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THE ROLE OF ENDOTHELIN-1 IN CIRCULATORY CHANGES DURING HYPODYNAMIC SEPSIS IN THE RAT

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ABSTRACT—Our objective was to investigate the significance of endogenous endothelin-1-induced systemic circulatory reactions during hypodynamic sepsis. In the first part of this study, we observed the changes in global hemodynamic parameters in Wistar rats after exogenous endothelin-1 administration in order to test an intervention strategy aimed at preventing the development of hypodynamic cardiovascular derangement during intraabdominal sepsis. Cardiac output, mean arterial blood pressure, and peripheral vascular resistance were recorded, and the endothelin-A receptor antagonist BQ-610 and the endothelin-B receptor antagonist IRL-1038 were used to investigate the role of receptor subtypes in circulatory changes. In addition, the effects of treatment with the novel endothelin-A receptor inhibitor ETR-P1/fl peptide were examined in endothelin-1-treated anesthetized rats. The injection of 1 nmol/kg endothelin-1 induced a significant rise in peripheral vascular resistance, a transient increase in mean arterial pressure, and a decrease in cardiac output. Administration of the endothelin-A receptor antagonist BQ-610 and ETR-P1/fl peptide increased cardiac output and decreased systemic vascular resistance in the controls and in animals treated with exogenous endothelin. In the second part of the study, the animals were instrumented for hemodynamic monitoring and randomized to undergo cecal ligation and perforation for 8 h or control laparotomy. Septic animals with cecal ligation and puncture were normotensive and hypodynamic, with a significantly increased total peripheral resistance throughout the 8 h observation period. ETR-P1/fl peptide treatment started after the induction of sepsis significantly increased cardiac output and decreased systemic vascular resistance almost to control levels. We conclude that endogenous endothelin-1 contributes significantly to the systemic hemodynamic alterations during hypodynamic circulatory response, and the inhibition of endothelin-A receptors may improve global hemodynamic status in this phase of sepsis.

INTRODUCTION

It is now recognized that endothelial cell-derived vasoconstrictor and vasodilator mediators play an important role in vascular control in pathologic processes and cause critical changes in systemic vascular resistance, blood pressure, and distribution of flow. The endothelins (ETs) are a family of powerful endogenous vasoconstrictor peptides produced by endothelial cells (1, 2). Three active isoforms (ET-1, ET-2, and ET-3) and two main receptor types (ET_A and ET_B) for ETs have been recognized and cloned to date (3–6). The receptors mediate different circulatory effects depending on their localization, but it is suggested that vasoconstriction is mediated predominantly via the ET_A receptors, while the activation of ET_B receptors elicits both vasodilator and vasoconstrictor responses in mammals (7–10).

Recent studies have demonstrated that the production of ET-1 rises rapidly during experimental and clinical endotoxemia and sepsis, which suggests that ET-1 may contribute to the regulation of vascular tone under these conditions (11–14). Sepsis and septic shock are the most common causes of death in the surgical intensive care unit. Although the acquisition of information about the role of ET-1 could have therapeutic consequences, the currently available experimental results are incompletely understood. Because of the continual transitions between hyper- and hypodynamic circulatory responses, it is difficult to compare data obtained in various progressive stages of sepsis. In addition, the question as to whether increased plasma levels of ET-1 are primary or secondary phenomena is still controversial.

Our main objective was to investigate the role of endogenous ET-1 in the systemic circulatory changes occurring during hypodynamic sepsis in anesthetized rats. In an attempt to gain further insight into this process, the characteristics of exogenous ET-1-induced systemic circulatory reactions with or without ET receptor antagonist pretreatment were investigated in order to establish a pattern for comparison with the circulatory consequences of intraabdominal sepsis. We determined the systemic hemodynamic effects of exogenous ET-1, which causes circulatory changes that are similar to those observed during hypodynamic sepsis. The involvement of ET_A and ET_B receptors was assessed by using the selective receptor antagonists BQ-610 and IRL-1038, respectively.

In the second part of our study, circulatory reactions were recorded in rats randomized to undergo cecal ligation and perforation (CLP) or control laparotomy. The increase in the RNA message of the ET precursor is apparent 6 h after an endotoxin challenge (14), and we therefore observed global hemodynamics for 8 h following the induction of intraabdominal sepsis by CLP. In addition, the effects of treatment with the novel ET_A receptor antagonist, ETR-P1/fl peptide, were investigated. ETR-P1/fl is a recently designed "antisense-homology box"-derived peptide, which exhibits potent anti-ET_A receptor

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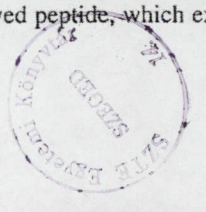

activity *in vitro* (15). Here, we present the first data on the effects of the ET receptor antagonist ETR-P1/fl peptide on the septic systemic vascular changes in the rat. Our results demonstrate that ET-1 is an instrumental component of the circulatory alterations during the hypodynamic phase of sepsis, and that these changes can be influenced significantly by an ET_A receptor-targeted treatment regimen.

