Endothelin receptor blockers reduce I/R-induced intestinal mucosal injury: role of blood flow

BERNA K. OKTAR,1 M. ALI GÜLPINAR,3 AYHAN BOZKURT,1 SALAH GHANDOUR,1 ŞULE ÇETINEL,2 HADI MOINI,3 BERRAK Ç. YEĞEN,1 SERPİL BILSEL,3 D. NEIL GRANGER,4 AND HIZIR KURTEL1

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

Received 28 September 2000; accepted in final form 24 August 2001

Oktar, Berna K., M. Ali Gülpinar, Ayhan Bozkurt, Salah Ghandour, Sule Çetinel, Hadi Moini, Berrak Ç. Yeğen, Serpil Bilsel, D. Neil Granger, and Hizir Kurtel. Endothelin receptor blockers reduce I/R-induced intestinal mucosal injury: role of blood flow. Am J Physiol Gastrointest Liver Physiol 282: G647–G655, 2002.—The aim of the present study was to assess the role of endothelin (ET) in ischemia-reperfusion (I/R)-induced mucosal injury. Mucosal permeability (51Cr-EDTA clearance) and tissue myeloperoxidase (MPO) activity were significantly increased after 30 min of ischemia followed by 30 min of reperfusion. The I/R-induced increases in mucosal permeability and polymorphonuclear leukocyte (PMN) infiltration were significantly attenuated by pretreatments with ETA (BQ-485) and/or ETB (BQ-788) receptor antagonists. Monoclonal antibody (MAb) directed against intercellular adhesion molecule-1 (ICAM-1; MAb 1A29) and superoxide dismutase (SOD) pretreatments significantly attenuated the increased mucosal permeability and PMN infiltration in a similar manner as with ET receptor antagonists. Superior mesenteric artery blood flow was significantly reduced during the reperfusion period. Both ET receptor antagonists caused a significant rise in blood flow compared with an untreated I/R group. In conclusion, our data suggest that ETA and/or ETB receptors, ICAM-1, and superoxide dismutase play an important role in I/R-induced mucosal dysfunction and PMN infiltration. Furthermore, ET is involved in the pathogenesis of postreperfusion-induced damage and beneficial effects of ET receptor antagonism are related to an improvement of disturbed blood flow during the reperfusion period.

mucosal permeability; mucosal injury; BQ-485; BQ-788; superoxide dismutase

THE NATURE OF INFLAMMATORY response after reperfusion of the ischemic intestine involves alterations in capillary filtration rate (lymph flow), vascular protein leakage, and interstitial edema. These changes are associated with accumulation of inflammatory cells and increases in several chemical mediators such as reactive oxygen metabolites (ROMs), platelet-activating factor (PAF), and leukotriene B4 (LTB4) (12). Although inflammatory cell-mediated microcirculatory disturbances seem to be an early event in ischemia-reperfusion (I/R)-induced mucosal injury, the mechanisms underlying these disturbances are not clearly understood. The potent and long-lasting vasoconstriction (37), increased polymorphonuclear leukocyte (PMN) infiltration (3), and microvascular and mucosal dysfunction (30) produced by endothelins (ET) raise the possibility that the peptide may contribute to the pathogenesis of I/R-induced gastrointestinal damage.

There are three major lines of evidence that implicate ET as a mediator of reperfusion-induced damage: 1) ET levels increase during the I/R period; 2) ET receptor antagonists provide significant protection (16, 29); and 3) reactivity of blood vessels to ET is enhanced during IR (37). Although evidence is available to support a role for ET-1 in the pathogenesis of I/R-induced gastrointestinal injury, several important issues need attention. First, the role of ET peptides in mediating I/R-induced increase in mucosal permeability is not known. 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 the circulation, causing sepsis with possible multiple organ failure (14). We recently demonstrated (30) that infusion of ET-1 into the superior mesenteric artery (SMA) causes a dose-dependent increase in mucosal permeability and PMN infiltration, indicating that ET-1 plays an important role in the induction of mucosal dysfunction. Therefore, whether ET contributes to the disturbances in the mucosal integrity observed after intestinal I/R should be resolved.

