Role of p38 MAPK pathway in 17β-estradiol-mediated attenuation of hemorrhagic shock-induced hepatic injury

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Submitted 30 May 2014; accepted in final form 8 November 2014

Hsu J-T, Chen T-H, Chiang K-C, Kuo C-J, Lin C-J, Yeh T-S. Role of p38 MAPK pathway in 17β-estradiol-mediated attenuation of hemorrhagic shock-induced hepatic injury. J Appl Physiol 118: 187–192, 2015. First published November 13, 2014; doi:10.1152/japplphysiol.00464.2014.—Although 17β-estradiol (E2) treatment following hemorrhagic shock or ischemic reperfusion prevents organs from dysfunction and injury, the precise mechanism remains unknown. We hypothesize that the E2-mediated attenuation of liver injury following hemorrhagic shock and fluid resuscitation occurs via the p38 mitogen-activated protein kinase (MAPK)-dependent heme oxygenase (HO)-1 pathway. To test this, male rats were treated with E2 alone and in combination with the p38 MAPK inhibitor SB-203580 (2 mg/kg) or estrogen receptor antagonist ICI 182,780 (3 mg/kg). At 2 h after hemorrhagic shock and fluid resuscitation, the liver injury markers were significantly increased compared with sham-operated control. Hemorrhagic shock resulted in a significant decrease in p38 MAPK phosphorylation compared with the shams. Administration of E2 following hemorrhagic shock normalized liver p38 MAPK phosphorylation, further increased HO-1 expression, and reduced cleaved caspase-3 levels. Co-administration of SB-203580 abolished the E2-mediated attenuation of the shock-induced liver injury markers. In addition, administration of chromium mesoporphyrin-IX chloride or ICI 182,780 abolished E2-mediated increases in liver HO-1 expression or p38 MAPK activation following hemorrhagic shock. Our results collectively suggest that the salutary effects of E2 on hepatic injury following hemorrhagic shock and resuscitation are in part mediated through an estrogen-receptor-related p38 MAPK-dependent HO-1 upregulation.

DESPITE NUMEROUS ADVANCES in intensive care medicine, profound shock, sepsis and severe damage leading to multiple organ failure remain the major causes of death in the injured host (25, 28, 29, 35). The liver has been shown to play an important role in the development of delayed organ dysfunction following traumatic damage and massive blood loss (18). The p38 mitogen-activated protein kinase (MAPK) has been reported to regulate inflammatory response in different cell types by various stimuli (22, 26). 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 mitogen-activated protein kinase kinase 3, an upstream kinase of p38 MAPK (20).

It has been shown that p38 MAPK activation leads to the induction of heme oxygenase (HO)-1 in the heart, intestine, and liver tissues (10, 19, 27). HO-1 confers protection against oxidative stress in vivo and in vitro, through antioxidative, antiapoptotic, and anti-inflammatory actions (1, 16, 30). Exogenous administration of HO-1 by gene transfer into rat’s lung prevents hyperoxia-induced lung injury through the modulation of neutrophil inflammation and lung apoptosis (21). Furthermore, overexpression of HO-1 attenuates the expression of adhesion molecules and reduces subsequent leukocyte-endothelial cell interactions and organ damage (24, 33).

A growing body of evidence indicates that hormonal mechanisms are involved in regulating posttraumatic organ injury (5, 6, 13, 32). Clinical and experimental studies suggest that females tolerate injury better than males (4). In this regard, we have found that hepatic injury is significant in male animals and ovariectomized females but not in proestrus females, a state with the highest plasma levels of E2 following hemorrhagic shock and fluid resuscitation (9, 15, 31). Studies also show that resistance of proestrus female rats to gut injury and gut-induced distant organ injury is greater than that observed in male rats (3). In this study, we hypothesize that the E2-mediated attenuation of liver injury following hemorrhagic shock occurs via the p38 MAPK-dependent HO-1 pathway. To test this, male rats were treated with E2 alone and in combination with the p38 MAPK inhibitor SB-203580 following hemorrhagic shock. The effects of these treatments were then examined on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, p38 MAPK, HO-1, cleaved caspase-3, interleukin (IL)-6, tumor necrosis factor (TNF)-α, cytokine-induced neutrophil chemoattractant (CINC)-1, and CINC-3 levels following trauma-hemorrhagic shock.

