Gender differences in small intestinal perfusion following trauma hemorrhage: the role of endothelin-1


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Endothelin-1 (ET-1), a 21-amino acid peptide produced by vascular endothelium, is a potent vasoconstrictor and a component of local regulation of vascular tone through its paracrine effect on underlying vascular smooth muscle (17, 45). Studies have demonstrated that there are significant sex differences at rest and stress-induced ET-1 release (40). In our primary experiments, we also found a significantly higher ET-1 level in intestinal tissue in males than females. Furthermore, our recent studies have shown that estradiol treatment prevents hepatic damage and alters portal response to ET-1 following T-H (47). Thus we hypothesized that the levels of ET-1 in regional tissues such as the intestine may be higher in males than in females or 17\(^\beta\)-estradiol (E2)-treated males following hemorrhagic shock. To test this hypothesis, the relationship between plasma levels of ET-1 and estrogen was examined. Moreover, the intestinal gene expression and peptide levels of ET-1 were determined in males, age-matched proestrus females, and E2-treated male rats. To determine the influence of ETs on the intestinal perfusion, an antagonist to the predominant ET\(_{A}\) receptor (BQ-123) was administered in an isolated intestinal perfusion system.

MATERIALS AND METHODS

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

**Animal model of T-H.** Harlan Sprague-Dawley male rats (275–325 g), age-matched proestrus female (200–225 g), and male rats were treated by subcutaneous implantation of E2 pellets (0.5 mg/21 days release, 7–14 days treatment; Innovative Research of America, Sarasota, FL). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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sota, FL) and fasted 16 h before the experiment but were allowed water ad libitum (5, 8). The animals were anesthetized with 1.5% isoflurane with air inhalation and underwent a 5-cm ventral midline laparotomy to induce tissue trauma before the onset of hemorrhage. Both femoral arteries and one femoral vein were cannulated with PE-50 tubing for bleeding, monitoring of mean arterial pressure, and fluid resuscitation. The animals were then restrained in a supine position, and the areas of incision were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) to minimize postoperative pain. After the procedure, anesthesia was removed, and when the mean arterial pressure reached ~120 mmHg, the animals were bled to a pressure of 40 mmHg (i.e., severe hypotension) within 10 min. The blood pressure of 40 mmHg was maintained by removing more blood until the animal was no longer able to maintain blood pressure (i.e., maximum bleed out). The blood pressure was then maintained at that level by infusing Ringer lactate (RL) intravenously until 40% of the shed blood volume was returned. The animals were resuscitated with four times the volume of maximum bleed out with RL over a period of 60 min at a constant rate. After resuscitation, the catheters were removed, the vessels were ligated, and skin incisions were closed with sutures. The animals were maintained conscious and without heparin injection throughout the hemorrhage and resuscitation procedure. Blood pressure was monitored with a blood pressure analyzer (Digi-Med, Louisville, KY), and the resuscitation perfusion pump was a Pump 11 (Harvard Apparatus, Holliston, MA). Sham-operated animals underwent the same surgical procedure but were neither bled nor resuscitated. The time required for maximum bleed out was ~45 min; the volume of maximum bleed out was ~60% of the calculated circulating blood volume.