Second, increased ET-1 formation and PMN accumulation are coexistent phenomena in I/R injury, and determination of a functional interaction between these variables is worthy of exploration in the gastrointestinal system. Third, although ET antagonism has been found to be consistently effective, relatively little is known about the contribution of ETB receptors to I/R-induced intestinal injury. Treatments with ET an-

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.
tibodies (21), ET-converting enzyme inhibitors (15), nonselective ET<sub>A</sub> and ET<sub>B</sub> antagonists (16), and the ET<sub>A</sub> receptor antagonist BQ-123 (29) all lead to improved posts ischemic tissue function in both rat and dog models of gastric and intestinal ischemic failure. However, the role of ET<sub>B</sub> receptors in the pathobiology of I/R injury has not been extensively studied, and this issue warrants attention. It is also noteworthy that ET may be involved in the incomplete restoration of blood flow seen after ischemia and that ET antagonism may provide significant protection against I/R-induced malperfusion. Accordingly, the objectives of this study were to determine whether: 1) ET<sub>A</sub> and/or ET<sub>B</sub> receptor antagonism attenuates or prevents the increased mucosal damage and PMN infiltration normally associated with intestinal I/R; 2) responses obtained from pretreatments with ET receptor antagonists correlate with the results elicited by anti-intercellular adhesion molecule-1 (ICAM-1) antibody and superoxide dismutase (SOD) pretreatments; and 3) any beneficial effects of ET receptor antagonism are related to an enhancement of blood flow during the reperfusion period.

MATERIALS AND METHODS

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 h and fed a standard diet and water ad libitum. Studies were approved by the Marmara University Animal Use and Care Committee. After 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 (Nihon 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 body temperature was maintained at 37°C by a heating pad. A midline abdominal incision was made, and the SMA was isolated and occluded with a micro-bulldog clamp for periods of 0 (sham controls), 5, 10, 20, and 30 min, followed by 30 min of reperfusion. At the end of the reperfusion period, blood samples were collected from the portal vein for subsequent determinations of plasma ET-1 levels. In sham-operated animals, the experimental protocol was performed in a similar fashion but SMA blood flow was not occluded.

Mucosal permeability measurements. Changes in mucosal permeability were assessed using the blood-to-lumen clearance of <sup>51</sup>Cr-EDTA (NEN, Boston, MA). Briefly, after an abdominal incision was made, a jejunal segment (at 15 cm distal to ligament of Treitz) 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, <sup>51</sup>Cr-EDTA (100 μCi) in saline was administered intravenously through the jugular vein as a bolus 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 I/R. Blood samples (0.3 ml) from the carotid artery were taken at the beginning and the end of experiments 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 <sup>51</sup>Cr-EDTA was calculated as (5) clearance = cpm<sub>pl</sub> × PR / 100/cpm<sub>pl</sub> × wt, where clearance of <sup>51</sup>Cr-EDTA is given in milliliters per minute per 100 g, cpm<sub>pl</sub> is counts per minute per milliliter of perfusate, PR is the perfusion rate, cpm<sub>pl</sub> is counts per minute per milliliter of plasma, and wt is weight of the intestine in grams.

In addition, the volume of luminal perfusate was measured at the times indicated above to determine alterations in net transmucosal fluid flux. This calculation was made by subtracting the amount of fluid entering the intestine from the amount collected from the distal end over a 10-min perfusion period. Net transmucosal fluid flux is expressed as milliliters per minute per 100 g of tissue weight. A positive value indicates net fluid absorption, and a negative value indicates net secretion.

In the untreated I/R group, mucosal permeability measurements involved a fixed duration of ischemia (30 min) followed by 30 min of reperfusion. In another group of animals, 5 min before the ischemia the ET<sub>A</sub> receptor antagonist BQ-485 (150 nmol/kg bolus and 10 nmol·kg<sup>-1</sup>·min<sup>-1</sup> infusion; donated by Banyu Pharmaceutical) and/or the ET<sub>B</sub> antagonist BQ-788 (150 nmol/kg bolus and 10 nmol·kg<sup>-1</sup>·min<sup>-1</sup> infusion; purchased from Sigma, St. Louis, MO) was administered and infusions were continued throughout the 30-min reperfusion period. Our preliminary experiments showed that 150 nmol/kg bolus administration of either ET<sub>A</sub> or ET<sub>B</sub> antagonists without continued infusion did not significantly alter I/R-induced responses in the small intestine. The doses of ET receptor antagonists used in the present study were comparable to the doses in studies obtaining significant protective effects in changes of hemodynamics in the rat small intestine (34) and liver (33) caused by endotoxemia. Additional mucosal permeability experiments were performed by using monoclonal antibody (MAb) directed against ICAM-1 (MAb 1A29; 2 mg/kg iv bolus; produced by Upjohn, Kalamaizo, MI) or SOD (10,000 U/kg bolus and 10,000 U·kg<sup>-1</sup>·h<sup>-1</sup> infusion; Sigma) pretreatments to compare with the results obtained from ET antagonists. The concentration of MAb used in this study was the amount needed to maximally reduce the leukocyte adherence and emigration induced by LTB<sub>4</sub> or PAF in rat mesenteric venules (23).