METHODS

Rat trauma-hemorrhagic shock model. Male (275–325 g) Sprague-Dawley rats were maintained in an animal room with 12:12-h light-dark cycle (lights on from 8:00 AM to 8:00 PM) and an ambient temperature of 22 ± 1°C. Food and water were available ad libitum. The use of experimental animals and procedures used in the study was approved by Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. Animals were fasted for 8 h before the experiment but had free access to water ad libitum. A nonheparinized
model of trauma-hemorrhagic shock was used as described previously (11, 12). At the time of the experiment, the rats were breathing in 100% O₂ and anesthetized by isoflurane inhalation (1.2%) using anesthesia vaporizer (VIP-3000, Paragon Medical, Coral Springs, FL) before the induction of soft-tissue trauma via only 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. Following surgery, the rats were breathing in 100% O₂, and the concentration of isoflurane was adjusted to 1.0% for 15 min with the rat’s arterial blood pressure (BP) returning 120–130 mmHg or heart rate more than 400 beats/min. Rats were then rapidly bled to a mean arterial BP of 40 mmHg within 10 min. The concentrations of isoflurane were adjusted to 0.8 and 0.6% at 10 and 20 min after starting withdrawal of blood, respectively. Hypotension (~90 min from the onset of bleeding) was maintained until the animals could no longer sustain a mean BP of 40 mmHg, unless additional fluid in the form of Ringer’s lactate was administered. Following this, the animals were resuscitated with four times the volume of the shed blood over 60 min with Ringer’s lactate.

Experimental groups. The sham-operated animals, treated with vehicle (cyclohexedrin iv, Sigma Chemical, St. Louis, MO), p38 MAPK inhibitor SB-203580 (2 mg/kg iv, Calbiochem, San Diego, CA), or estrogen receptor antagonist ICI 182,780 (3 mg/kg ip, Tocris Cookson, Ballwin, MO) underwent the groin dissection and femoral artery and vein cannulation, but neither hemorrhage nor resuscitation was carried out (n = 6). Animals subjected to trauma-hemorrhagic shock were administered vehicle, E₂ (1 mg/kg iv, Sigma) alone or E₂ plus SB-203580, HO-1 inhibitor chromium mesoporphyrin-IX chloride (CrMP; 2.5 mg/kg ip, Frontier Scientific, Logan, UT), or ICI 182,780 at the beginning of the resuscitation (n = 6 in each group). Following resuscitation, the catheters were removed, the vessels were ligated, and the skin incisions were closed with sutures. The animals were killed at 2 h after the end of resuscitation or the sham operation. The liver tissues were harvested and stored at −70°C in a freezer. A blood sample was analyzed to determine the levels of serum markers, including ALT and AST by a multianalyzer (T600-210 Automatic Analyzer, Hitachi, Japan).

Measurement of MPO activity. Equal weights (100 mg wet wt) of liver 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 centrifuging at 17,000 g at 4°C for 10 min, and the supernatants were stored at −7°C. Protein content in the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). The samples were incubated with a substrate 3-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 μl of 50 mM phosphate buffer, 3 μl substrate solution (containing 20 mg/ml 3-dianisidine hydrochloride), and 3 μl H₂O₂ (20 mM). A sample (10 μl) was added to each well to start the reaction. Standard MPO (Sigma) was used in parallel to determine the MPO activity in the sample. The reaction was stopped by adding 3 μl sodium azide (30%). Light absorbance at 460 nm was read. MPO activity was determined by using the curve obtained from the standard MPO.

Western blot analysis. Approximately 0.1 g of freshly collected liver 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). Tissue lysates were centrifuged at 17,000 g for 20 min at 4°C, and an aliquot of the supernatant was used to determine the protein concentration (Bio-Rad DC Protein Assay). The lysates (40 μg per lane) were then mixed with 4× sodium dodecyl sulfate (SDS) sample buffer and were electrophoresed on the 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred electro-