Preparation of isolated small intestine and measurement of intestinal perfusion flow. At 2 h postresuscitation, the animals were anesthetized again with isoflurane (1.5% with air) and the anesthesia was maintained by injection of pentobarbital sodium (30 mg/kg body wt) via the femoral vein. Animals underwent an isolated small intestinal perfusion model as described previously with minor modification (5). Briefly, the small intestine was isolated and perfused without removal from the abdominal cavity. The branches of blood vessels to and from the cecum, ascending colon, and transverse colon were then ligated. Five minutes after intravenous injection of 0.3 ml heparin solution (500 U), the superior mesenteric artery and portal vein were cannulated with a PE-50 and PE-90 catheter, respectively. Although the rat was still alive, the isolated intestine was perfused with 95%O2-5%CO2 oxygenated Krebs-Ringer-HCO3 buffer (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 2.4 H2O, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.026 Ca-EDTA, and 11.1 d-glucose, with 0.22 g/l Na-pyruvate, pH 7.4) at 37°C through the superior mesenteric artery catheter (PE-50). The rat was killed by overdose of pentobarbital sodium via femoral vein. It has been reported that resistance blood vessels (i.e., small arteries and arterioles), rather than large arteries, play a major role (90%) in maintaining peripheral resistance. Therefore, changes in perfusion rate in isolated small intestinal preparations reflect the reactivity of small arteries and arterioles in the intestine. The perfusion flow (ml/min·100 g−1 intestinal tissue wt) was measured from the portal vein at a constant perfusion pressure (80 mmHg) produced by a raised perfusate reservoir. Perfusion pressure was determined by a blood pressure analyzer (Digi-Med) that was placed in line immediately before the superior mesenteric artery inlet cannula. In another set of animals, intestinal perfusion flow was also measured under the perfusion buffer containing 10 ng/ml of ETA receptor antagonist (BQ-123, A.G. Scientific, San Diego, CA). The isolated intestinal perfusion resistances were calculated by the perfusion pressure divided by perfusion flow rate (mmHg·ml−1·min−1·100 g−1).

Determination of plasma level of ET-1 and estradiol. Blood samples were obtained from the femoral artery and centrifuged at 1,600 g for 15 min at 4°C to separate plasma. Plasma was immediately frozen (~80°C) until assayed. The assay for ET-1 and estradiol was performed by using a commercially available Endothelin-1 enzyme immunometric assay kit (Assay Designs, Ann Arbor, MI) and estradiol EIA kit (Cayman Chemical, Ann Arbor, MI).

Intestinal gene expression and peptide levels of ET-1. The mRNA levels of ET-1 in intestinal tissue were determined by real-time PCR. Total RNA was isolated from total intestinal tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. cDNA was generated from the total RNA samples by using a transcription kit (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, CA). Each real-time PCR reaction was performed in a mix of 10-μl reaction mixture containing 20 ng of cDNA, 2× PCR Master Mix (Applied Biosystems), and each probe and primer set. TaqMan Gene Expression assays (Applied Biosystems) for ET-1 were purchased as probe and primer sets. The reaction mixture was denatured for 1 cycle of 2 min at 50°C, 10 min at 95°C, and incubated for 40 cycles (denaturing for 15 s at 95°C and annealing and extending for 1 min at 60°C) using ABI Prism 7900HT (Applied Biosystems). All samples were tested in triplicate, and average values were used for quantification. 18S RNA was used as an endogenous control. Analysis was performed using SDS 2.1 software (Applied Biosystems) according to the manufacturer’s instruction. The comparative CT method (ΔΔCT) was used for quantification of gene expression. The calibrator sample was designed as the most highly expressed time point for each gene and, therefore, has set an expression of one. Thus all samples are expressed as the fold expression of this value. To decide the calibrator sample, preliminary experiments were performed to ensure that amplification efficiencies for the target genes and 18S were equivalent.

Peptide levels of ET-1 in intestinal tissue were also examined. The intestinal tissue was sampled, immediately placed in liquid nitrogen, and stored at ~80°C until assayed. Tissue samples (100 mg wet wt) were homogenized in 1 ml PBS (pH 7.4) containing aprotinin (Sigma, St. Louis, MO) in an ice bath and centrifuged at 12,000 g for 20 min at 4°C. The supernatant was analyzed by using a commercially available endothelin-1 enzyme immunometric assay kit (Assay Designs).

Statistical analysis. All data are presented as means ± SE. One-way ANOVA and Tukey’s test were employed for the comparison between each group. Correlation between plasma ET-1 and estradiol was evaluated by Pearson’s correlation analysis. The differences were considered significant at P < 0.05.

RESULTS

Intestinal perfusion flow following T-H. As shown in Fig. 1A, the intestinal perfusion flow in sham-operated males was significantly lower than proestrus females and E2-treated males and was significantly decreased after T-H; however, there were no significant changes in proestrus females and E2-treated males under such conditions. The lower sham level of intestinal perfusion flow in males was significantly elevated by treatment with the blockade of ETA receptor, BQ-123. This increase was similar to sham levels in proestrus females but still lower than in E2-treated males. In male rats treated with E2 in the T-H group, the use of BQ-123 significantly increased the perfusion flow. Moreover, the decreased intestinal perfusion flow in males after T-H was also attenuated by treatment with BQ-123 (Fig. 1B).

