that the HO product CO plays a central role in the preservation of hepatic microcirculation under such conditions (4, 27, 28, 30). In addition, intestinal induction of HO-1 has been shown not only to improve local mesenteric circulation but to also prevent distant organ response of hemorrhagic shock (11). Previous studies from our laboratory (13, 45) showed salutary effects of estrogen and its derivatives in restoring organ functions after trauma-hemorrhage. Furthermore, it has been demonstrated that trauma-hemorrhage-induced HO activity in the liver of female proestrous rats with elevated plasma estradiol level exceeded that of male counterparts (36). This gender dimorphism in enzyme production and activity correlated with decreased organ damage and improved hepatic function. In the present study, estradiol was found to induce a further increase in HO enzyme expression and activity in both the heart and the liver after trauma-hemorrhage. This upregulation in HO was associated with attenuation of organ damage and restoration of cardiac and hepatic functions. How-
ever, administration of the HO inhibitor CrMP with estradiol prevented the estradiol-induced restoration of organ functions. It could therefore be concluded that the salutary effects of estradiol are mediated, at least in part, by the HO pathway.

HO-derived products can mediate vasodilatation and improve rheological conditions via multiple mechanisms. Similar to NO, CO has capacity to activate soluble guanylate cyclase and induce vasodilatation via cGMP (23). An additional mechanism of improved tissue perfusion may be the CO-mediated activation of the Ca\(^{2+}\)/H\(^{+}\) dependent K\(^+\) (K\(\text{Ca}\)) channels. In this regard, K\(\text{Ca}\) channels in the vasculature serve as regulatory elements of membrane potential, and hence, vascular tone and reactivity. The activation of K\(\text{Ca}\) channels leads to hyperpolarization of the smooth muscle cells, resulting in decreased vascular contractility (40). Thus CO-mediated K\(\text{Ca}\) activation may lead to a reduced responsiveness to vasoconstrictive agents. Furthermore, CO can improve tissue perfusion not only by hampering the vasoconstrictive signal but also by down-regulating the production of endothelin (22). It is therefore possible that estrogen improves organ functions by one or more of the mechanisms described above.

Leukocyte activation can also deteriorate microcirculation. Activated leukocytes, either by attaching to the vessel wall or by inducing perivascular edema, can significantly reduce circulation (10). Therefore, reduction of neutrophil activation may significantly improve organ functions after trauma-hemorrhage (17). In this regard, estradiol has been shown to reduce neutrophil infiltration in the liver after ischemia (7). Our studies support this observation because proestrous female rats with elevated estradiol levels responded with reduced leukocyte priming and activation after trauma-hemorrhagic shock (35); hence estradiol-mediated inhibition of leukocyte activation may contribute to its protective effects after an ischemic insult (29). It has also been reported that HO-1 can reduce the expression of adhesion molecules and may therefore also prevent subsequent leukocyte-endothelial cell interactions (33). It has also been shown that CO inhalation decreases leukocyte rolling and adhesion and prevents liver ischemia (26). Furthermore, it has been reported that the other heme degradation product, biliverdin, reduces ICAM-1 expression in the ischemic intestine (25). Nonetheless, Wunder et al. (43) emphasized that HO-mediated alterations rather than reduced adhesion molecule expression in hepatic leukocyte activation play an important role in regulating sinusoidal diameter. Because the CO-mediated inhibition of platelet aggregation has also been evidenced, it is plausible that HO activity can prevent thrombus formation and subsequent proinflammatory mediator release effectively (6). Although the net contribution of the individual degradation products to the inhibition of leukocyte-endothelial cell interactions has not been evaluated, the relevance of HO-1 upregulation in hemorrhage-induced leukocyte

![Fig. 6. Representative blots showing the effect of estradiol treatment on liver HO-1 (A) and HO-2 (B) protein levels after trauma-hemorrhage. At the end of the resuscitation, estradiol (1 mg/kg iv) was administered. Rats were killed at 24 h after trauma-hemorrhage, and HO-1 and HO-2 protein levels were determined in liver tissue homogenates. Blots were analyzed densitometrically; densitometric values obtained from several animals were pooled and are shown in bar graphs. Data are presented as means ± SE (n = 4 animals in Sham group; n = 6 animals in T-H and T-H + E\(_2\) groups). *P < 0.05 vs. Sham group.](http://ajpheart.physiology.org/)