**Determination of cytokines and chemokines levels.** IL-6, TNF-α, CINC-1, and CINC-3 levels in liver tissues were determined using the enzyme-linked immunosorbent assay kits (R&D, Minneapolis, MN), according to the manufacturer’s instructions. Briefly, the samples (50 mg) 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). Tissue lysates were centrifuged at 17,000 g for 20 min at 4°C, and an aliquot of the supernatant was used to determine the protein concentration (Bio-Rad DC Protein Assay). The lysates (40 μg per lane) were then mixed with 4× sodium dodecyl sulfate (SDS) sample buffer and were electrophoresed on the 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred electro-

**Fig. 1.** Serum asparate aminotransferase (AST; A) and alanine aminotransferase (ALT; B) levels at 2 h after sham and trauma-hemorrhagic shock (TH). Animals were treated with vehicle, 17β-estradiol (E₂), SB-203580 (SB), or ICI 182,780 (ICI) alone, or E₂ plus SB, chromium mesoporphyrin-IX chloride (CrMP), or ICI. Values are shown as means ± SE of 6 animals in each group. *P < 0.05 vs. other groups; #P < 0.05 vs. sham.

*J Appl Physiol* • doi:10.1152/japplphysiol.00464.2014 • www.jappl.org
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 (50 μl) was assayed for IL-6, TNF-α, CINC-1, and CINC-3 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC Protein assay). The concentration of these proinflammatory mediators is expressed in picograms per milligram protein in each sample.

**Statistical analysis.** All data are expressed as means ± SE. Comparison between the groups were performed using one-way ANOVA and Tukey’s test, and differences were considered significant at P < 0.05.

**RESULTS**

**Alterations of liver injury markers.** Serum AST and ALT levels were significantly higher in the hemorrhagic shock rats compared with the sham-operated rats treated with vehicle, SB-203580, or ICI 182,780 (Fig. 1). E2 treatment attenuated the shock-induced increase in AST and ALT levels. Administration of SB-203580, CrMP, or ICI 182,780 following hemorrhagic shock abolished E2-induced decreases in AST and ALT levels. The ALT levels in the E2-treated hemorrhagic shock rats were higher than in the shams.

**Liver tissue MPO activity.** Liver tissue MPO activity was increased in the vehicle-treated hemorrhagic shock rats compared with the shams (Fig. 2). The increase in the liver tissue MPO activity following hemorrhagic shock was normalized by E2. Coadministration of SB-203580, CrMP, or ICI 182,780 following hemorrhagic shock abolished the E2-mediated decrease in the MPO activity following hemorrhagic shock.

**Activation of liver p38 MAPK.** Hemorrhagic shock resulted in a significant decrease in the p38 MAPK activation compared with the shams. Administration of E2 normalized the hemorrhagic shock-induced reduction in the p38 MAPK activation, which was blocked by the coadministration of SB-203580 or ICI 182,780, but not altered by the coadministration of CrMP. There was no change in the total p38 MAPK protein expression among the sham and hemorrhagic shock rats (Fig. 3).

**Liver HO-1 protein levels.** Liver HO-1 protein levels lead to marked increases following hemorrhagic shock rats compared with shams. Administration of E2 following hemorrhagic shock further increased the liver HO-1 protein expression compared with the hemorrhagic shock rats treated with vehicle, which was inhibited by the coadministration of SB-203580, CrMP, or ICI 182,780 (Fig. 4).
Administration of SB-203580 abolished the E2-mediated increase in p38 MAPK phosphorylation and rises in HO-1 protein levels following hemorrhagic shock. Furthermore, administration of CrMP or ICI 182,780 also abolished E2-mediated increases in liver HO-1 protein expression or p38 MAPK activation following hemorrhagic shock. These findings collectively suggest that E2 prevents hemorrhagic shock-induced liver injury through an estrogen-receptor related p38 MAPK-dependent HO-1 upregulation.

HO-1, a rate-limiting enzyme in the catabolism of heme, is among the most critical protective mechanisms activated during the deleterious, pathological effects of the low-flow states, and it is thought to act as a protective agent in many organs against injury (8, 21, 34). Upregulation of HO-1 reduces neutrophil infiltration and attenuates organ damage (19, 34). Similar to the findings of the other investigators, our results indicate that the administration of E2 following hemorrhagic shock resulted in a further increase in the liver HO-1 expression. However, overexpression of HO-1 appears to be p38 MAPK independent in this setting, as hemorrhagic shock led to a marked decrease in p38 MAPK phosphorylation. Treatment of E2 following hemorrhagic shock induced a further increase in liver HO-1 levels. In this situation, liver HO-1 overexpression would seem to occur via the p38 MAPK pathway, as the administration of the p38 MAPK inhibitor SB-203580 abolished the E2-induced increase in the HO-1 level following hemorrhagic shock. Our findings collectively suggest that the salutary effects of E2 on liver injury are mediated via the p38 MAPK-dependent HO-1 pathway.

