Mechanism of estrogen-mediated intestinal protection following trauma-hemorrhage: p38 MAPK-dependent upregulation of HO-1

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Hsu J-T, Kan W-H, Hsieh C-H, Choudhry MA, Schwacha MG, Bland KI, Chaudry IH. Mechanism of estrogen-mediated intestinal protection following trauma-hemorrhage: p38 MAPK-dependent upregulation of HO-1. Am J Physiol Regul Integr Comp Physiol 294: R1825–R1831, 2008. First published April 23, 2008; doi:10.1152/ajpregu.00112.2008.—p38 MAPK has been reported to regulate the inflammatory response in various cell types via extracellular stimuli. p38 MAPK activation also results in the induction of heme oxygenase (HO)-1, which exerts potent anti-inflammatory effects. Although studies have shown that 17β-estradiol (E2) prevented organ dysfunction following trauma-hemorrhage, it remains unknown whether p38 MAPK/HO-1 plays any role in E2-mediated attenuation of intestinal injury under those conditions. To study this, male rats underwent trauma-hemorrhage (mean blood pressure ~40 mmHg for 90 min) followed by fluid resuscitation. At the onset of resuscitation, rats were treated with vehicle, E2 (1 mg/kg body wt), the p38 MAPK inhibitor SB-203580 (2 mg/kg body wt) or E2 plus SB-203580. Two hours thereafter, intestinal myeloperoxidase (MPO) activity and lactate, TNF-α, IL-6, ICAM-1, cytokine-induced neutrophil chemoattractant (CINC)-1, and macrophage inflammatory protein (MIP)-2 levels were measured. Intestinal p38 MAPK and HO-1 protein levels were also determined. Trauma-hemorrhage led to an increase in intestinal MPO activity and lactate, TNF-α, IL-6, ICAM-1, CINC-1, and MIP-2 levels. This was accompanied with a decrease in intestinal p38 MAPK activity and increase in HO-1 expression. Administration of E2 normalized all the above parameters except HO-1, which was further increased following trauma-hemorrhage. Administration of SB-203580 with E2 abolished the E2-mediated restoration of the above parameters as well as the increase in intestinal HO-1 expression following trauma-hemorrhage. These results suggest that the p38 MAPK/HO-1 pathway plays a critical role in mediating the salutary effects of E2 on shock-induced intestinal injury.

p38 MAPK inhibitor; CINC-1; MIP-2; MPO

The gut is considered a critical organ in the development of organ dysfunction following traumatic injuries and severe blood loss (22). Multiple organ dysfunction or failure, secondary to a systemic inflammatory response, remains the major cause of mortality after trauma (1, 21, 24, 30). The p38 MAPK has been reported to regulate inflammatory response in different cell types by various stimuli (10, 37). Studies have shown that the inflammatory response to hyperoxia-induced lung injury in epithelial cells (A549) increased in the presence of p38 MAPK inhibitor SB-203580 or if those cells were transiently transfected with dominant negative mutants of MKK3, an upstream kinase of p38 MAPK (28). Additional studies have suggested that activation of p38 MAPK reduces TNF-α production in various cell types (10, 26).

An increasing body of evidence shows that p38 MAPK activation leads to the induction of heme oxygenase (HO)-1 (10, 37). HO-1 confers protection against oxidative stress in vivo and in vitro, through antioxidative, antiapoptotic, and anti-inflammatory actions (26, 27). Exogenous administration of HO-1 by gene transfer into rat lung mediates potent anti-inflammatory effects in the lung (27). Moreover, overexpression of HO-1 reduces the expression of adhesion molecules and prevents subsequent leukocyte-endothelial cell interactions and organ damage (32, 38).

Studies have shown that reduction of neutrophil infiltration following trauma-hemorrhage attenuated organ injury (19, 38). Neutrophil movement and migration are mediated by multiple adhesion molecules and proinflammatory mediators. ICAM-1 assumes a central role in firm adhesion of neutrophils to the vascular endothelium and is markedly upregulated following trauma-hemorrhage (8, 38, 39). The influx of neutrophils to the inflammatory sites is also driven by locally produced cytokines/chemokines (30). In this regard, TNF-α and IL-6 play important roles in the pathophysiology of intestinal ischemia/reperfusion injury (14, 36). In addition to cytokines, the cysteine-X-cysteine chemokines such as cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2) also activate and attract neutrophils (13, 20). A significant decrease in the influx of neutrophils in rat inflammation models has been observed via the use of antibodies to neutralize CINC-1 and MIP-2 (14, 29).

