Estrogen prevents intestinal inflammation after trauma-hemorrhage via downregulation of angiotensin II and angiotensin II subtype I receptor

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Chen J, Yang S, Hu S, Choudhry MA, Bland KI, Chaudry IH. Estrogen prevents intestinal inflammation after trauma-hemorrhage via downregulation of angiotensin II and angiotensin II subtype I receptor. Am J Physiol Gastrointest Liver Physiol 295: G1131–G1137, 2008.—Although angiotensin II (Ang II) plays a key role in development of organ ischemia-reperfusion injury, it remains unclear whether it is involved in development of intestinal injury following trauma-hemorrhage (T-H). Studies have shown that 17β-estradiol (E2) administration following T-H improves small intestinal blood flow; however, it is unclear whether Ang II plays a role in this E2-mediated salutary effect. Male Sprague-Dawley rats underwent laparotomy and hemorrhagic shock (removal of 60% total blood volume, fluid resuscitation after 90 min). At onset of resuscitation, rats were treated with vehicle, E2, or E2 and estrogen receptor antagonist ICI 182,780 (ICI). A separate group of rats was treated with Ang II subtype I receptor (AT1R) antagonist losartan. At 24 h after T-H, plasma Ang II, IL-6, TNF-α, intercellular adhesion molecule (ICAM)-1, cytokine-induced neutrophil chemotactant (CINC)-1 and CINC-3 levels, myeloperoxidase (MPO) activity, and AT1R expression were determined. T-H significantly increased plasma and intestinal Ang II, IL-6, TNF-α levels, intestinal ICAM-1, CINC-1, CINC-3 levels, MPO activity, and AT1R protein compared with shams. E2 treatment following T-H attenuated increased intestinal MPO activity, Ang II level, and AT1R protein expression. ICI administration abolished the salutary effects of E2. In contrast, losartan administration attenuated increased MPO activity without affecting Ang II and AT1R levels, Thus Ang II plays a role in producing small intestine inflammation following T-H, and the salutary effects of E2 on intestinal inflammation are mediated in part by Ang II and AT1R downregulation.

The small intestine is one of the most susceptible organs to injury induced by trauma-hemorrhagic shock. Previous studies have shown that intestinal injury occurs during hemorrhagic shock and persists despite fluid resuscitation (15). Impaired intestinal perfusion during resuscitation results in persistent mucosal hypoxia and subsequent loss of mucosal integrity. This loss of gut mucosal integrity has been implicated in the pathogenesis of multiple organ dysfunction syndrome (32). A number of studies have demonstrated that the enhanced secretion of proinflammatory cytokines by mast cells, dendritic cells, and macrophages is an important factor in the initiation and perpetuation of intestinal inflammation (8). These proinflammatory cytokines recruit other immune cells including neutrophils, thereby increasing leukocyte trafficking and intestinal permeability (18, 30, 38, 41).

Previous studies have shown that 17β-estradiol (E2) administration following trauma-hemorrhage decreases plasma and intestinal endothelin (ET)-1 levels and improves small intestine blood perfusion under those conditions (2). E2 also reduces neutrophil accumulation in the gut via estrogen receptor (ER)-mediated process. In addition to reduction of neutrophil accumulation, E2 also upregulates heme oxygenase-1 expression and protects the organs against dysfunction and injury (36) in males following trauma-hemorrhage.

