Endothelin-1-induced PMN infiltration and mucosal dysfunction in the rat small intestine

BERNA K. OKTAR,1 TAMER COSKUN,1 AYHAN BOZKURT,1 BERRAK Ç. YEĞEN,1 MERAL YÜKSEL,2 GONÇAGÜL HAKLAR,2 SERPIL BILSEL,2 FEHIME BENLI AKSUNGAR,2 ŞULE ÇETINEL,3 D. NEIL GRANGER,4 AND HIZIR KURTEL1

Departments of 1Physiology, 2Biochemistry, and 3Histology, Marmara University School of Medicine, 81326 Haydarpasa, Istanbul, Turkey; and 4Department of Molecular and Cellular Physiology, Louisiana State University, Shreveport, Louisiana 71130

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Oktar, Berna K., Tamer Coskun, Ayhan Bozkurt, Berrak Ç. Yeğen, Meral YükseL Goncağül Haklar, Serpil Bilsel, Fehime Benli Aksungar, Sule Çetinel, D. Neil Granger, and Hizir Kurtel. Endothelin-1-induced PMN infiltration and mucosal dysfunction in the rat small intestine. Am J Physiol Gastrointest Liver Physiol 42: 483–491, 2000.—The objectives of this study were to characterize the effects of endothelin (ET)-1 on intestinal mucosal parameters and to assess the contribution of polymorphonuclear leukocytes (PMNs), intercellular adhesion molecule-1 (ICAM-1), and a platelet-activating factor (PAF) to the mucosal dysfunction induced by ET-1. Different concentrations of ET-1 (100, 200, and 400 pmol/kg) were infused into the superior mesenteric artery for 10 min, and tissue samples were obtained 30 min after terminating the infusion. ET-1 administration significantly elevated tissue myeloperoxidase activity, plasma carbonyl content, and tissue chemiluminescence intensity, indicating that ET-1 produces PMN infiltration and oxidant stress. Blood-to-lumen clearance of 51Cr-EDTA was significantly increased after ET-1 infusion (400 pmol/kg). Monoclonal antibodies against ICAM-1 (1A29, 2 mg/kg), antineutrophil serum, and PAF antagonist (WEB-2086, 10 mg/kg) attenuated the mucosal barrier dysfunction induced by ET-1. Overall, our data indicate that ET-1 causes PMN accumulation, oxidant stress, and mucosal dysfunction in the rat small intestine and that ET-1-induced mucosal dysfunction involves a mechanism that includes a role for PMNs, ICAM-1, and PAF.

oxidant stress; mucosal permeability; intercellular adhesion molecule-1; platelet-activating factor

THE REGULATION OF THE MUCOSAL microcirculation is intimately involved in the maintenance of mucosal integrity; hence, the local release of vasoactive mediators from endothelial cells of the microvasculature plays a significant modulatory role. Endothelin (ET)-1, an extremely potent vasoconstrictor peptide with 21 amino acid residues, was originally identified in the culture supernatant of porcine endothelial cells (38). ET-1 is considered to have a physiological role in the control of gastrointestinal function (32, 35). In addition, ET-1 has been implicated in the pathogenesis of various microvascular disturbances, including liver ischemia-reperfusion injury (29), hemorrhagic shock-induced gastric mucosal injury (26), and ethanol-induced gastric mucosal damage (25). The involvement of ET-1 in the pathogenesis of various microvascular disturbances was mainly supported by the observations that ET-1 levels increase during these experimental conditions and that ET-1 inhibitors provide a significant level of protection against microvascular disturbances. To evaluate the role of ET-1 in mediating gastrointestinal disease, several investigators have examined alterations in microvascular permeability induced by ET-1 infusion. Exogenous ET-1 administration produces a marked increase in vascular tone and significant elevation in protein extravasation in the gastric microvasculature, leading to substantial gastric mucosal injury in the rat (1, 9). In another study, direct assessment of the effects of ET-1 on intestinal microcirculation was obtained by using a fluorescence videomicroscopy system (3). It has been shown that reductions in subserosal lymphatic capillary density and mucosal functional capillary density are among the earliest microcirculatory consequences of systemic ET-1 administration at low doses. Higher doses of the peptide produce marked decreases in perfusion parameters in all layers of the small intestine. Moreover, it has been demonstrated that ET-1 induces significant necrotic and hemorrhagic lesions in the small intestine that can be partially inhibited by superoxide dismutase, catalase, a platelet-activating factor (PAF) inhibitor, and a calcium antagonist (nicardipine), indicating that reactive oxygen metabolites (ROM), PAF, and possibly polymorphonuclear leukocytes (PMNs) are involved in the injury process induced by ET-1 (28).