Tissue myeloperoxidase measurements. Tissue-associated myeloperoxidase (MPO) activity was determined in 0.2- to 0.5-g samples obtained from the intestinal segment that was used for mucosal permeability measurements. The tissue samples were homogenized in 10 vols of ice-cold potassium phosphate buffer (20 mM K<sub>2</sub>HPO<sub>4</sub>, 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 resuspended with an equivalent volume of 50 mM K<sub>2</sub>HPO<sub>4</sub>, containing 0.5% (wt/vol) hexadecyltrimethylammonium hydroxide (Sigma). MPO activity was assessed by measuring the H<sub>2</sub>O<sub>2</sub>-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<sup>-1</sup> at 460 nm and 37°C (4).

ET assay. Blood samples were collected from the portal vein at 30 min of reperfusion after 5, 10, 20, or 30 min of ischemia in chilled polypropylene tubes containing 1 mg/ml EDTA and 500 KIU/ml aprotinin (Trasyloil; Bayer). The blood samples were centrifuged at 4°C, and the plasma samples
were stored at −80°C until they were assayed. Immunoreactive ET-1 concentration was assayed using a commercially available RIA kit (RIK 6901; Peninsula Laboratories). Media was extracted with Sep-Pak C₁₈ cartridges and evaporated by blowing nitrogen gas. The reconstituted solution was applied to the RIA. According to the manufacturer, the intra-assay coefficient of variation is <5% and the interassay coefficient of variation is <15%. The cross-reactivity of the kit with human Big ET-1 is 17% and with human ET-2 and -3 is 7%.

Blood flow measurements. In groups used for hemodynamic studies, blood flow in the SMA was measured by the ultrasonic transit time technique as previously described (8). A 1RB 1-mm diameter probe (Transonic Systems, Ithaca, NY) was used. The probe houses two ultrasonic transducers and a fixed acoustic reflector. The transducers pass ultrasonic signals back and forth, alternately intersecting the flowing liquid in upstream and downstream directions. A short length (5 mm) of the SMA was isolated proximal to the superior mesenteric nerve plexus for flow probe placement. All fatty tissue was removed from the isolated segment of the vessels to avoid obstruction of the ultrasonic signal. A microbulldog clamp was placed around the SMA just distal to the nerve plexus to obtain complete mechanical occlusion of blood flow. The flowmeter (model T106; Transonic Systems) subtracts the downstream transit time estimating the volume of blood flow, which is expressed in milliliters per minute per 100 g of tissue. Mean arterial pressure (MAP) was recorded simultaneously with the blood flow using a Nihon Kohden multichannel recorder (model AP-621G). Resistance of SMA (expressed as mmHg·ml⁻¹·min⁻1·100 g) was calculated by dividing MAP in millimeters of mercury by blood flow. Hemodynamic studies were performed in untreated animals exposed to 30 min of I/R or in animals treated with ETₐ and/or ETₐ receptor antagonists before intestinal I/R.

Histological analysis. Samples from the standardized regions of the duodenum, jejunum, and ileum were fixed with 10% 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 0–4 grading scale was used as follows: 0 = normal histology; 1 = slight disruption of the surface epithelium; 2 = epithelial cell loss injury at villus tip; 3 = mucosal vasocongestion, hemorrhage, and focal necrosis with loss of more than one-half of villi; and 4 = damage extending to more than half of villi. The final score for each animal was determined from the means of scores of sections obtained from duodenum, jejunum, and ileum. All assessments of damage were performed by an observer unaware of the treatment.

Statistics. All values are reported as means ± SE. One-way analysis of variance with the Newman-Keuls post hoc test was used to determine whether data from different groups were statistically different. \( P < 0.05 \) was considered statistically significant.