Angiotensin II (Ang II) is an important vasoconstrictor during hypovolemia and may contribute to shock-induced hypoxic/ischemic organ damage (27). Ang II also stimulates a wide variety of proinflammatory responses including increased leukocyte rolling and adhesion, production of oxidative stress, and induction of cysteine-x-cysteine chemokine expression (3, 28, 47). It has been shown that the Ang II subtype-I receptor (AT1R) antagonist significantly inhibits the intestinal mucosal injury induced by ischemia-reperfusion in rats (40). The AT1R mediates many biological effects of the renin-angiotensin system (RAS), such as vasoconstriction, water and sodium retention, free radical release, and cell growth (9). Furthermore, AT1R antagonist prevents irreversible tissue injury and improves outcome from stroke in animal experiments (35). Recent studies have shown that E2 deficiency caused AT1R overexpression in vivo, leading to enhanced biological effects of the RAS. A recent in vitro study has shown that E2 downregulates AT1R mRNA expression and AT1R protein in vascular smooth muscle cells isolated from ovariectomized rats (21). Furthermore, E2 has been shown to inhibit Ang II-induced cell proliferation, ET-1 gene expression, reactive oxygen species (ROS) generation, extracellular signal-regulated kinase phosphorylation, and activator protein-1-mediated reporter activity in vascular smooth muscle cells (13).

Our previous studies have shown that treatment of animals with E2 following trauma-hemorrhage prevents intestinal tissue damage under those conditions (45). Nonetheless, it is unclear whether E2 protects against intestinal injury following trauma-hemorrhage via regulation of Ang II. Accordingly, we tested the hypothesis that the E2 modulates Ang II in the small intestine following trauma-hemorrhage and thus produces its salutary effects under those conditions.
MATERIALS AND METHODS

Animals. Male adult (250–300 g) Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in this study. Rats were allowed to acclimatize in the animal facility for 1 wk before the experiments. All experiments were performed in adherence with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Experimental procedures. A nonheparinized rat trauma-hemorrhagic shock model was used in this study. Rats were fasted overnight before the experiment but allowed water ad libitum. Trauma-hemorrhage was induced as described previously (43). Briefly, the rats were anesthetized using 1.5% isoflurane (Aittane; Minrad, Bethlehem, PA) inhalation and underwent a 5-cm midline laparotomy to induce soft-tissue trauma before the onset of hemorrhage. The abdomen was then closed in layers and catheters (polyethylene, PE-50 tubing; Becton-Dickinson, Franklin Lakes, NJ) were placed in both femoral arteries and the right femoral vein. The wounds were bathed with 1% lidocaine (Elkkins-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 rats 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 that MAP until 40% of the shed blood volume was returned in the form of Ringer’s lactate and were then resuscitated with four times the volume of shed blood with Ringer’s lactate over 60 min. Following resuscitation, the catheters were removed, the vessels were ligated, and skin incisions closed with sutures. The sham-operated animals underwent the same surgical procedure but were neither bled nor resuscitated. The animals were returned to their cages and allowed food and water ad libitum and euthanized at 24 h after resuscitation.

Chemicals and antibodies. β-cyclodextrin and water-soluble E2 were purchased from Sigma Chemical (St. Louis, MO); ICI 182,780 (ICI) was purchased from Tocris Bioscience (New England, Ellsiville, MO). Losartan was provided by Merck (Rathway, NJ) as a gift. All of the prepared agents were stored at −80°C until use. Anti-rAT1R polyclonal and anti-rAT1R GAPDH monoclonal antibodies were purchased from AbCam (Cambridge, MA).

Experimental groups. Animals were randomly divided into eight groups: 1) sham + vehicle; 2) sham + losartan; 3) sham + E2; 4) sham + ICI + E2; 5) trauma-hemorrhage + vehicle; 6) trauma-hemorrhage + losartan; 7) trauma-hemorrhage + E2; and 8) trauma-hemorrhage + ICI + E2. Vehicle cyclodextrin (20 mg/kg) or E2 (1 mg/kg) was administered intravenously at the time of sham operation or at the beginning of resuscitation. The ER antagonist ICI (3 mg/kg) was given intraperitoneally at the time of sham operation or at the beginning of resuscitation.

