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

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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. After a 5-cm midline laparatomy, male rats underwent hemorrhagic shock (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) alone, or E2 plus p38 MAPK inhibitor SB-203580 (2 mg/kg), HO-1 inhibitor chromium mesoporphyrin-IX chloride (2.5 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. Coadministration 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.

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) levels and liver tissue myeloperoxidase (MPO) activity, p38 MAPK, HO-1, and caspase-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

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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 −70°C in a freezer. 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 o-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 o-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-
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).

![Fig. 2. Liver tissue myeloperoxidase (MPO) activity at 2 h after sham or TH. Animals were treated with vehicle, E2 alone, or E2 plus SB, CrMP, or ICI. Values are means ± SE of 6 animals in each group. *$P < 0.05$ vs. other groups.](image)

![Fig. 3. Expression of total and phosphorylated (activated) p38 MAPK (p-p38) in the liver at 2 h after sham and TH. Animals were treated with vehicle, E2 alone, or E2 plus SB or ICI. Blots obtained from several experiments were analyzed using densitometry. The densitometric values were pooled from animals in each group and are shown as means ± SE of 6 animals in each group. *$P < 0.05$ vs. other groups.](image)

![Fig. 4. Liver heme oxygenase (HO)-1 protein expression at 2 h after sham or TH. Animals were treated with vehicle, E2 alone, or E2 plus SB, CrMP, or ICI. Blots obtained from several experiments were analyzed using densitometry. The densitometric values were pooled from animals in each group and are shown as means ± SE of 6 animals in each group. *$P < 0.05$ vs. other groups.](image)
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 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, 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 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). In this study, we found that E2-induced attenuation of liver injury following hemorrhagic shock is partly via increases in p38 MAPK activation, which were blocked by estrogen receptor antagonist ICI 182,780. These findings thus suggest that E2 binds to its receptor and in turn mediates the activation of p38 MAPK.

It should be noted that the inflammatory response to 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

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. E\textsubscript{2} administration
normalized p38 MAPK phosphorylation and cytokine production
by Kupffer cells and splenic macrophages in rats following hemorrhagic shock. Thus these findings suggest that E\textsubscript{2} has a
unique property of normalized p38 MAPK activation whether it
is upregulated or downregulated following hemorrhagic shock.
Therefore, E\textsubscript{2} treatment will not result in a detrimental
inflammatory reaction in rats following hemorrhagic shock.

Our laboratory’s previous studies have shown that administra-
tion of E\textsubscript{2} prevented hemorrhagic shock-induced cardiac
dysfunction and attenuated plasma inflammatory mediators’
levels (7, 8, 14). Therefore, the salutary effects of E\textsubscript{2} on the
liver following hemorrhagic are a result of the systemically
improved cardiac function by E\textsubscript{2}. Nonetheless, since E\textsubscript{2} treat-
ment following hemorrhagic shock increased liver p38 MAPK
activation, further upregulated HO-1 expression, and attenu-
ated liver inflammatory mediators’ production, it is possible
that E\textsubscript{2} also exerts a direct action on the liver. Additional
studies are necessary to elucidate the precise mechanism by
which E\textsubscript{2} 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 E\textsubscript{2} 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 E\textsubscript{2} 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 E\textsubscript{2},
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 E\textsubscript{2} 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 E\textsubscript{2} or SB-203580 alone in the shams and SB-203580 or
ICI 182,780 alone in hemorrhagic shock animals 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
E\textsubscript{2} 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 mecha-
nism of the salutary effects of E\textsubscript{2} on the liver and the
contribution of p38 MAPK/HO-1 in minimizing liver damage
following hemorrhagic shock remain unclear, our study pro-
vides evidence that p38 MAPK-dependent upregulation of
HO-1 may be critical in reducing hemorrhagic shock-induced
liver injury.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.-T.H., T.-H.C., K.-C.C., and T.-S.Y. conception and
design of research; J.-T.H. performed experiments; J.-T.H., T.-H.C., K.-C.C.,
C.-J.K., C.-J.L., and T.-S.Y. analyzed data; J.-T.H., T.-H.C., K.-C.C., C.-J.K.,
C.-J.L., and T.-S.Y. interpreted results of experiments; J.-T.H. prepared figures;
of manuscript.

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