Although there are several possible mechanisms by which ET-1 may contribute to mucosal damage, to date there are no published data that assess the role of ET-1 in the integrity of the intestinal epithelial barrier. It is...
well established that loss of restrictive mucosal function is closely associated with the severity of intestinal damage and that leaky intestinal mucosa leads to transmural movement of toxic factors into circulation, causing sepsis and possibly multiple organ failure (12). The status of the mucosal barrier can be assessed quantitatively using a $^{51}$Cr-labeled EDTA molecule. Administration of $^{51}$Cr-EDTA results in an almost instant equilibration across the vasculature (into interstitium) but is greatly restricted by the epithelial layer (4). Hence, the epithelium is the limiting barrier to the movement of $^{51}$Cr-EDTA, and increased clearance of this molecule from blood to lumen suggests an impairment in the integrity of the epithelial layer. As a potent vasoconstrictor and an inflammatory agent, it is possible that ET-1 may be an important modulator of events leading to intestinal mucosal barrier dysfunction.

In addition to the vasoactive properties of ET-1, there are several reports in the literature that address the potential role of ET-1 in modulating leukocyte-endothelial cell interactions. It has been shown that ET-1 enhances superoxide generation from human neutrophils stimulated by chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine, whereas neutrophils stimulate endothelial cell production of ET-1 in vitro (17). It has also been shown that ET-1 stimulates neutrophil adhesion to cultured endothelial cells and increases the expression of adhesion molecules on both the neutrophil surface and endothelial cells (13, 23). The participation of ET in an interaction between leukocytes and the endothelium is further supported by in vivo studies demonstrating that ET-1 infusion causes leukocyte rolling and adherence in submucosal venules of the intestinal microcirculation (3). These data raise the possibility that PMNs may be important modulators of the sequel associated with inflammation of the small intestine induced by ET-1. Overall, the objectives of our study were 1) to characterize the effects of ET-1 on intestinal mucosal parameters, including tissue PMN accumulation, oxidant stress, and the permeability of mucosal membranes, and 2) to assess whether altered mucosal barrier function induced by ET-1 is associated with PMNs, intercellular adhesion molecule-1 (ICAM-1), and PAF.

MATERIALS AND METHODS

Animals. Wistar Albino rats of both sexes (250–300 g) were kept in a room at a constant temperature of 22 ± 2°C with light-dark cycles of 12:12 h and fed a standard diet and water ad libitum. Studies were approved by the Marmara University Animal Use and Care Committee. Following an overnight (18 h) fast, the rats were anesthetized with urethane (1.2 g/kg) and a tracheotomy was performed to facilitate breathing. The right carotid artery was cannulated for arterial pressure recording (Nikon Kohden polygraph model AP-621G). The right jugular vein was also cannulated for the injection of various compounds. A thermometer was inserted into the rectum, and the body temperature was maintained at 37°C by a heating pad. A midline abdominal incision was made, and small intestine was exteriorized to facilitate cannulation of the abdominal aorta. A catheter was inserted from the bifurcation of common iliac arteries into the abdominal aorta up to the opening of the superior mesenteric artery. ET-1 (Sigma Chemical, St. Louis, MO) was dissolved in saline, and doses of 100, 200, and 400 pmol/kg were infused at 0.1 ml/min through the intra-aortic catheter for 10 min. Control animals received the vehicle (0.1% bovine serum albumin) instead of ET-1. Thirty minutes after the administration of ET-1 or the vehicle, tissue samples were obtained from duodenal, jejunal, and ileal segments and stored at −70°C for subsequent biochemical determinations. Heparinized blood samples were obtained from the jugular vein at the beginning of the experiment and from the portal vein at the end of the experiment. The blood was immediately centrifuged, and plasma was stored at −70°C for the determination of the protein carbonyls.