It is well established that gender can influence immune and cardiovascular functions after injury (3, 14, 18). Furthermore, clinical and experimental studies suggest that females tolerate injury better than males (9, 11, 12). Studies have also shown that administration of 17β-estradiol (E2) in male rats following trauma-hemorrhage improves depressed cardiac function and attenuates hepatic injury under those conditions (15, 34). Interestingly, our recent studies have shown that E2-mediated cardioprotection following trauma-hemorrhage is via activation of p38 MAPK (15). Additional studies have also shown that HO-1 upregulation contributes to the salutary effects of E2 on cardiac function (34). Nonetheless, it remains unknown whether p38 MAPK/HO-1 plays any role in the E2-mediated attenuation of intestinal injury following trauma-hemorrhage. The aim of this study, therefore, was to determine whether the salutary effects of E2 on the intestine following trauma-hemorrhage are mediated via p38 MAPK-dependent HO-1 upregulation.

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**MATERIALS AND METHODS**

*Rat trauma-hemorrhagic shock model.* Male (275–325 g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were fasted overnight before the experiment but were allowed water ad libitum. All experiments were performed in adherence with National Institutes of Health Guidelines for the Care and Use of Experimental Animals and our protocol was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. A nonheparinized model of trauma-hemorrhage was used as described previously (15). Briefly, rats were anesthetized by isoflurane inhalation prior to the induction of soft tissue trauma via 5-cm midline laparotomy. The abdomen was closed in layers, 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 (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to reduce postoperative pain. Rats were then placed into a Plexiglas box in a prone position and allowed to awaken, after which time they were rapidly bled to a mean arterial blood pressure of 35–40 mmHg within 10 min. This degree of hypotension was maintained until the animals could no longer keep a mean blood pressure of 35 mmHg unless additional fluid in the form of Ringers lactate was administered. This time was defined as maximum bleed-out, and the amount of withdrawn blood was recorded. Following this, the rats were maintained at mean blood pressure of 35–40 mmHg until 40% of the maximum bleed-out volume was returned in the form of Ringers lactate (~90 min from the onset of bleeding). The animals were then resuscitated with four volumes the time of the shed blood over 60 min with Ringers lactate. Sham-operated animals underwent the same groin dissection, which included ligation of the femoral arteries and vein, but neither hemorrhage nor resuscitation was carried out. Animals subjected to trauma-hemorrhage were allocated randomly into four groups receiving intravenously either vehicle (cyclo- dextrin; Sigma, St. Louis, MO), E2 (1 mg/kg body wt; Sigma), p38 MAPK inhibitor SB-203580 (2 mg/kg body wt; Calbiochem, San Diego, CA), or p38 MAPK inhibitor SB-203580 at the beginning of resuscitation. Following resuscitation, the catheters were removed, the vessels were ligated, and the skin incisions closed with sutures. The animals were killed at 2 h after the end of resuscitation or sham operation.

*Measurement of intestinal myeloperoxidase activity.* All reagents were purchased from Sigma. Myeloperoxidase (MPO) activity in homogenates of the whole intestine (proximal jejunum) was determined as described previously (38). Briefly, equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were cleared by centrifugation at 17,000 g at 4°C for 10 min, and the supernatants were stored at −80°C. Protein content in the samples was determined using the DC Protein Assay (Bio-Rad). The lysates (50 μg per lane) were then mixed with SDS sample buffer and were electrophoresed on 4 –12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred electrophoretically onto nitrocellulose membranes (Invitrogen). The membranes were immunoblotted with antibodies against p38 MAPK, phospho-p38 MAPK (Cell Signaling Technology, Beverly, MA), HO-1 (Stressgen Bioreagents, Ann Arbor, MI), or GAPDH (Abcam, Cambridge, MA). The membranes were then incubated with horse-radish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody for detection of bound antibodies by enhanced chemiluminescence (Amersham, Piscataway, NJ). Mouse monoclonal GAPDH antibody was used to determine GAPDH as the loading control. Signals were quantified using Chemilumager 5500 imaging software (Alpha Innotech, San Leandro, CA).