MATERIALS AND METHODS

The experiments were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research of Szent-Györgyi Albert Medical University.

Surgical procedure

38 male Wistar rats (weighing 250 ± 15 g) were deprived of food, but not water, for 12 h prior to the experiments. The animals were anesthetized with sodium pentobarbital (60 mg/kg body weight intraperitoneally) and placed in a supine position on a heating pad, and tracheotomy was performed. The right jugular vein was cannulated with PE50 tubing for fluid administration. The left common carotid artery was exposed, and a thermistor-tip catheter was advanced into the ascending aorta to measure cardiac output (CO) by a thermodilution technique. The right femoral artery was cannulated for mean arterial pressure (MAP) and heart rate (HR) measurements. CO, HR, and MAP values were recorded, and the total peripheral resistance (TPR) was calculated by means of a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary). Following a midline laparotomy, the animals were divided into two experimental series.

Experimental protocol

Two series of experiments (with nonseptic controls and septic animals) were performed to investigate the effects of ET_A receptor antagonism in hypodynamic sepsis. All animals received a continuous infusion of isotonic saline at a rate of 3 mL/h/kg. After surgery, the animals were allowed to stabilize for 30 min, and the cardiovascular parameters were then recorded to establish the baseline variables. The ET_A and ET_B receptor antagonists alone were given intravenously (i.v.) to normal rats in pilot studies.

In the first series of experiments, ET-1 was administered alone or in combination with the ET receptor antagonists. In group 1 (n = 6), a solution of 1 nmol/kg ET-1 (Alexis Corp., Laufelfingen, Switzerland) was infused i.v. into the systemic circulation with a syringe pump, and the hemodynamic parameters were then observed for a further 70 min.

In groups 2–5 (n = 6 in each group), the ET_A receptor antagonist BQ-610 (homopiperidinyl-carbonyl-Leu-D-Trp(CHO)-D-Trp-OH (16), Alexis Corp., Laufelfingen, Switzerland, 100 nmol/kg (17)), ETR-P1/fl peptide (VLNLCALSVDRYRAVASWRVI, Kurabo Ltd., Osaka, Japan, 100 nmol/kg), or the ET_B receptor blocker IRL-1038 (Cys11-Cys15-ET-1 (11–21), Alexis Corp., 100 nmol/kg (18, 19)) was infused i.v. into the systemic circulation over 10 min. In these groups, 10 min after the end of IRL-1038, BQ-610, or ETR-P1/fl peptide pretreatment, ET-1 in a 1 nmol/kg dose was infused i.v. over 10 min, and the circulatory effects were observed for another 70 min.

In the second set of experiments, the animals were instrumented as above and were randomized after laparotomy.

In group 6 (n = 6), the base of the cecum was exteriorized, ligated, and punctured six times (CLP) with a 20-gauge needle. Upon completion of the surgical procedure, the abdomen was closed and the cardiovascular parameters were registered during a further 8 h period. In pilot studies, we established that this injury results in reproducible circulatory changes throughout the experiments.

In group 7 (n = 6), 100 nmol/kg ETR-P1/fl peptide was infused i.v. 10 min after CLP.

In group 8 (n = 6), a sham operation was performed, and the abdomen was closed without CLP.

During the experimental period, MAP, HR, CO, and TPR were measured every 30 min.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within groups. Time-dependent differences from the baseline were analyzed by Dunn's method. Differences between groups were analyzed with the Mann-Whitney rank-sum test (between two groups) and Kruskal-Wallis one-way analysis of variance on ranks (among groups). p values $< .05$ were considered significant.

RESULTS

The resting hemodynamic parameters were similar in each of the groups studied. Administration of the ET_A receptor antagonist ETR-P1/fl peptide and BQ-610 induced a transient, approximately 10% decrease in MAP and a 10% increase in CO, while the HR values did not change significantly. The ET_B receptor antagonist IRL-1038 did not exert any significant effect on these parameters.

Infusion of 1 nmol/kg ET-1 induced an immediate significant rise by 250% in TPR (Fig. 1). After 30 min, MAP had