Mucosal permeability measurements. Changes in mucosal permeability were assessed using the blood-to-lumen clearance of $^{51}$Cr-EDTA (obtained from NEN, Boston, MA). Briefly, after an abdominal incision was made and the abdominal aorta was cannulated, a jejunal segment (at 15 cm distal to Treitz ligament) was isolated and cannulated at both ends using Silastic tubing. The luminal contents were removed by perfusion with warmed isotonic saline solution (pH 7). Both renal pedicles were ligated to prevent rapid excretion of the radioisotope marker into urine. After the surgery was completed, $^{51}$Cr-EDTA (100 μCi) in saline was administered intravenously as a bolus through the jugular vein followed by a 20-min equilibration period, during which the isolated intestinal segment was perfused with saline at a rate of 0.5 ml/min but no clearance measurements were taken. All perfusates were warmed so that their temperature at the point of entering the intestine was 37°C. After the equilibration period, luminal perfusate was collected over 10-min periods before and after the administration of various doses of ET-1 (100, 200, and 400 pmol·kg$^{-1}$·min$^{-1}$). Blood samples (0.3 ml) were taken at the beginning and the end of experiments from the carotid artery and centrifuged (15,000 rpm) for 5 min. The amount of radioactivity in the plasma and the perfusate was then determined by gamma spectroscopy. At the end of the experiment, the isolated segment of small intestine was removed, rinsed, and weighed. The plasma-to-lumen clearance of $^{51}$Cr-EDTA was calculated as

$$\text{clearance} = \frac{\text{cpm}_{\text{pl}} \times \text{pr} \times 100}{\text{cpm}_{\text{pl}} \times \text{wt}}$$

where clearance of $^{51}$Cr-EDTA is given in milliliters per minute per 100 g, cpm$_{\text{pl}}$ is counts per minute per milliliter of perfusate, pr is the perfusion rate, cpm$_{\text{pl}}$ is counts per minute per milliliter of plasma, and wt is weight of the intestine in grams.

In a group of animals, three intraperitoneal injections of antineutrophil serum (ANS; Accurate Chemical, Westbury, NY) were given at 12-h intervals to render the animals neutropenic before the infusion of ET-1. Neutropenia was confirmed by counting the circulating PMNs on a hemocytometer using acetic acid-crystal violet stain before and after the injections of ANS before surgery, which was performed 3–6 h after the last injection. Another approach to investigating the role of PMNs on ET-1-induced alterations in mucosal permeability involved the administration of a monoclonal antibody (MAB) directed against ICAM-1 (MAB 1A29, 2.0 mg/kg iv; Upjohn, Kalamazoo, MI) 5 min before the infusion of ET-1. The concentration of MAB used in this study was the amount needed to maximally reduce the leukocyte adherence and emigration induced by leukotriene B$_4$ or PAF in rat mesenteric venules (18).

Another strategy for studying ET-1-induced alterations in mucosal permeability involved the use of a specific PAF receptor antagonist (WEB-2086). PAF is a major inflamma-
tory mediator that has been implicated in the infiltration of PMNs. Animals were pretreated with WEB-2086 (10 mg/kg iv bolus) 5 min before the infusion of ET-1, and the permeability measurements were obtained during and after the infusion of ET-1 (400 pmol·kg⁻¹·min⁻¹) for 60 min.

**Endothelin assay.** Portal plasma ET-1 levels were measured in control and intra-arterial ET-1-treated groups at a dose of 400 pmol·kg⁻¹·min⁻¹. Blood samples were drawn from the portal vein at the end of each experiment and were collected in chilled polystyrene tubes containing 1 mg/ml EDTA and 500 KIU/ml aprotinin (Trasylo; Bayer). The blood samples were centrifuged at 4°C, and the plasma samples were collected and stored at −80°C until assay. Plasma levels of ET-1 were measured with a sandwich immunoperoxidase technique (BBE-5; R&D Systems). According to the manufacturer, in-ter-assay coefficient of variation is <4.6% and inter-assay coefficient of variation is <6.5%. Sensitivity of the kit was 0.25 pg/ml. The cross-reactivity of the kit with big ET-1 is <1% and with ET-3 was <14%.

**Tissue myeloperoxidase measurements.** Tissue-associated myeloperoxidase (MPO) activity was determined in 0.2- to 0.5-g samples. The tissue samples were homogenized in 10 vol of ice-cold potassium phosphate buffer (20 mM K₂HPO₄, pH 7.4). The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mM K₂HPO₄ containing 0.5% (wt/vol) hexadecyltrimethylammonium hydroxide. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of o-dianisidine·2HCl. One unit of enzyme activity is defined as the amount of the MPO present that causes a change in absorbance of 1.0/min at 460 nm and 37°C (2).