*Western blot analysis.* Intestinal tissues from each rat 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 (Sigma). Tissue lysates were centrifuged at 17,000 g for 20 min at 4°C and an aliquot of the supernatant was used to determine protein concentration (DC Protein Assay, Bio-Rad). The lysates (50 μg per lane) were then mixed with 4× SDS sample buffer and were electrophoresed on 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred electrophoretically onto nitrocellulose membranes (Invitrogen). The membranes were immunoblotted with antibodies against p38 MAPK, phospho-p38 MAPK, TNF-α, IL-6, ICAM-1, CINC-1, and MIP-2 levels. TNF-α, IL-6, ICAM-1, CINC-1 (R&D, Minneapolis, MN), and MIP-2 (Biosource, Camarillo, CA) levels in the intestine were determined using enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions. Briefly, the samples were homogenized in 0.5 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). The homogenates were centrifuged at 17,000 g for 20 min at 4°C and the supernatant was assayed for TNF-α, IL-6, ICAM-1, CINC-1, and MIP-2 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC Protein assay) and the cytokines and chemokines levels were normalized to the protein contents in the homogenates.

*Statistical analysis.* Results are presented as means ± SE (n = 4–6 rats/group). ANOVA and Tukey’s test were employed for comparison among groups, and differences were considered significant at P < 0.05.

**RESULTS**

*Intestinal MPO activity.* Trauma-hemorrhage produced a significant increase in intestinal MPO activity in vehicle-treated animals compared with sham controls (Fig. 1). Administration of E2 prevented the trauma-hemorrhage-induced increase in intestinal MPO activity. To determine whether E2 reduces intestinal MPO activity following trauma-hemorrhage via a p38 MAPK-dependent pathway, a group of animals was treated with the p38 MAPK inhibitor SB-203580 along with E2. The results indicated that coadministration of SB-203580 abolished the E2-induced attenuation in intestinal MPO activity. Furthermore, the intestinal MPO activity was not affected further in trauma-hemorrhage rats treated with SB-203580. E2 did not alter intestinal MPO activity in sham-operated rats.

*Intestinal lactate levels.* Intestinal lactate levels were markedly elevated in the vehicle-treated trauma-hemorrhage rats compared with shams and the levels were normalized by administration of E2 (Fig. 2). Coadministration of SB-203580 abolished the E2-induced reduction in intestinal lactate levels following trauma-hemorrhage. No change was observed in the intestinal lactate levels in SB-203580-treated trauma-hemorrhage rats. Moreover, there was no significant differ-
ence in intestinal lactate levels in sham animals treated with vehicle or E2.

**Intestinal p38 MAPK.** Trauma-hemorrhage induced a marked decrease in the phosphorylation of p38 MAPK compared with shams (Fig. 3). E2 treatment following trauma-hemorrhage increased intestinal p38 MAPK phosphorylation to values similar to shams. The increase in p38 MAPK phosphorylation by E2 following trauma-hemorrhage was abolished by coadministration of SB-203580. The phosphorylation of p38 MAPK was not altered in trauma-hemorrhage rats treated with vehicle or E2. No difference in total p38 MAPK protein expression was observed among trauma-hemorrhage and sham-operated rats.

**HO-1 expression in the intestine.** Intestinal HO-1 expression was significantly increased following trauma-hemorrhage in rats compared with shams (Fig. 4). Administration of E2 following trauma-hemorrhage induced a further increase in intestinal HO-1 protein expression compared with vehicle-treated trauma-hemorrhage rats. The increase in HO-1 expression induced by E2 was, however, abolished by coadministration of SB-203580. Administration of SB-203580 alone following trauma-hemorrhage did not change the expression of HO-1. Moreover, there was no difference in HO-1 expression in sham animals treated with vehicle or E2.
Intestinal TNF-α and IL-6 levels. As shown in Fig. 5, trauma-hemorrhage significantly increased TNF-α and IL-6 levels in the intestine. Administration of E2 prevented the trauma-hemorrhage-induced increase in these cytokines, which was abolished by coadministration of SB-203580. In contrast, SB-203580 in the absence of E2 did not alter intestinal TNF-α and IL-6 levels in trauma-hemorrhage rats. Furthermore, no change was observed in intestinal TNF-α and IL-6 levels in vehicle- or E2-treated sham animals.