RESULTS

ET-1 levels. The purpose of our first experiments was to examine the effect of varying duration of ischemia on ET-1 levels. ET-1 levels were measured in portal plasma samples obtained at 30 min of reperfusion after different periods of ischemia (5, 10, 20, 30 min). ET-1 concentration in the sham-operated group in the portal plasma was 28.16 ± 5.15 pg/ml. Among the I/R groups, ET-1 levels were found to be significantly elevated only in the 30-min ischemia group (122.78 ± 25.88 pg/ml) compared with the sham-operated group. Shorter durations of ischemia did not significantly change the ET-1 levels (data not shown). All further studies were done using 30-min ischemia followed by 30-min reperfusion. Pretreatment of animals with anti-ICAM MAb (1A29) or SOD significantly reduced the elevated ET-1 levels after I/R (Fig. 1).

Mucosal permeability. Figure 2 shows the time-dependent changes in mucosal permeability during 30 min of ischemia (Fig. 2A) and 30 min of reperfusion (Fig. 2B). In sham-operated animals, basal \(^{51}\text{Cr}-\text{EDTA} \) clearance did not significantly change throughout the entire study period. Clearance values significantly increased after the induction of ischemia and remained elevated at approximately four times the preischemia values for the next 30 min. Pretreatment of animals with ETₐ receptor antagonist (BQ-485) reduced the elevated clearance values during the entire measurement period of ischemia. Similarly, pretreatment of animals with ETₐ receptor antagonist alone (BQ-788), ETₐ plus ETₐ receptor antagonists, anti-ICAM MAb 1A29, or SOD reduced the mucosal permeability values, which were significantly decreased at 20 and 30 min after occlusion of SMA.

Reperfusion of the ischemic bowel after 30 min of ischemia significantly increased the mucosal permeability (~6- to 25-fold) throughout the reperfusion period compared with the corresponding sham-operated group \((P < 0.01)\). Pretreatment of animals with SOD significantly attenuated the reperfusion-induced increases in mucosal permeability during the entire measurement period of reperfusion. Similarly, MAb 1A29 pretreatment reduced the clearance values during the

---

Fig. 1. Plasma endothelin (ET)-1 concentrations of portal blood in sham-operated \((n = 7)\) and untreated ischemia-reperfusion \((\mathrm{I/R})\); 30-min ischemia + 30-min reperfusion; \(n = 10\) groups and in animals pretreated with monoclonal antibody (MAb) against intercellular adhesion molecule-1 (anti-ICAM; \(n = 6\)) or superoxide dismutase (SOD; \(n = 6\)) before the induction of I/R. Data are expressed as means ± SE. \(*P < 0.05\) compared with sham-operated group; \(P < 0.05\) compared with untreated group.

AJP-Gastrointest Liver Physiol • VOL 282 • APRIL 2002 • www.ajpgi.org
reperfusion period, during which significant decreases were observed at 10 and 30 min of reperfusion. Comparable decreases in mucosal permeability during reperfusion were also observed in animals pretreated with ET_A and ET_B receptor antagonists. When both antagonists were administered together, permeability measurements were further reduced to values that were significantly different compared with the measurements obtained from ET_A or ET_B receptor antagonists alone.

Transmucosal fluid flux. Net fluid flux in the sham group was $-0.18 \pm 0.31 \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1}$, indicating slight fluid secretion into the intestinal lumen. Net fluid loss into the bowel lumen significantly increased after 30 min of ischemia ($-2.1 \pm 0.33 \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1}; P < 0.05$) and 30 min of reperfusion ($-1.7 \pm 0.4 \text{ml} \cdot \text{min}^{-1} \cdot \text{100 g}^{-1}; P < 0.05$) compared with the sham-operated group, indicating the presence of either reduced absorptive capacity or increased secretion. When the relationship between the net transmucosal water flux and blood-to-lumen clearances of $^{51}$Cr-EDTA was investigated, no correlation was found between these parameters ($r = -0.14$). Pretreatment with MAb 1A29, SOD, or ET receptor antagonists did not significantly alter the I/R-induced increases in fluid secretion (data not shown).

MPO activity. Figure 3 compares the tissue-associated MPO responses to I/R in untreated animals and in animals pretreated with ETA receptor antagonist BQ-485, ET_B receptor antagonist BQ-778, anti-ICAM MAb 1A29, or SOD. I/R alone produced a significant increase in jejunal MPO activity compared with the sham-operated group ($P < 0.01$). The response to I/R was significantly reduced ($P < 0.05$) with all above-mentioned pretreatments compared with the untreated I/R group, indicating that PMNs might be an important regulator of I/R-induced mucosal injury. MPO activity obtained from coadministration of ET_A and ET_B receptor antagonists was found to be significantly different ($P < 0.05$) from the anti-ICAM, SOD, and ETA receptor antagonist pretreatment groups.