**Assessment of oxidant stress.** Protein oxidation was quantified using the interaction between 2,4-dinitrophenylhydrazine (DNP; Sigma) and the carbonyls generated from the oxidative modification of proteins to yield a chromophore that absorbs strongly at 380 nm. Briefly, protein in the aliquots (0.5 ml) was precipitated by the addition of 20% TCA. Precipitated protein was collected by centrifugation and resuspended in 0.5 ml of 10 M DNP in 2 M HCl. The samples were incubated for 1 h at 25°C with occasional mixing. Protein was then precipitated by the addition of 20% TCA and collected by centrifugation, and the pellet was washed three times with 1 ml of an ethanol-ethyl acetate (1:1) solution to remove any unreacted DNP. The protein precipitant was solubilized by the addition of 1 ml of 1 N NaOH, and the absorbance was determined at 380 nm. The carbonyl content was calculated assuming a molar extinction coefficient of 22,000 (21).

Chemiluminescence measurements were done using a liquid scintillation counter (Tricarb 1500; Packard Instruments) in out-of-coincidence mode with a single active photomultiplier tube. Fresh intestinal samples were gently transferred to precounted glass scintillation vials containing phosphate buffered saline at pH 7.4 supplemented with HEPES (0.02 mol/l). Luminescence was recorded at room temperature after the addition of 0.2 mM lucigenin (bis-N-methylacridinium nitrate; Sigma) or 0.2 mM luminol (5-amino-2,3-dihydro-1,4-phenthalazinedione; Sigma). Counts were obtained at 1-min intervals over a period of 60 min, and each point was calculated as counts per minute for milligram of tissue after subtraction of scintillation vial background. The results were expressed as area under the curve, with the integration of the area by the trapezoidal rule (36).

**Tissue analysis.** Samples from the standardized regions of the duodenum, jejunum, and ileum were fixed with neutral buffered formalin and processed by routine techniques before embedding in paraffin wax. Sections (4 mm) were stained with hematoxylin and eosin and examined under a light microscope. For histological assessment of intestinal injury, a 0–3 grading scale was used as follows: 0 = having normal histology; 1 = subepithelial vasocongestion and epithelial cell loss, injury at villus tips; 2 = mucosal congestion, hemorrhage, and focal necrosis, with loss of more than half of villi; and 3 = having damage extending to submucosa or transmucosa. All assessments of damage were performed by an observer unaware of the treatment.

**Statistics.** All values are reported as means ± SE. Student’s t-test and one-way analysis of variance with the Newman-Keuls post hoc test were used to determine whether data from different groups were statistically different. P < 0.05 was considered statistically significant.

**RESULTS**

Close intra-arterial administration of ET-1 at various doses did not cause any significant change in mean arterial pressure. The mean arterial pressures before and after ET-1 infusion (400 pmol·kg⁻¹·min⁻¹) were 90.34 ± 3.45 mmHg and 95.84 ± 4.92 mmHg, respectively, which slowly declined to 83.13 ± 7.8 mmHg 30 min later. In permeability experiments, the mean arterial pressure significantly decreased at 60 min following 400 pmol·kg⁻¹·min⁻¹ ET-1 administration. Treatment with ANS, ICAM-1 MAb, or PAF receptor antagonist WEB-2086 did not change the arterial pressure values compared with the ET-1 group alone.

The basal ET-1 concentration in the portal plasma was 6.37 ± 3.05 pg/ml. In the ET-1-treated group, intra-arterial infusion of ET-1 at a dose of 400 pmol·kg⁻¹·min⁻¹ resulted in an ~25-fold increase (162.25 ± 16.72 pg/ml) in portal blood ET-1 levels at 30 min after its administration.

Figure 1 illustrates the changes in MPO values induced by administration of different doses of ET-1. In the control group, MPO activities were 139.33 ± 14.9,
control animals, basal $^{51}$Cr-EDTA clearance did not significantly change throughout the 1-h measurement period. ET-1 increased the mucosal permeability in a dose-dependent fashion, in which significant elevations in mucosal permeability were observed at a dose of 400 pmol·kg$^{-1}$·min$^{-1}$. Clearance values significantly increased during the infusion period, leveled at ~2.5 times the preperfusion values for the next 20 min, and then reached the peak permeability values (~6.6-fold) at the end of the experiment.