![Fig. 7. Effect of estradiol on HO-1 gene expression in the heart (A) and the liver (B) after trauma-hemorrhage. At the end of the resuscitation, estradiol (1 mg/kg iv) was administered. Rats were killed at 24 h after trauma-hemorrhage, and HO-1 gene expression was determined by real-time PCR in heart and liver tissue. Data are presented as means ± SE (n = 3–4 animals/group) and are relative quantities. The mean value of the sham group’s expression was set to 1; endogenous control was 18S). *P < 0.05 vs. Sham group; #P < 0.05 vs. T-H group.](http://ajpheart.physiology.org/)
activation has been clearly evidenced (20). Although we examined estradiol-induced HO upregulation and not leukocyte activation/inactivation, a previous study clearly indicates the ability of HO to inhibit expression of adhesion molecules in endothelial cells (33). These studies collectively lead us to conclude that HO may be a significant effector mechanism by which estradiol attenuates leukocyte activation after trauma-hemorrhagic shock.

Enhancement of antioxidant capacity has been reported to be an effective therapy for the treatment of hemorrhagic shock (24, 31). It should be noted, however, that bilirubin, the degradation product of HO activity, also has a potent antioxidant activity. Although HO-1-mediated bilirubin production is limited, the oxidized bilirubin (biliverdin) can be regenerated by biliverdin reductase, forming a potent redox cycle (1). In support of this hypothesis, it has been reported that HO upregulation protects mitochondrial function and prevents ATP depletion after oxidative stress (5). Because superoxide anion acts as a vasoconstrictor, neutralization of this free radical may also serve to improve organ perfusion (14). Moreover, HO-1-derived bilirubin can inhibit selectin expression and leukocyte adhesion (37). Thus it appears that HO activity has multiple potentials for improving tissue perfusion and protecting cellular damage, and this may play a significant role in the salutary effects of estradiol after trauma-hemorrhage.

The functional significance of the involvement of NO in the HO-1 induction has been established (46). Evidence also suggests that NO increases HO-1 via the induction of HO-1 transcription and stabilization of HO-1 mRNA (8). It is also possible that there is a common regulation of NO and HO-1 because both endothelial nitric oxide synthase (eNOS) and HO-1 are compartmentalized and functionally influenced by caveolae (15). Caveolae, the bulb-shaped invaginations of plasma membrane, are implicated in many cellular processes, such as transport functions and signal transduction. Furthermore, caveolins are basic structural and regulatory components of the caveolae. Similar to the way that eNOS and caveolin-1 association in caveolae maintains eNOS in an inactive state, caveolin-1 expression results in decreased HO activity (15). Thus the potential of estradiol to decrease the expression of caveolin-1 suggests a direct link between estradiol and HO-1-mediated effects (41).

In summary, our results indicate that induction of HO-1 is an important mechanism by which estradiol improves cardiac and hepatic functions after trauma-hemorrhage. Blockade of HO pathways and the associated deterioration of the examined parameters suggest that the improvement in organ perfusion is mediated by HO-derived bioactive compounds. Although the precise mechanism of the salutary effects of estradiol on organ functions and the contribution of HO pathways in improving organ functions after trauma-hemorrhage remain unclear, our study provides additional evidence that HO-1 upregulation serves as a significant effector mechanism in the maintenance of cardiac and liver function after trauma-hemorrhage. We observed that estradiol administration upregulates HO activity in heart and liver and prevents cardiac and hepatic dysfunction at 24 h after trauma-hemorrhage. However, whether such attenuation in cardiac and liver function is due to an early upregulation of HO or to a sustained elevation in HO activity remains to be established. In our previous study, we observed (36) that at 5 h after resuscitation HO-1 expression reached significantly higher levels in the livers of female animals with high estrogen than in the livers of their male counterparts. The sustained amelioration of organ functions after estradiol treatment and the prevention of these beneficial effects with the early administration of the HO inhibitor CrMP suggest that estradiol-mediated HO upregulation is likely to play a significant role in the beneficial effects of estradiol on cardiac and hepatic function after trauma-hemorrhage. Nonetheless, because estradiol can mediate its effects in multiple ways, we do not consider HO-1 upregulation to be the exclusive effect of estradiol. Furthermore, we propose that a better understanding of the relationship between estrogen and HO-1 systems may enable us to develop new therapeutic modalities for hemorrhagic shock.

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