Alteration and correlation of plasma levels of ET-1 and estradiol. As shown in Fig. 2A, the plasma levels of ET-1 significantly increased following T-H among three experimental groups. The increase of ET-1 following T-H in males was significantly higher than that in females or E2-treated males. Furthermore, Fig. 2B shows that the plasma levels of E2 in males were significantly lower than in females and E2-treated...
males. T-H had no influence in plasma level of E2 among three experimental groups (Fig. 2B).

When the data from all groups (male, proestrus female, and E2-treated male rats following T-H) were included, plasma levels of ET-1 were inversely correlated to plasma levels of E2 according to the Pearson’s correlation analysis ($r^2 = 0.604$, $P < 0.05$; Fig. 3).

Intestinal tissue ET-1 gene expression and peptide levels of ET-1. The intestinal ET-1 gene expression determined by real-time PCR is shown in Fig. 4A. The results show that the ET-1 gene expression in sham-operated males was significantly higher than female shams and sham-operated E2-treated males. Although ET-1 gene expressions were slightly increased compared with sham by T-H, there was no significant difference among all experimental groups following T-H.

Although intestinal peptide levels of ET-1 were significantly elevated by T-H in all experimental groups, the increased intestinal peptide level of ET-1 was significantly higher in males than that in females and E2-treated males following T-H (Fig. 4B).

**DISCUSSION**

Gender influences the function and responsiveness of the cardiovascular system under normal and pathophysiological conditions (14, 15, 28, 31, 36). Previous data from our laboratory have shown that females in the proestrus stage of the estrus cycle maintain cardiovascular function and immune responses compared with male rats following T-H (3, 5, 26, 27). Moreover, endothelial dysfunction did not develop in the intestine of proestrus females but was observed in the male intestine after T-H. These results collectively suggest that maintenance of vascular endothelial function in proestrus females following T-H might be a potential mechanism respon-
ANOVA and Tukey’s-test: Hence, it appears that the proestrus females, and E2-treated males were attenuated by the selective ETA-receptor blocker BQ-123. Thus, the precise mechanism responsible for gender differences in small intestinal perfusion following T-H remains unclear. ET-1 is a potent vasoconstrictor and a component of local regulation of vascular tone (17, 45). Furthermore, it has been reported that there is a sex difference in rest and stress-induced ET-1 release (40). The aim of the present study was to investigate the role of ET-1 in the gender-dependent small intestinal perfusion response following T-H. The influence of ET-1 on intestinal perfusion was also investigated ex vivo in isolated intestines from male, proestrus female, and E2-treated male rats following T-H.

We observed marked gender differences in the intestinal perfusion pattern following T-H. Male rats showed significant reduction in intestinal perfusion flow following hemorrhagic shock compared with proestrus females and E2-treated males. However, these differences among the responses of the males, proestrus females, and E2-treated males were attenuated by the specific ET\(_A\)-receptor blocker BQ-123. Thus, it appears that the alterations in the intestinal perfusion flow are related to the potent vasoconstrictor peptide ET-1, and the levels correlate with plasma level of estradiol. It could therefore be concluded that ET-1 plays a significant role on intestinal perfusion failure following T-H in males. Because E2 can modulate this vasoconstrictor effect of ET-1, these findings may partially explain the previously observed salutary effect of estrogen in improving intestinal perfusion following T-H shock in males.