Figure 3 compares the tissue MPO responses to ET-1 administration (400 pmol·kg$^{-1}$·min$^{-1}$) in untreated animals and in animals treated with ET-1 plus ICAM-1-specific MAb 1A29, ANS, or PAF receptor antagonist WEB-2086. Tissue samples were obtained from the perfused jejunal segments at the end of permeability measurements. Tissue-associated MPO activity significantly increased in animals treated with ET-1. Pretreatment with MAb 1A29 and ANS significantly reduced PMN infiltration elicited by ET-1 administration, indicating that both approaches to inhibition of

Table 1. Formation of plasma carbonyl groups and tissue luminol- and lucigenin-enhanced chemiluminescence intensities in the jejunum of control and ET-1-treated rats

<table>
<thead>
<tr>
<th>n</th>
<th>Change in Carboxyl Formation, mmol/ml plasma</th>
<th>Luminol-Enhanced Chemiluminescence, AUC</th>
<th>Lucigenin-Enhanced Chemiluminescence, AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>11.94 ± 5.21</td>
<td>20,379 ± 2,794</td>
</tr>
<tr>
<td>ET-1 100</td>
<td>6</td>
<td>11.43 ± 3.14</td>
<td>34,228 ± 6,126</td>
</tr>
<tr>
<td>ET-1 200</td>
<td>9</td>
<td>21.11 ± 3.72</td>
<td>30,629 ± 5,344</td>
</tr>
<tr>
<td>ET-1 400</td>
<td>9</td>
<td>27.20 ± 3.48*</td>
<td>70,864 ± 6,730**</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Endothelin (ET-1) was administered at doses of 100 (ET-1 100), 200 (ET-1 200) and 400 (ET-1 400) pmol·kg$^{-1}$·min$^{-1}$. *$P < 0.05$ and **$P < 0.01$ compared with control group. AUC, area under the curve.

133.91 ± 12.75, and 129.39 ± 18.1 U/g in the duodenal, jejunal, and ileal segments, respectively. Basal MPO activities in the duodenum, jejunum, and ileum were found to be not statistically different. Intra-arterial administration of ET-1 at various doses increased the magnitude of the PMN infiltration in all segments of small intestine in a dose-dependent fashion, with the 400 pmol·kg$^{-1}$·min$^{-1}$ dose exhibiting the greatest response. All intestinal segments responded to 400 pmol/kg ET-1 with a significant increase in MPO activity.

Table 1 summarizes the changes in plasma carbonyl content and the tissue chemiluminescence intensity in response to administration of various doses of ET-1. In plasma samples, formation of carbonyl groups, which is an indicator of protein oxidation, significantly increased after ET-1 infusion at a dose of 400 pmol·kg$^{-1}$·min$^{-1}$. Luminol- and lucigenin-enhanced tissue chemiluminescence intensities were significantly increased in animals treated with 400 pmol·kg$^{-1}$·min$^{-1}$ of ET-1 compared with control values, indicating the enhanced ROM production in the intestinal samples.

Figure 2 illustrates the time-dependent effects of ET-1 infusions (100, 200, and 400 pmol·kg$^{-1}$·min$^{-1}$) on intestinal blood-to-lumen clearance of $^{51}$Cr-EDTA. In
PMN accumulation were effective. The effectiveness of ANS in reducing the number of PMNs was also confirmed in the peripheral blood samples. Neutrophil counts before and after the injections of ANS were 3.68 ± 1.1 × 10⁶ and 0.95 ± 0.53 × 10⁶ cells/ml blood, respectively (P < 0.01 compared with pretreatment values; data not shown). On the other hand, PAF receptor antagonist did not significantly alter the elevated MPO activities normally observed after ET-1 administration.

Figure 4 shows the time-dependent changes in mucosal permeability values assessed in ET-1-treated (400 pmol/kg) and ET-1 plus MAb 1A29-, ANS-, or WEB-2086-treated rats. MAb 1A29 pretreatment lowered the clearance values compared with the ET-1-treated group alone. This decrease in permeability was especially evident during the first 30 min of the experiment, reaching statistical significance at 0, 10, and 20 min after ET-1 administration. Similarly, pretreatment of animals with ANS reduced the ET-1-induced increase in mucosal permeability in which significant decreases were observed at 0 and 20 min after ET-1 infusion. Administration of PAF receptor antagonist WEB-2086 reduced the elevated clearance values during the entire measurement period; however, significant decreases were observed between 30 and 60 min of measurement.