Preparation of blood samples. The animals were anesthetized with isoflurane 24 h after sham operation or resuscitation in the trauma-hemorrhage groups, and blood was obtained via cardiac puncture using a syringe coated with heparin (Abraxis Pharmaceutical Products, Winooski, VT) according to the manufacturer’s instructions (45). In brief, ~0.2 g of small intestine tissue was suspended in 1 ml lysis buffer [0.5% hexadecyltrimethyl-ammonium bromide (HETAB) in 50 mM PBS, pH 6.0] and homogenized by sonication on ice for 30 s, twice, then centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was transferred into new tubes and aliquoted. The protein concentration of supernatant was measured by Power Wave (Bio-Tek, Winooski, VT). For standard, 5 U of MPO standard (Sigma-Aldrich) were dissolved in 1 ml of cold double-distilled H2O2 (20 mg O-dianisidide dihydrochloride in 1 ml water) diluted to 2.5 U. 1.25 U, 0.625 U, 0.3125 U, and 0 U. Then, 290 µl of 50 mM PBS, 3 µl of O-dianisidide dihydrochloride solution, and 3 µl of 20 mM H2O2 were added to each well; next, either 10 µl of standard or sample was added to each well. After 5 min, the reaction was stopped by the addition of 3 µl of 30% sodium azide. Light absorbance at 460 nm was read by Power Wave. MPO activity was determined by using the curve obtained from the standard MPO. All solutions including 0.5% HETAB, 20 mM H2O2, and O-dianisidide dihydrochloride were freshly prepared.

Statistical analysis. Data are presented as means ± SE (n = 4–6 rats/group). Statistical differences among groups were determined by one-way ANOVA followed by Tukey’s test. The differences were considered significant if the P value was < 0.05.

RESULTS

Ang II levels in plasma and small intestine. As shown in Fig. 1, the level of Ang II in plasma and small intestine
increased significantly ($P < 0.05$) at 24 h following trauma-hemorrhage. Treatment of animals with E2 normalized Ang II levels in plasma and small intestine to sham levels. The salutary effect of E2 on Ang II levels in plasma and small intestine was abolished by ER antagonist ICI. In contrast, administration of AT1R antagonist losartan following trauma-hemorrhage did not produce any effect on Ang II levels in plasma or small intestine.

Alteration of AT1R expression in small intestine following trauma-hemorrhage. AT1R protein levels in small intestine increased significantly following trauma-hemorrhage (Fig. 2, $P < 0.05$). Treatment of animals with losartan did not influence intestinal AT1R expression following trauma-hemorrhage. In contrast, administration of E2 significantly decreased intestinal AT1R expression in trauma-hemorrhage animals compared with the vehicle-treated trauma-hemorrhage group ($P < 0.05$). The salutary effect of E2 on AT1R expression following trauma-hemorrhage was abolished by coadministration of ICI.

Cytokine levels in plasma and small intestine. Trauma-hemorrhage led to a significant increase in systemic and small intestine IL-6 (Fig. 3) and TNF levels (Fig. 4) compared with shams. Treatment of animals with losartan lowered IL-6 and TNF levels following trauma-hemorrhage, but these levels remained significantly higher than shams (Figs. 3 and 4; $P < 0.05$). In contrast, administration of E2 following trauma-hemorrhage normalized the increase in systemic and small intestine IL-6 and TNF levels, which was abolished by coadministration of ICI (Figs. 3 and 4).

Intestinal CINC-1, CINC-3, and ICAM-1 levels. Trauma-hemorrhage significantly increased intestinal CINC-1, CINC-3, and ICAM-1 levels ($P < 0.05$ compared with shams). Treatment of the animals with losartan normalized intestinal...
CINC-1 and CINC-3 levels and significantly lowered ICAM-1 levels following trauma-hemorrhage (Fig. 5). In contrast, administration of E2 following trauma-hemorrhage normalized CINC-1, CINC-3, and ICAM-1 levels (Fig. 5). Coadministration of ICI abolished the effect of E2 on intestinal CINC-1, CINC-3, and ICAM-1 levels in trauma-hemorrhage groups (Fig. 5).