Microscopic assessment of intestinal injury. The degree of I/R-induced mucosal damage expressed as a microscopic damage score is shown in Fig. 4. In the sham-operated group, microscopic assessment of the tissue revealed slight abnormalities, including disruption of the surface epithelium and epithelial cell loss in some areas. I/R caused significant ($P < 0.05$ vs. sham

![Image](http://ajpgi.physiology.org/Download/10.1152/ajpgi.00165.2001)

Fig. 2. Effect of pretreatments with ET_A receptor antagonist BQ-485 (n = 7), ET_B receptor antagonist BQ-778 (n = 7), ET_A + ET_B receptor antagonists (BQ-485 + BQ-778; n = 6), anti-ICAM-1 MAb (n = 6), or SOD (n = 6) on $^{51}$Cr-EDTA clearance across intestinal mucosa during ischemia (A) and ischemia (30 min) followed by reperfusion (B). Data are expressed as means ± SE. *$P < 0.05$, **$P < 0.01$ compared with corresponding time point of sham-operated group; \( ^*P < 0.05, \ ^{^*}P < 0.01 \) compared with corresponding time point of untreated ischemia or reperfusion group; \( ^{\circ}P < 0.05 \) compared with corresponding time point of BQ-485 + BQ-788 group.

![Image](http://ajpgi.physiology.org/Download/10.1152/ajpgi.00165.2001)

Fig. 3. Intestinal myeloperoxidase activities in sham-operated group (n = 7) or in I/R groups without any treatment (untreated; n = 10) or treated with ET_A receptor antagonist BQ-485 (n = 7), ET_B receptor antagonist BQ-778 (n = 7), ET_A + ET_B receptor antagonists (BQ-485 + BQ-778; n = 6), anti-ICAM-1 MAb (n = 6), or SOD (n = 6). Data are expressed as means ± SE. \( ^*P < 0.01 \) compared with sham; \( ^{\circ}P < 0.05, \ ^{^*}P < 0.01 \) compared with untreated group; \( ^{\circ}P < 0.05 \) compared with BQ-485 + BQ-788 group.

AJP-Gastrointest Liver Physiol • VOL 282 • APRIL 2002 • www.ajpgi.org
pretreatment, the decrease in blood flow was evident only at 10 min of reperfusion ($P < 0.05$ compared with baseline). However, after BQ-485 pretreatment, the decrease in blood flow was evident at 15, 20, 25, and 30 min of reperfusion ($P < 0.05$ and 0.01, compared with baseline). Overall, BQ-485 administration reversed the reductions in blood flow compared with the untreated I/R group, in which significant elevations were observed at 15, 20, 25, and 30 min of reperfusion ($P < 0.05$ and 0.01). Pretreatment of animals with BQ-788 caused a significant rise in blood flow at the beginning of reperfusion ($P < 0.05$ compared with baseline), and then blood flow values leveled off at approximately the baseline values during the rest of the experiment. The blood flow measurements in this group were significantly higher at all time points compared with the corresponding time points of the untreated I/R group ($P < 0.05$ and 0.01). Similarly, administration of ETA plus ETB antagonists significantly elevated the reductions in blood flow at 20, 25, and 30 min of reperfusion ($P < 0.05$ and 0.01) compared with the untreated I/R group. There was no statistical significance between groups pretreated with ETA and/or ETB receptor antagonists. Similar results were obtained from the resistance data, in which I/R-induced increases in SMA resistance were significantly reduced by both ET receptor antagonists (Fig. 5B).
DISCUSSION

The results of the present study demonstrate that: 1) I/R-induced mucosal dysfunction and PMN infiltration are significantly attenuated by the blockade of ET receptors; 2) anti-ICAM-1 MAb and SOD pretreatments significantly attenuated the increased mucosal permeability and PMN infiltration in a similar manner as ET receptor antagonists; and 3) administration of ET_A and/or ET_B receptor antagonists largely prevented the reperfusion-induced reduction in blood flow. Our findings suggest that ET modulates responses to I/R and that beneficial effects of ET receptor antagonism are related to an improvement in the disturbed blood flow during the reperfusion period.