Figure 5 shows light micrographs of the small intestine from rats infused with ET-1 (400 pmol·kg⁻¹·min⁻¹) or ET-1 plus ANS, WEB-2086, or MAb 1A29. In the ET-1-administered group, severe lesions were most commonly observed and consisted of disruption of more than half of the villi, with diffuse microvascular congestion and hemorrhagic damage (Fig. 5A). With the use of ANS, WEB-2086, or MAb 1A29, severe mucosal destruction induced by ET-1 was partially prevented, and instead of these lesions, lifting of epithelial cell layer, injury at villus tips, mucosal lymphangiectasia, and submucosal edema were commonly observed (Fig. 4, B–D). The degree of mucosal damage expressed by the histological index was significantly lowered by the pretreatment with ANS, WEB-2086, or MAb 1A29 for each compared with those receiving ET-1 alone (Table 2).

DISCUSSION

Local intra-arterial administration of ET-1 induces extensive hemorrhagic damage in the rat gastrointestinal tract that is morphologically similar to human ischemic bowel necrosis (28). The effect of ET-1 has been attributed to the potent vasoactive properties of this peptide. In addition, increased vascular permeability in the gastrointestinal organs has been reported in response to ET-1 administration (9). Recently, a number of studies have shown that ET-1 is a neutrophil-activating agent causing the accumulation of neutrophils to various vascular beds, including lung, kidneys, and heart (8, 15, 23). However, there has not been a systematic analysis of the mucosal alterations that are produced by ET-1. In the present study, we have employed a rat model of ET-1-induced intestinal injury to assess the effects of ET-1 on intestinal mucosal parameters. The results of our study suggest that infusion of ET-1 into the superior mesenteric artery causes a dose-dependent increase in PMN accumulation, elevates mucosal permeability to ⁵¹Cr-EDTA molecules, and produces marked changes in the formation of ROMs.

In an attempt to determine whether ROMs contribute to the pathogenesis of ET-1-induced mucosal injury, we determined the plasma levels of carbonyls and the tissue chemiluminescence intensity. The reactions of endogenous proteins with ROMs lead to the oxidative modification of plasma proteins. These observations are consistent with the previously published report demonstrating that pretreatment with superoxide dismutase/catalase attenuates ET-1-induced bowel damage (28). Although there is circumstantial evidence that supports the role of ROMs, the enhanced O₂⁻ production after ET-1 administration has not been demonstrated. Luminol- and lucigenin-dependent chemiluminescence is a very sensitive means of detecting ROM levels in biological media (34). Luminol-enhanced chemiluminescence has often been used to study the MPO-dependent oxidative process and the generation of hydrogen peroxide and hypochlorous acid in particular (5). On the other hand, lucigenin responds more specifically to superoxide than luminol (6). Using
Fig. 5. Light micrographs of the small intestine from rats treated with ET-1 (400 pmol·kg⁻¹·min⁻¹) alone (A) or pretreated with anti-ICAM-1 monoclonal antibody (B), ANS (C), or PAF receptor antagonist (WEB-2086: D) before ET-1 administration. Note the extensive mucosal necrosis with disruption of more than half of the villi, hemorrhage, and microvascular congestion (A). In rats that received MAb 1A29, ANS, or WEB-2086 before ET-1, mucosal destruction was partially prevented, and only lifting of epithelial cell layer, injury at villus tips, mucosal lymphangiectasia, and submucosal edema were observed (B–D). Hematoxylin and eosin staining. Magnification = ×200.
Table 2. Histological assessment of intestinal mucosal damage induced by ET-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Histological Damage Score</th>
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<tr>
<td>ET-1</td>
<td>2.16 ± 0.105</td>
</tr>
<tr>
<td>ET-1 + anti-ICAM</td>
<td>1.83 ± 0.105</td>
</tr>
<tr>
<td>ET-1 + ANS</td>
<td>1.73 ± 0.062***</td>
</tr>
<tr>
<td>ET-1 + WEB-2086</td>
<td>1.74 ± 0.166**</td>
</tr>
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Values are means ± SE; n = 4. The 0–3 grading scale for assessing injury was 0 = normal; 1 = injury to villus tips; 2 = loss of more than half of villi; and 3 = damage extending to submucosa or transmucosa. ET-1 was administered at a dose of 400 pmol·kg⁻¹·min⁻¹ alone or with a monoclonal antibody against intracellular adhesion molecule-1 (anti-ICAM), antineutrophil serum (ANS), or platelet-activating factor antagonist WEB-2086. For each animal, 3 intestinal segments (duodenum, jejunum, and ileum) were evaluated using histological scoring, and mean values were obtained for each animal. *P < 0.05, **P < 0.01 compared with ET-1 alone.