It is well recognized that the predominant adrenergic response during hemorrhagic shock is sympathoadrenal reaction (16). A combination of hypotension, hypovolemia, hypoperfusion, and tissue ischemia following hemorrhagic shock constitutes potent stimuli for the release of stress hormones such as epinephrine and norepinephrine. Yanagisawa et al. (45) reported that epinephrine induces the production of ET-1 in cultured porcine aortic endothelial cells. Rapid elevation of plasma ET-1 levels after hemorrhage is also evidenced (11, 35), supporting the suggestion that stress-hormone release during hemorrhagic shock is a major determinant of the elevation of plasma ET-1 levels. Indeed, in our experiments, T-H induced a simultaneous elevation of plasma and intestinal ET-1 levels, suggesting the contribution of ET-1 to the development of splanchnic perfusion failure after hemorrhagic shock (5, 6). Sharma et al. (37) reported that the subsequent increase of vascular resistance and fall in tissue perfusion after hemorrhage are due to the increased endothelin production. Michida et al. (24) have also reported that endogenously released ET-1 impairs microcirculation and plays an important role in the pathogenesis of hemorrhagic shock-induced gastric mucosal damage. It should be noted, however, that despite the amelioration of the decreased perfusion flow mediated by BQ-123 treatment in males, the intestinal perfusion flow was still significantly lower than in the E2-treated males both at the baseline and posthemorrhage time points. This indicates the existence of additional effector mechanisms in the estradiol-mediated vasoregulation during rest and stress conditions. For instance, our recent study has shown that estradiol treatment prevents hepatic damage and alters portal response to ET-1 following T-H (47). Moreover, studies have shown that besides producing vasoconstriction via activation of ETA and ETB\(_2\) receptors, ET-1 also can induce vasodilatation via activation of ETB\(_1\) receptors via nitric oxide and prostacyclin release (25).

Wang et al. (44) have shown that the expression of the predominant subtype of ET receptor ET\(_A\) was decreased after treatment with E2 in vascular smooth muscle cells. Therefore, estrogen may regulate ET receptor functions, which may also be one mechanism of the salutary effect of this sex steroid.

Our present data indicated that E2 modulates this vasoconstrictor effect of ET-1; however, the precise mechanism still remains unclear. Previous data showed that 17\(\beta\)-estradiol reduces mRNA expression and peptide release of ET-1 in endothelial cells in vitro (1, 9, 18). In our experiments, intestinal gene expression and peptide levels of ET-1 in sham-operated males were significantly higher than in females and in E2-treated counterparts, but there was no significant difference in ET-1 gene expression following T-H between the different groups. Nonetheless, our results have shown that both plasma and intestinal ET-1 peptide are increased significantly in males compared with proestrus females and E2-treated males following T-H.

Doi et al. (13) have shown that acute hypoxia enhanced the expression of ET-1 and ET-converting enzyme-1 (ECE-1) in rat aorta. Furthermore, studies have reported that estrogen can modulate ECE-1 functions. For instance, Tan et al. (39) demonstrated that estrogen downregulates plasma ET-1 levels by inhibiting the ET-1 mRNA expression and functional ECE activity. Rodrigo et al. (32) have shown that vascular ECE-1 mRNA expression decreases in response to estrogens. Because the present study did not examine such a relationship between estrogen and ECE-1, it is possible that intestinal ECE-1 activity or expression is altered following T-H. Because there were apparent gender differences in the intestinal perfusion pattern following T-H, we conducted our study in male, proestrus female, and E2-treated male rats at 2 h following sham operation and T-H. Data are presented as means ± SE and compared by 1-way ANOVA and Tukey’s-test. *P < 0.05 vs. sham in the same group; #P < 0.05 vs. corresponding male sham-operated group. RQ, relative quantity.

Fig. 4. The intestinal gene expressions (A) and peptide levels (B) of ET-1 in male, proestrus female, and E2-treated male rats at 2 h following sham operation and T-H. Male rats showed significant reduction in intestinal perfusion flow following T-H.

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significantly lower intestinal peptide levels of ET-1 following T-H in proestrus females and in E2-treated males, it strongly indicates that estradiol reduces ET-1 peptide production following T-H. We therefore propose that the sustained vasoconstriction induced by T-H is related to the increased ET-1 production, which, in turn, could be counteracted by estradiol-mediated mechanisms.

In conclusion, these results indicate that the endogenous vasoconstrictor peptide ET-1 appears to play an important role on intestinal perfusion failure following T-H shock in males. Because a high level of E2 can modulate this vasoconstrictor peptide ET-1 appears to play an important role on intestinal perfusion following T-H shock in males. Because estradiol has the potential ability to restore impaired tissue perfusion following T-H, this hormone may be a useful therapeutic adjunct for reducing elevated endothelin production following low-flow conditions.

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REFERENCES