**Intestinal MPO activity following trauma-hemorrhage.** Neutrophil accumulation was evaluated by the measurement of tissue-associated MPO activity in small intestine tissue homogenates. As indicated in Fig. 6, small intestine MPO activity significantly increased following trauma-hemorrhage ($P < 0.05$). Losartan administration significantly reduced the increase of MPO in small intestine ($P < 0.05$) following trauma-hemorrhage; however, the MPO activity remained higher than shams and E2-treated trauma-hemorrhage animals ($P < 0.05$). Administration of E2 significantly attenuated the increase in small intestine MPO activity after trauma-hemorrhage, which was abolished by coadministration of ICI.

**DISCUSSION**

The gut is considered a critical organ in the development of the delayed organ dysfunction in patients suffering from traumatic injuries and severe blood loss (11, 19). Gut ischemia is frequently encountered in trauma, shock, cardiovascular surgery, and organ transplantation (4). Splanchnic hypoperfusion is a characteristic feature in the cardiovascular response to hemorrhagic shock (24) and can cause hypoxia of the intestinal mucosa and subsequent increase in intestinal permeability (7). Our present studies indicate that plasma Ang II, IL-6, and TNF levels and intestinal Ang II, IL-6, TNF, CINC-1, CINC-3, and ICAM-1 levels markedly increased at 24 h following trauma-hemorrhage. This was accompanied with an increase in small intestine MPO activity. Administration of losartan at the beginning of resuscitation attenuated the increase in those inflammatory markers but did not affect the trauma-hemorrhage-induced increase in AT1R expression in the intestine. In contrast, administration of a single dose of E2 at the beginning of resuscitation normalized all the above parameters to levels observed in sham animals. Administration of E2 also prevented the trauma-hemorrhage-induced increase in intestinal AT1R expression. The salutary effects of E2 were receptor mediated since administration of ICI along with E2 following trauma-hemorrhage abolished the salutary effects of E2 on the above parameters (46). These studies collectively suggest that the salutary effects of E2 on intestinal injury following trauma-hemorrhage are mediated via downregulation of AT1R.

Ang II is an important vasoconstrictor during hypovolemia and thus may contribute to shock-induced ischemic organ damage (27). The overall function of the RAS is to maintain extracellular fluid and electrolyte homeostasis, as well as to regulate vascular tone and blood pressure (3, 23). Ang II also stimulates a wide variety of proinflammatory responses such as increased induction of proinflammatory chemokines and cyto-
kines (25, 28, 47), expression of adhesion molecules (26), activation of NF-κB (31), and production of oxidative stress (10). Moreover, Ang II increases the level of hypoxia-inducible factor-1α expression via a ROS-dependent activation of the phosphatidylinositol 3-kinase pathway (29). Ultimately, Ang II causes vascular injury and organ damage. In fact, several lines of evidence have implicated that inhibition of Ang II may protect against ischemia-reperfusion-induced tissue injury in the heart, brain, liver, kidney, and small intestine (1, 5, 17, 42). Previous studies have shown that the increase in Ang II is more prominent in the splanchnic circulation than in the systemic circulation during major aortic surgery and hypovolemia (44). This further suggests that Ang II plays a more critical role in the gastrointestinal system than in other organ systems in critical situations. Moreover, it has been shown that the splanchnic vasculature has extraordinarily high concentrations of Ang II receptors (27). The highest angiotensin-converting enzyme (ACE) mRNA, ACE protein, and activity within the intestine have been found in the brush-border membrane fraction in the proximal to mid region of the rat small intestine (6). Functional AT1Rs have been found in the rat ileum and duodenum (34). Other studies have shown that the AT1R antagonist inhibited the development of intestinal mucosal injury induced by ischemia-reperfusion in rats (40). Furthermore, ACE-I inhibition, which decreases the production of angiotensin II, has been shown to decrease postischemic injury (14, 26a, 28). AT1R blockade reduces cerebral ischemia-reperfusion injury in part by attenuating inflammatory processes (35). Studies using in vitro techniques have also shown that E2 downregulated AT1R mRNA expression and AT1R protein in vascular smooth muscle cells isolated from ovariec-
tomized rats (21) and inhibited Ang II-induced cell proliferation and ET-1 gene expression studies (13).