both techniques, we showed that ET-1 increases the photoemission observed with chemiluminescence in the rat small intestine, suggesting increased ROM production after ET-1 administration. The source of ROMs in this model of intestinal injury may include the activation of parenchymal cell-associated xanthine oxidase and/or activation of granulocyte-associated NADPH oxidase from granulocytes that normally reside in the intestinal mucosa. The association of xanthine oxidase-derived free radicals with ischemia-reperfusion injury has been well documented in many studies (11). Miura et al. (28) have recently shown that local infusion of ET-1 at 1 nmol/kg significantly decreases intestinal blood flow to 15% of control without changing systemic arterial pressure. It is conceivable that local microcirculatory disturbances induced by ET-1 could cause the activation of xanthine oxidase, an event that increases ROM production.

In addition to vasoactive properties, several ET-1-related mechanisms have been described as PMN properties, including Ca²⁺ mobilization (24), O₂⁻ production (17), adhesion to endothelial cells, and aggregation (10). Investigators have shown that increased ET-1 formation and PMN accumulation are coexisting phenomena in pathological circumstances such as ischemia-reperfusion injury (8) and endotoxin-induced microcirculatory disturbance (27). In the present study, by measuring the tissue-associated MPO activities, PMN infiltration was monitored in duodenal, jejunal, and ileal samples. We have demonstrated that intra-arterial administration of ET-1 at a dose of 400 pmol·kg⁻¹·min⁻¹ significantly increases the number of PMNs in all regions of small intestine, indicating a possible role of PMNs in ET-1-induced intestinal damage. Our results also indicate that the magnitude of PMN infiltration does not differ significantly between the regions of the small intestine exposed to the same doses of ET-1. The mechanisms by which ET-1 increases PMN accumulation in the small intestine remain unclear, but several possibilities have been proposed, including a direct activating effect of ET-1 on PMNs. ET-1 induces neutrophil adhesion to plastic surfaces, suggesting a predominant effect of ET-1 on the neutrophil site of the adhesion. It has been also shown that ET-1 stimulates neutrophil adhesion to cultured endothelial cells by an effect on the expression of CD18 and CD11b on the neutrophil surface and ICAM-1 on the endothelial cells (13, 23). Recent work by Boros et al. (3) provides direct in vivo evidence of ET-1-mediated leukocyte-endothelial cell interactions by demonstrating that ET-1 induces leukocyte rolling and adherence through a predominantly ET₂ receptor-mediated mechanism in the submucosal venules of the intestinal microcirculation. The authors proposed that microvascular flow-related alterations (low shear stress) and secondary mediators released during microvascular impairment may stimulate leukocyte-endothelial cell interactions. In that study, however, the dose of ET-1 that was used to induce leukocyte adhesion did not significantly increase microvascular permeability. Furthermore, it remains unclear whether ET-1-induced leukocyte adhesion eventually triggers the destructive properties of PMNs, leading to tissue injury. Lopez-Belmonte and Whittle (22) recently demonstrated that the ET-1-induced increase in gastric vascular albumin leakage is unaffected by pretreatment with antineutrophil serum, indicating that neutrophils are not involved in the gastric vascular dysfunction induced by ET-1. Although ET-1 is implicated as an important modulating agent for PMN functions, it is not entirely clear whether or not PMNs are a prerequisite for ET-induced tissue injury. The observation that ET-1 infusion significantly increases the mucosal permeability to a ⁵¹Cr-EDTA molecule at a dose of 400 pmol·kg⁻¹·min⁻¹ indicates that ET-1 may be an important modulator of events leading to intestinal mucosal barrier dysfunction. It is conceivable that permeability changes induced by ET-1 may be associated with activated tissue PMNs. To assess this possibility, we compared the mucosal permeability elicited by ET-1 in ANS-pretreated animals with those which received ET-1 infusion alone. ANS significantly reduced the ET-1-induced increases in both tissue MPO activity and mucosal permeability values, and significant differences in mucosal permeability were observed during the early measurement periods. The results of this analysis suggest that tissue PMNs are the important mediators of ET-1-induced mucosal dysfunction. Another approach to investigating the role of PMNs in ET-1-induced permeability changes involved the pretreatment of animals with the ICAM-1-specific MAb 1A29 before ET-1 infusion. ET-1-induced ICAM-1 expression has been previously demonstrated by investigators who showed that ET-1 increases ICAM-1 expression on the surface of brain microvascular cells and on the aortic endothelial cells (13). The administration of MAb 1A29 significantly lowered the clearance values during the first 30 min of our experiments compared with the ET-1 group alone. The antiadhesive effect of MAb 1A29 has been confirmed by tissue MPO measurements, which were found to be significantly less than untreated values. Furthermore, the degree of mucosal damage expressed by the histological index was found to be significantly lower in both ANS- and
MAb 1A29-treated animals than in untreated animals, indicating that PMNs play an important role in the induction of injury by ET-1. However, the observation that pretreatment with ANS and MAb 1A29 partially reduces the alterations in mucosal barrier function indicates that PMN-independent mechanisms must also be considered.