Intestinal ICAM-1, CINC-1, and MIP-2 levels. Trauma-hemorrhage markedly increased intestinal ICAM-1 expression, which was normalized by treatment with E2 (Fig. 6). Coadministration of SB-203580 abolished the E2-induced reduction in ICAM-1 expression following trauma-hemorrhage. In addition, intestinal CINC-1 and MIP-2 levels were significantly increased in vehicle-treated rats following trauma-hemorrhage (Fig. 7). E2 administration prevented the trauma-hemorrhage-mediated increase in intestinal CINC-1 and MIP-2 levels, which was abolished by coadministration of SB-203580. In contrast, the above parameters were not affected in SB-203580-treated trauma-hemorrhage rats. In sham-operated rats, E2 did not alter intestinal ICAM-1, CINC-1, and MIP-2 levels (Figs. 6 and 7).

DISCUSSION

Our results indicate that at 2 h following trauma-hemorrhage, intestinal MPO activity, lactate, TNF-α, IL-6, ICAM-1, CINC-1, and MIP-2 levels are significantly increased. Administration of E2 following trauma-hemorrhage normalized the trauma-hemorrhage-induced increase in the above parameters. Intestinal p38 MAPK activation was significantly decreased following trauma-hemorrhage and was normalized by E2 administration following trauma-hemorrhage. Intestinal HO-1 expression was increased by trauma-hemorrhage compared with shams. E2 induced a further increase in HO-1 expression compared with vehicle-treated rats following trauma-hemorrhage. Coadministration of p38 MAPK inhibitor SB-203580 following trauma-hemorrhage abolished the E2-mediated ef-
ffects. These results collectively suggest that the salutary effects of E2 on the intestine following trauma-hemorrhage are in part mediated via p38 MAPK-dependent HO-1 upregulation.

A number of studies have shown that p38 MAPK activation contributes to the protection of cell/tissue responses to a variety of stimuli (2, 10, 26, 31). For instance, in human monocytes the increase in p38 MAPK activation is associated with the alcohol-induced attenuation of TNF-α production and augmentation of IL-10 secretion (10). The inflammatory response to hyperoxia-induced lung injury in epithelial cells is increased after administration of SB-203580 (28). p38 MAPK phosphorylation is also reported to be cardioprotective following ischemic preconditioning or trauma-hemorrhage (15, 25). Furthermore, studies have indicated that p38 MAPK activation positively regulates mucosal recovery in ischemic-injured porcine ileum (31) and protects glomerular epithelial cells against complement-mediated cell injury (2). In accordance with these findings, our results suggest that the E2-mediated attenuation of intestinal injury following trauma-hemorrhage is via intestinal p38 MAPK phosphorylation.

Upregulation of HO-1 plays a central role in the protection of cells/tissue against various pathophysiological conditions such as hemorrhagic shock, ischemia, oxidative stress, and endotoxemia (4, 34, 38). Multiple mechanisms are involved in the protection of HO-1 from pathophysiological conditions. For example, carbon monoxide, one of the main byproducts of the catabolism of heme by HO-1, activates soluble guanylate cyclase and induces vasodilatation via cGMP (23). Studies have also shown that HO-1-mediated tissue protection may be due to the protection of E2 on the intestine following trauma-hemorrhage (3). Thus, our previous results also have suggested that administration of E2 following trauma-hemorrhage increased intestinal p38 MAPK phosphorylation, upregulated HO-1, and decreased intestinal proinflammatory mediator production, it is possible that E2 also exerts a direct action on the intestine. The present study also indicates that intestinal lactate levels were significantly increased following trauma-hemorrhage. These results further support our previous findings that trauma-hemorrhage lead to a significant reduction in intestinal blood flow (40). Moreover, our results also show that E2 administration normalized the trauma-hemorrhage-induced increased intestinal lactate levels suggesting that E2 treatment improved intestinal perfusion under those conditions. Similar to the present findings, Ba et al. (3) have also found that intestinal perfusion was significantly reduced in males following trauma-hemorrhage, which was restored by E2 treatment. Furthermore, intestinal perfusion was also maintained in proestrus females (which have high E2 levels) following trauma-hemorrhage (3). Thus, even though the effect of E2 on intestinal structure (i.e., histological examination) or intestinal function/integrity (i.e., permeability etc.) were not carried out in this study, it appears that the salutary effects of E2 on intestine are likely due to a decrease in intestinal MPO activity and lactate levels. Nonetheless, additional studies are needed to elucidate the precise mechanism by which E2 attenuated intestinal injury following trauma-hemorrhage.