In the present study, intestinal injury was assessed by the measurement of the small intestinal MPO activity, a marker of neutrophil content. Neutrophils are the principal cells involved in host defense against acute bacterial and fungal infections (16). Neutrophil movement and migration are mediated by multiple adhesion molecules on the neutrophils, endothelial cell surfaces, and chemotactic factors. ICAM-1 is an important mediator in the firm adhesion of neutrophils to the vascular endothelium and is upregulated strongly following trauma-hemorrhagic shock (22). With regard to chemokines, rat CINC-1 and CINC-3 are members of the IL-8 family and are potent chemotactic factors for neutrophils (20, 37). Chemotaxis of neutrophils is an important, functional response to chemokines and is a key event in the recruitment of neutrophils during inflammation. In addition, it has been reported that pretreatment with anti-CINC-1 monoclonal antibodies attenuates reperfusion injury in the small intestine in association with a reduction of TNF-α and MPO content, and thereby prolongs survival (39). Our previous studies indicate that CINC-1 and macrophage inflammatory protein-2 levels correlated with intestinal MPO activity following trauma-hemorrhage (33). The present study is consistent with these findings and indicates that administration of the AT1R antagonist decreases small intestinal CINC-1, CINC-3, and ICAM-1 levels and MPO activity. These results indicate that AT1R is involved in the development of trauma-hemorrhage-induced intestinal inflammation.

There is increasing evidence indicating a role for E2 in the modulation of proinflammatory mediator ICAM-1 expression and chemokine production in shock states (12). Previous studies have shown that E2 administration following trauma-hemorrhage improves cardiac function and decreases intestinal neutrophil infiltration. It is therefore possible that the salutary effects of E2 on small intestine following trauma-hemorrhage are a result of the systemically improved cardiac function by E2. Our present results showed that administration of E2 normalized the trauma-hemorrhage-induced increase in intestinal CINC-1, CINC-3, and ICAM-1 levels and inhibited the increase in intestinal inflammation (45). Moreover, the results also showed that the small intestinal Ang II level and AT1R expression increased in trauma-hemorrhage animals. Administration of AT1R antagonist losartan decreased intestinal TNF and ICAM-1 levels and MPO activity without changing Ang II level and AT1R expression, thus indicating that Ang II and AT1R are involved in the intestinal inflammation following trauma-hemorrhage. Since a group with administration of losartan and E2 was not included in this study, it remains unclear whether the combination of these two agents has additional salutary effects compared with either of these agents alone. Nonetheless, since administration of E2 normalized plasma and intestinal Ang II levels, downregulated intestinal AT1R expression, and prevented small intestinal inflammation following trauma-hemorrhage, it is unlikely that the combination of these two agents would have produced additional salutary effects. Furthermore, ER antagonist ICI 182,780 abolished the effect of E2 on Ang II and AT1R. Moreover, although the effects of E2 on intestinal structure and function (histology, absorption, and permeability) were examined in this study, it appears that the salutary effects of E2 on intestine are likely mediated in part due to downregulation of Ang II and AT1R and a decrease of intestinal MPO activity. It could, however, be argued that we should have also measured mRNA levels of cytokines and chemokines since they are secretory factors and may not be appropriately detected by ELISA. Since RNA was not extracted from intestine in this study, we cannot correlate plasma results of chemokines and cytokines exclusively with the intestine in this study.

In conclusion, our data demonstrated that modulation of Ang II and AT1R expression plays an important role in the salutary effects of E2 on small intestine injury following trauma-hemorrhage.

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GRANTS

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