The lack of a significant effect of lower doses of ET-1 on mucosal permeability as well as tissue PMN accumulation and oxidant stress indicates that 400 pmol·kg⁻¹·min⁻¹ is a dose that produces marked inflammatory changes in the intestinal mucosa. In our experiments, 400 pmol·kg⁻¹·min⁻¹ intra-arterial infusion of ET-1 resulted in an ~25-fold increase in portal blood ET-1 levels at 30 min after its administration. It is generally accepted that ET-1 is mainly released from the endothelial cell abluminally (30), and serum concentrations may be poor indicators of actual local tissue concentrations in pathophysiological conditions. However, a prolonged, ~15- to 30-fold increase in plasma ET levels has also been reported after endotoxin-induced microcirculatory disturbances in the rat small intestine and liver (7, 27), which were comparable to increased levels of ET-1 in the present study.

To investigate the role of PAF in ET-1-induced alterations in mucosal permeability, we pretreated the rats with PAF receptor antagonist WEB-2086 before ET-1 infusion. Our results indicate that administration of WEB-2086 significantly lowers the ET-1-induced changes in clearance values without altering the ET-1-induced increase in tissue MPO measurements. These results are consistent with the previous observations indicating that PAF antagonists inhibit ET-1-induced intestinal damage (28) and gastric vascular leakage of albumin, an effect that is suggested to be neutrophil independent (22). Recent work from Miura et al. (27) indicates that increased PAF production in endotoxin-induced microcirculatory disturbance is closely associated with increased ET-1 levels through the ET₄ receptor in rat small intestine. Because ET₄ receptor antagonist BQ-123 is effective in attenuating the endotoxin-induced production of PAF, the authors suggest that ET-1 may directly induce PAF production and/or that PAF production is increased as a consequence of mucosal damage induced by ET-1. Although PAF is known as a potent mediator of the PMN accumulation, considering the effect of WEB-2086 on time course changes in mucosal permeability (especially evident during the second half of the experiment) and its inefficacy on elevated tissue MPO activity normally observed after ET-1 administration, it is conceivable that there may be other factors that are responsible for ET-1-induced PMN accumulation in the small intestine. Moreover, it is also possible that PMN-independent systems are activated (e.g., protease release) as the injury process continues and that tissue factors are released such as PAF or cytokines, which may directly or indirectly cause the release of toxic metabolites (14, 16, 19). Finally, it is likely that an agent that activates Ca²⁺ influx into cells also exerts a major effect on other inflammatory cells, including mast cells and platelets, all of which can release substances that may cause microvascular dysfunction (20). Recent work from Shigematsu et al. (31) indicates that ET-1 plays an important role in mast cell-mediated responses via ETA receptors in intestinal anaphylaxis. Yamamura et al. (37) have demonstrated that cultured mouse bone marrow-derived mast cells are capable of releasing histamine and leukotriene C₄ in response to ET-1 stimulation via ETA receptors. They also found that ET-1 is one of the most potent histamine-releasing factors in mouse peritoneal mast cells discovered to date. Additional studies are required to test the validity of these mechanisms on ET-1-induced intestinal mucosal dysfunction.

In summary, we have demonstrated that neutrophil depletion and blockage of ICAM-1 attenuates the increased mucosal permeability and microscopic damage observed in the rat small intestine following ET-1 administration. This indicates that neutrophils play an important role in mediating the mucosal dysfunction elicited by ET-1. Moreover, our results also demonstrate that ROM production and mucosal barrier dysfunction are important properties of ET-1-induced intestinal inflammation. Finally, increased mucosal permeability observed in the rat small intestine following ET-1 administration involves a mechanism that includes a role for PAF.

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