We found that in the studied I/R periods only 30-min ischemia followed by 30 min of reperfusion led to a significant increase in portal plasma ET-1 levels. Shorter durations of ischemia followed by the same period of reperfusion did not significantly change plasma ET-1 levels. This indicates that duration of ischemia is an important factor in regulating ET metabolism. Our results are consistent with those of previous investigators who reported that hypoxia and ischemia are the most potent stimulants of ET production (22). The increased plasma ET-1 concentration may reflect either decreased clearance or increased production of this peptide. It was demonstrated previously that ET-1 injected intravenously is mainly taken up by the lung, the liver, and the kidney (9, 10, 32). Therefore, in the present study elevated ET-1 levels in portal blood after I/R would be associated with increased ET-1 production rather than decreased clearance of plasma ET-1.

The results of our study support the view that ROMs and leukocyte-endothelial cell adhesion are involved in I/R injury. First, we found that I/R-induced increases in mucosal permeability can be significantly reduced by pretreatments with both SOD and anti-ICAM MAb. Second, we found that administration of SOD and MAb 1A29 attenuated the I/R-induced increases in PMN infiltration and microscopic damage. These results are consistent with the hypothesis that superoxide generation and leukocyte-endothelial cell interactions are involved in the pathobiology of I/R injury. We have also demonstrated that a comparable protection against I/R injury can be obtained when animals are pretreated with ET_A and ET_B receptor antagonists. Both antagonists were effective in attenuating I/R-induced increases in mucosal permeability as well as PMN infiltration and microscopic injury, indicating that ET is an important modulator of I/R-induced tissue injury. Additionally, I/R-induced alterations in these parameters were further attenuated by combined administration of antagonists, indicating an additive effect.

The observation that ICAM-1 MAb and SOD pretreatments significantly reduced the elevated ET-1 levels after I/R indicates that superoxide radical and leukocyte-endothelial cell interactions are related to I/R-induced ET-1 production. Because hypoxia seems to be an important contributing factor in ET-1 production and release, it is conceivable that enhanced microvascular disturbance as a result of impaired release of endogenous vasodilators or increased leukocyte-endothelial cell interactions would exacerbate ET-1 production, leading to enhanced tissue injury. Coincident with the rise in superoxide formation after I/R is a significant decrease in the concentration of nitric oxide (NO), a potent vasodilator produced by endothelial cells. It has been suggested that the balance between locally produced relaxing factors (e.g., NO) and ET-1 plays an important role in the maintenance of mucosal microcirculation (36). Evidence also suggests that PMNs are involved in the inadequate reperfusion of microvessels in many postischemic organs despite the presence of adequate perfusion pressure, a phenomenon known as “no reflow” (1). Thus it is possible that the activated and damaged endothelial cells in the intestinal microcirculation caused by hypoxia and/or leukocyte adherence may further enhance local production of ET-1. This could explain why SOD and MAb 1A29 pretreatments reduce the increased ET-1 levels associated with I/R. Alternatively, several other factors including ROMs, cytokines, and PMNs related to I/R may mediate ET-1 production by vascular endothelium. It has been shown that ROMs generated from hypoxanthine/xanthine oxidase cause an increase in ET receptor density in cultured stellate cells, specifically in the ET_B receptor subtype, and this effect is coupled to the enhanced release of ET-1 from these cells (11). Cytokines such as interleukin-1 and tumor necrosis factor, both of which may be upregulated by I/R, stimulate ET-1 production and release from endothelial cells (35). PMNs per se can influence local levels of ET, either by producing or degrading the peptide (20). The exact roles of these mechanisms in I/R-induced changes in ET-1 production require further attention.