Activation of p38 MAPK pathway by E2 has been observed in different cells/tissues (7, 10, 26). For example, our laboratory’s previous studies have shown that E2 induced the activation of p38 MAPK in the heart and intestine (7, 10). Studies have also shown that E2 normalized the hemorrhagic shock-induced decrease in p38 MAPK activation in the splenic macrophages (26). Furthermore, the action of E2 on p38 MAPK phosphorylation is observed to be mediated by estrogen receptor-dependent mechanisms (17, 26). For example, p38 MAPK activation is upregulated in hemorrhagic shock and fluid resuscitation is organ or cell specific (26, 33). For example, p38 MAPK activation is upregulated in Kupffer cells, whereas it is downregulated in splenic macrophages (26). This was accompanied with an increase in inflammatory mediators’ production in Kupffer cells and a decrease in p38 MAPK phosphorylation.

**DISCUSSION**

Our present results indicate that liver injury markers and inflammatory cytokines and chemokines were significantly increased following hemorrhagic shock and resuscitation. Administration of E2 after hemorrhagic shock markedly improved these parameters and normalized liver p38 MAPK phosphorylation. Although hemorrhagic shock per se also led to an increased HO-1 protein expression compared with sham controls, these effects were further enhanced by the addition of E2. Administration of SB-203580 abolished the E2-mediated increase in p38 MAPK phosphorylation and rises in HO-1 protein levels following hemorrhagic shock. Furthermore, administration of CrMP or ICI 182,780 also abolished E2-mediated increases in liver HO-1 protein expression or p38 MAPK activation following hemorrhagic shock. These findings collectively suggest that E2 prevents hemorrhagic shock-induced liver injury through an estrogen-receptor related p38 MAPK-dependent HO-1 upregulation.

**Liver-cleaved caspase-3 levels.** Liver-cleaved caspase-3 levels were markedly increased in rats following hemorrhagic shock compared with shams. Administration of E2 prevented the hemorrhagic shock-induced increase in cleaved caspase-3, which was abolished by the coadministration of SB-203580, CrMP, or ICI 182,780 (Fig. 5). Liver IL-6, TNF-α, CINC-1, and CINC-3 levels. As shown in Table 1, levels of IL-6, TNF-α, CINC-1, and CINC-3 in the liver were significantly elevated following hemorrhagic shock compared with the shams. Administration of E2 attenuated the hemorrhagic shock-induced increase in the liver levels of IL-6, TNF-α, CINC-1, and CINC-3, which were reversed by the coadministration of SB-203580, CrMP, or ICI 182,780.

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**Table 1. Levels of liver inflammatory mediators in each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-6, pg/mg protein</th>
<th>TNF-α, pg/mg protein</th>
<th>CINC-1, pg/mg protein</th>
<th>CINC-3, pg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>55.50 ± 3.34</td>
<td>53.50 ± 2.83</td>
<td>116.00 ± 4.68</td>
<td>54.17 ± 2.93</td>
</tr>
<tr>
<td>TH + vehicle</td>
<td>127.17 ± 9.07*</td>
<td>123.83 ± 10.92*</td>
<td>195.50 ± 9.16*</td>
<td>98.50 ± 4.71*</td>
</tr>
<tr>
<td>TH + E2</td>
<td>56.33 ± 2.50</td>
<td>53.00 ± 2.38</td>
<td>113.67 ± 6.66</td>
<td>54.50 ± 3.54</td>
</tr>
<tr>
<td>TH + E2 + SB</td>
<td>121.67 ± 10.52*</td>
<td>116.67 ± 8.12*</td>
<td>204.67 ± 4.67*</td>
<td>97.17 ± 4.53*</td>
</tr>
<tr>
<td>TH + E2 + CrMP</td>
<td>129.17 ± 9.00*</td>
<td>109.17 ± 11.20*</td>
<td>208.67 ± 8.13*</td>
<td>102.00 ± 6.46*</td>
</tr>
<tr>
<td>TH + E2 + ICI</td>
<td>125.33 ± 10.25*</td>
<td>119.83 ± 9.77*</td>
<td>203.33 ± 9.29*</td>
<td>98.17 ± 4.58*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 animals in each group. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; CINC, cytokine-induced neutrophil chemoattractant. Animals subjected to trauma-hemorrhagic shock (TH) were treated with vehicle, 17β-estradiol (E2) alone, E2 plus SB-203580 (SB), E2 plus chromium mesoporphyrin-IX chloride (CrMP), or E2 plus ICI 182,780 (ICI). *P < 0.05 vs. sham or TH + E2.
in these cytokines in splenic macrophages. E2 administration normalized p38 MAPK phosphorylation and cytokine production by Kupffer cells and splenic macrophages in rats following hemorrhagic shock. Thus these findings suggest that E2 has a unique property of normalized p38 MAPK activation whether it is upregulated or downregulated following hemorrhagic shock. Therefore, E2 treatment will not result in a detrimental inflammatory reaction in rats following hemorrhagic shock.