The present study examined only a single time point, i.e., 2 h after treatment, and thus, it remains unclear whether the salutary effects are sustained for longer periods of time, i.e., 24 h after treatment. In this regard, our previous studies have shown that if the improvement in organ functions by any pharmacological agent were evident early after treatment, then those beneficial effects were sustained for prolonged intervals, and they also improved the survival of animals (7). Furthermore, our previous studies also have suggested that administration of a single dose of E2 immediately after trauma-hemorrhage maintains intestinal integrity at 24 h after trauma-hemorrhage (39). Thus, although a time point other than 2 h was not examined in this study, based on our previous results, it would appear that the salutary effects of E2 would be evident, even if one measured those effects at another time point following trauma-hemorrhage and resuscitation.

It could also be argued that our study did not provide direct evidence of the beneficial effects of HO-1 on the intestine...
following trauma-hemorrhage. In this regard, our previous studies have shown that administration of HO enzyme inhibitor (chromium-mesoporphyrin) abrogated the salutary effects of E\textsubscript{2} or flutamide (a testosterone receptor antagonist) on cardiac function as well as on intestinal injury following trauma-hemorrhage (34, 38). Previous studies have also indicated that the effects of E\textsubscript{2} on HO-1 expression in the heart or liver were decreased/restored the altered immunological functions and improved/restored the altered immunological functions and decreased the mortality of animals following trauma-hemorrhage (28). Treatment of rats with HO enzyme inhibitor or determination of HO-1 activity was therefore not employed in this study to demonstrate that point. An additional issue that can be raised is that we should have administered SB-203580 alone in sham animals to determine whether that per se has any adverse effects. In this regard, since our previous study has shown that administration of SB-203580 alone did not influence cardiac function or p38 MAPK phosphorylation in sham-operated rats (16), administration of SB-203580 alone was therefore not carried out in this study.

**Perspectives and Significance**

Our previous results have shown that administration of E\textsubscript{2} following trauma-hemorrhage improved/restored cardiac output, myocardial contractility, and hepatic and pulmonary function under those conditions. Furthermore, our studies have shown that E\textsubscript{2} administration after trauma-hemorrhage also improved/restored the altered immunological functions and decreased the mortality of animals following trauma-hemorrhage and induction of subsequent sepsis. The present results indicate that E\textsubscript{2} administration after trauma-hemorrhage also attenuated intestinal injury and downregulated proinflammatory mediators. Our present studies also suggest that the salutary effects of E\textsubscript{2} are mediated in part through p38 MAPK-dependent HO-1 upregulation. Support for this suggestion comes from the findings that administration of p38 MAPK inhibitor following trauma-hemorrhage abolished the salutary effects of E\textsubscript{2} on the intestine. These findings have major implications for the potential utility of E\textsubscript{2} as a clinical adjunct to hemorrhagic shock. However, since E\textsubscript{2} can mediate its effects in multiple ways, we do not propose the activation of p38 MAPK is the exclusive action of E\textsubscript{2} following trauma-hemorrhage. Since E\textsubscript{2} produces various beneficial effects by multiple pathways, it is important to further characterize the molecular mechanism by which this hormone improves and maintains various cell and organ functions following low flow conditions. We hope in our future studies to pinpoint the precise mechanism by which E\textsubscript{2} produces the above-mentioned salutary effects following low flow conditions. A better understanding of the relationship between E\textsubscript{2} and other signaling pathways should enable us to develop new therapeutic modalities for the treatment of hemorrhagic shock.

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