Although the involvement of ET receptors in I/R injury does not explain the mechanisms of injury itself or how the injury is initiated, it is possible that ETs may be involved in the I/R-induced PMN infiltration that results in subsequent tissue injury. This is supported by the observation that ET receptor antagonists used in the present study to attenuate I/R injury were also effective in reducing I/R-induced PMN infiltration. Several reports in the literature address the potential role of ET in modulating PMN functions. It has been demonstrated that administration of ET-1 into the SMA causes PMN infiltration in the rat small intestine and increases mucosal permeability to the $^{51}$Cr-EDTA molecule. When animals are rendered neutropenic with anti-neutrophil serum, ET-1-induced increases in mucosal permeability are significantly attenuated, indicating that PMNs are important mediators of ET-1-induced mucosal dysfunction (30). Recent work by Boros et al. (3) provides direct in vivo evidence that ET-1 induces leukocyte rolling and adherence through a predominantly ETA receptor-mediated mechanism in the submucosal venules of the intestinal microcirculation. It has been proposed that ET-induced alterations in microvascular flow (low shear stress) and secondary
mediators (e.g., PAF, LTB₄) released during microvascular impairment may stimulate leukocyte-endothelial cell interactions, leading to PMN infiltration. However, in the study of Boros et al. (3), the ETB receptor antagonist was partially effective in reducing ET-1-induced leukocyte adherence in the intestinal microcirculation. Importance of the ETA receptor has also been implicated by Massberg et al. (28), demonstrating that ET-1-induced perfusion failure and mucosal damage in the rat small intestine are markedly reduced by an ETA receptor antagonist. In contrast, an ETB receptor antagonist was not effective. Our observations suggest that both ETA and ETB receptors are involved in the pathogenesis of I/R-induced PMN infiltration and mucosal dysfunction. It should be emphasized that induction of mucosal injury by exogenously infused ET-1 is expected to be different from the action of endogenously released ETs. Furthermore, ET peptides other than ET-1 may also be involved in the injury process.

It is conceivable that fluid secretion into the lumen may promote a leaky mucosal barrier, thereby increasing mucosal permeability. It has been suggested that ETs may participate in the changes of intestinal transport across the gut wall through their effects on the release of mast cell mediators or neurotransmitters. It has been demonstrated that treatment with ETA receptor antagonist attenuates the increase in net water outflux, histamine release, and serum rat mast cell protease II concentrations in rats with anaphylaxis in the jejunal mucosa (34). Direct effects of ETs on ion transport have also been proposed by investigators showing that ETA and ETB receptors regulate Na⁺ and Cl⁻ transport by different mechanisms in the colonic mucosa of rats (19). In the present study, net fluid loss into the bowel lumen was significantly increased after I/R. However, there was no correlation between transmucosal fluid flux and permeability measurements. Furthermore, I/R-induced increases in net fluid flux into the bowel lumen were not altered by pretreatments with ET receptor antagonists, SOD, or anti-ICAM MAb. These results suggest that mechanisms other than net water flux must be considered to explain the beneficial effects of ET receptor antagonists or SOD and anti-ICAM on mucosal permeability.

The incomplete restoration of blood flow after ischemia is thought to be an important contributing factor to reperfusion-induced injury (13). Obstruction of microvessels by vasoconstriction is one possible factor that explains the decreased blood flow after I/R. We found that there was a significant reduction in SMA blood flow during reperfusion. Coincident with the decrease in blood flow during reperfusion there was also a significant rise in vascular resistance. The increase in vascular resistance may in fact be a result of enhanced ET activity, because ET receptors are associated with vasoconstriction. It is generally excepted that ETA receptors expressed in smooth muscle are associated with vasoconstriction and are more selective for ET-1 than other peptides. ETB receptors are non-selective and interact with ET-1 and ET-3 with equal affinity. ETB receptors expressed on endothelial cells cause a short-lasting release of NO and prostacyclin that is associated with a transient vasodilator response, especially on intraluminal stimulation with ET (2, 6, 25). On the other hand, ETB receptors expressed in vascular smooth muscle are associated with ET-induced vasoconstriction (17, 26). In the perfused mesenteric vasculature of the rat, D’Orléans-Juste et al. (7) showed that the vasoconstrictor effects of ET-1 are mediated predominantly by the activation of ETA receptors. ETB receptor agonists induce weak constrictions of the venous mesenteric vasculature and are inactive in the arterial site. In another study, Kurose et al. (24) investigated the microvascular responses to ET-3 in rat mesentery under fluorescence microscopy. Exogenous administration of ET-3 induced arteriolar constriction in a dose-dependent manner and increased the macromolecular leakage in mesenteric venules. ETA receptor antagonism was effective in inhibiting the arteriolar constriction, whereas ET-3-induced elevation in vascular permeability was unaffected by the ETA receptor antagonist. These observations indicate that the differential susceptibility to regional vascular beds is an important issue that requires further attention because ET-induced arteriolar vasoconstriction would increase precapillary resistance and consequently decrease capillary pressure, whereas venular constriction would lead to elevated capillary pressure, thereby facilitating fluid and protein extravasation.