Our laboratory’s previous studies have shown that administration of E2 prevented hemorrhagic shock-induced cardiac dysfunction and attenuated plasma inflammatory mediators’ levels (7, 8, 14). Therefore, the salutary effects of E2 on the liver following hemorrhagic shock are a result of the systemically improved cardiac function by E2. Nonetheless, since E2 treatment following hemorrhagic shock increased liver p38 MAPK activation, further upregulated HO-1 expression, and attenuated liver inflammatory mediators’ production, it is possible that E2 also exerts a direct action on the liver. Additional studies are necessary to elucidate the precise mechanism by which E2 prevented the hemorrhagic shock-induced liver injury.

The present study examined only a single time point, i.e., 2 h after treatment, and thus it remains unknown whether the beneficial 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 (2). Furthermore, previous studies also have suggested that administration of a single dose of E2 immediately following hemorrhagic shock prevented organ injury at 24 h after trauma-hemorrhage (33). Thus, although a time point other than 2 h was not examined in this study, based on our previous findings, it would appear that the salutary effects of E2 would be evident, even if one measured those effects at another time point following hemorrhagic shock and fluid resuscitation.

It can be argued that we should have administered E2, SB-203580, or ICI 182,780 alone in sham or hemorrhagic shock animals to determine if either of those per se has any adverse effects. In this regard, previous study has shown that administration of E2 alone in sham-operated animals and SB-203580 or ICI 182,780 alone in hemorrhagic shock animals did not affect the levels of liver tissue proinflammatory mediators, liver enzymes, or liver injury markers (9, 19, 23). Administration of E2 or SB-203580 alone in the shams and SB-203580 or ICI 182,780 alone in hemorrhagic shock animal was, therefore, not carried out in this study. In addition, since there were no differences in the liver injury markers (AST and ALT) among the shams treated with vehicle, SB-203580, or ICI 182,780 in the present study, we did not further investigate the other parameters and signaling pathway.

In summary, our results have indicated that administration of E2 following hemorrhagic shock and resuscitation upregulated p38 MAPK-dependent HO-1 expression and attenuated liver injury. Blockade of p38 MAPK activation or estrogen receptor and the associated deterioration of the examined parameters suggest that reduction of neutrophil infiltration in the liver is, in part, mediated through an estrogen-receptor related, p38 MAPK-dependent HO-1 pathway. Although the precise mechanism of the salutary effects of E2 on the liver and the contribution of p38 MAPK/HO-1 in minimizing liver damage following hemorrhagic shock remain unclear, our study provides evidence that p38 MAPK-dependent upregulation of HO-1 may be critical in reducing hemorrhagic shock-induced liver injury.

ACKNOWLEDGMENTS

We thank Shu-Fang Huang for assistance in the preparation of the figures.

GRANTS

This investigation was supported by the grant of Chang Gung Medical Research Program (CMRPG3A0171 and CMRPG3A0172) and Ministry of Science and Technology (MOST 103-2314-B-182-047).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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Estrogen Attenuates Shock-Induced Liver Injury • Hsu J et al.


J Appl Physiol • doi:10.1152/japplphysiol.00464.2014 • www.jappl.org