The ETB receptor has recently been implicated in pathophysiological conditions such as endotoxemia by investigators demonstrating that the ETA receptor antagonist did not attenuate circulatory failure and liver dysfunction during endotoxemia but the ETB antagonist was effective in the septic shock model (33). The authors suggested that the observed effects of ETB receptor antagonist on liver integrity were due to an improvement in hepatic blood flow secondary to inhibition of presinusoidal constriction.

In the present study, pretreatment with ETA and/or ETB receptor antagonists caused a significant rise in blood flow compared with the untreated I/R group, indicating that ET is involved in the pathogenesis of postreperfusion damage through microcirculatory disturbances. There was no additive effect of ETA plus ETB receptor antagonists on hemodynamic parameters, probably because each antagonist alone produced a complete restoration of blood flow and resistance. However, after combined administration of ETA and ETB receptor antagonists an additive effect was observed on I/R-induced increases in tissue PMN infiltration, mucosal permeability, and microscopic damage, suggesting that in addition to blood flow restoration, other mechanisms during reperfusion may also be involved in the protective effects of these antagonists. The vasoconstriction produced by ET receptors is related to the ability of these receptors to stimulate phospholipase C, which leads to the formation of inositol triphosphate and diacylglycerol. The former increases the intracellular calcium concentration, which in turn causes the vasoconstriction (27). On the other hand, these receptors may mediate many other com-
plex cellular actions, producing important physiological effects such as increased inflammatory mediator production, microvascular permeability, PMN activation, and release of vasodilator or vasoconstrictor substances (25). Therefore, individual mechanisms of ETA and ETB receptors in mediating decreased intestinal injury after I/R remain to be determined.

It was recently demonstrated in mechanically perfused ex vivo segments of canine stomach that the magnitude of ET-1-induced vasoconstriction in the stomach is enhanced after I/R (37). In that study, the vascular responses to ET-1 were significantly greater after I/R compared with the response under control conditions. Because administration of SOD during reperfusion attenuated the augmented response to ET-1, it has been suggested that generation of ROMs contributes to altered gastric vascular reactivity to ET-1. Thus it is conceivable that, in addition to increased release of ETs, increased sensitivity of vessels to this peptide during reperfusion may also account for the reperfusion-induced microcirculatory disturbances. Either way, we demonstrated that ET receptors are related to an improvement in the disturbed blood flow during the reperfusion period.

Vascular beds throughout the body exhibit elevated blood flow period after transient occlusion of an artery. This phenomenon of vasodilatation after transient ischemia-reactive hyperemia has been clearly demonstrated in the intestinal circulation (31). In the present study, the reperfusion period was associated with a decrease in blood flow and an increase in vascular resistance that were particularly apparent after 15 min of reperfusion. We observed that the ETB receptor antagonist elevated the postischemic blood flow compared with its baseline and that elevation in blood flow was significantly higher than that of the untreated group throughout the reperfusion period. However, this initial hyperemic response was not statistically significant after ETA receptor antagonist pretreatment. Instead, it presented a delayed effect on blood flow during the reperfusion period. Although reactive hyperemic response has been suggested to be beneficial in some inflammatory conditions such as damage induced by topical irritants (18), its role in I/R injury is not clear. Moreover, it is difficult to suggest that ETB receptors are more potent in the control of postischemic blood flow than ETA receptors. It should be emphasized that the distribution and function of ET receptor subtypes in different organs and pathological conditions remain uncertain.

In conclusion, our results suggest that both ETA and ETB receptors play important roles in I/R-induced mucosal dysfunction and PMN infiltration. In addition, because administration of ETA and ETB receptor antagonists largely prevented the reperfusion-induced reduction in blood flow, our findings indicate that ET are involved in the pathogenesis of reperfusion-induced damage and that the beneficial effects of ET receptor antagonism are related in part to an enhancement of blood flow during the reperfusion period.

We thank Dr. Julian Panes (Dept of Gastroenterology, Hospital Clinic, University of Barcelona, Spain) for the generous gift of ICAM-1 monoclonal antibody (Mab 1A29). This work was supported by National Institutes of Health Grants TW-00755 and HL-26441. Preliminary data were presented at the 100th Annual Meeting of the American Gastroenterological Association, May 16–19, 1999, Orlando, FL (see Ref. 30a).

REFERENCES
18. Holzer P, Pabst MA, Lippe IT, Peskar BM, Peskar BA, Livingston EH, and Guth PH. Afferent nerve-mediated pro-

AJP-Gastrointest Liver Physiol • VOL 282 • APRIL 2002 • www.ajpgi.org