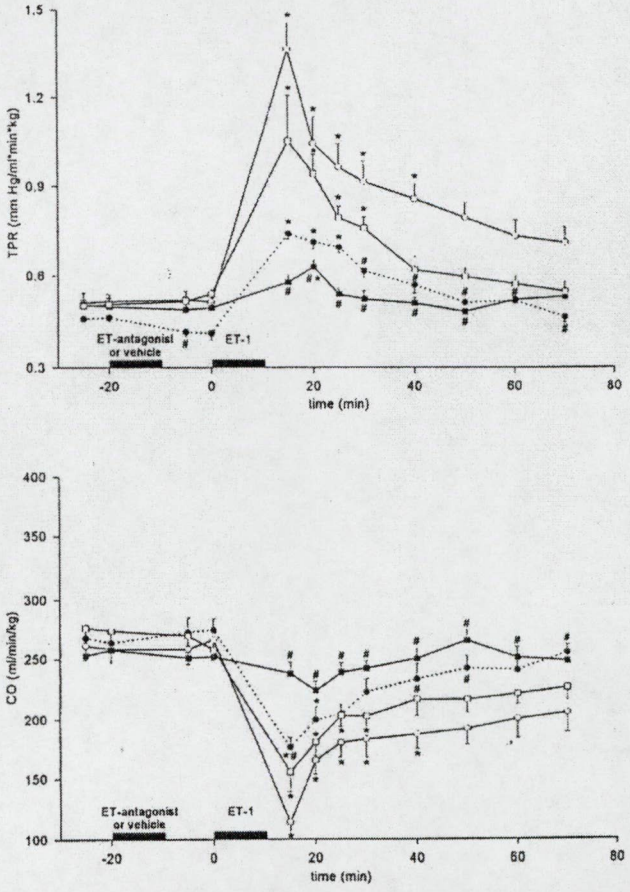

FIG. 1. **Changes in the TPR (*upper panel*) and CO (*lower panel*) in rats treated with 1 nmol/kg ET-1 (○) and in animals that received an ET receptor antagonist pretreatment.** □: IRL-1038 + ET-1 pretreatment (n = 5); ●: ETR-P1/fl peptide + ET-1 pretreatment (n = 5); ■: BQ-610 + ET-1 pretreatment (n = 5). Values are means ± standard error. $^{\#}p < .05$ vs. ET-1-treated group; $^{*}p < .05$ vs. baseline value (Kruskal-Wallis one-way analysis of variance on ranks and Friedman test).

TABLE 1. **Hemodynamic data: Effects of ET-A and ET-B receptor antagonists on exogenous ET-1-induced circulatory responses**

Treatment						
ET-1	ET receptor antagonist	Measurement times	n	CO (mL/min/kg)	TPR (mmHg/mL·min·kg)	MAP (mmHg)
+	–	Baseline	6	258 ± 11.1	0.51 ± .02	131 ± 3.8
+	–	15 min	6	113 ± 7.3*	1.36 ± .09*	153 ± 8
+	–	30 min	6	183 ± 15.3*	.90 ± .07*	163 ± 8.3*
+	–	70 min	6	206 ± 16.8	.71 ± .05	143 ± 7.5
+	ETR-P1/f1	Baseline	5	264 ± 8.6	.46 ± .01	122 ± 6.1
+	ETR-P1/f1	15 min	5	177 ± 8.5*‡	.74 ± .02*	130 ± 3.2
+	ETR-P1/f1	30 min	5	223 ± 10.6	.62 ± .02‡	136 ± 2.1‡
+	ETR-P1/f1	70 min	5	256 ± 6.8‡	.46 ± .02‡	118 ± 6.7
+	BQ 610	Baseline	5	258 ± 5.5	.50 ± .02	127 ± 3.3
+	BQ 610	15 min	5	238 ± 9.3‡	.58 ± .02‡	138 ± 7.2
+	BQ 610	30 min	5	243 ± 6‡	.52 ± .03‡	126 ± 4.4‡
+	BQ 610	70 min	5	249 ± 9.8	.53 ± .02	131 ± 4.3
+	IRL 1038	Baseline	5	274 ± 12	.50 ± .02	137 ± 1.6
+	IRL 1038	15 min	5	156 ± 16*	1.06 ± .15*	155 ± 4.2
+	IRL 1038	30 min	5	203 ± 8.7*	.77 ± .04*	153 ± 6
+	IRL 1038	70 min	5	227 ± 10.1	.55 ± .04	123 ± 6.8

Values are expressed as means ± standard error.
*$p < .05$ versus baseline value.
‡$p < .05$ versus ET-1 treated group.

increased from 131 to 163 mmHg (Table 1). This hypertension was associated with a significant decrease in CO from 258 mL/min/kg (baseline value) to 183 mL/min/kg.

Pretreatment with ETR-P1/fl peptide and BQ-610 significantly attenuated the ET-1-induced increase in MAP and reduced the decrease in CO. Similarly, at 40 min, IRL-1038 effectively diminished the elevation in MAP, but this pretreatment did not influence the ET-1-induced fall in CO (Table 1). The increase induced in TPR by ET-1 was reduced by BQ-610 and ETR-P1/fl pretreatment, whereas IRL-1038 was ineffective (Fig. 1).

CLP induced a significant fall in CO after 90 min (from 283 ± 25 mL/min/kg to 246 ± 24 mL/min/kg), which developed progressively over the following 3 h and remained depressed until the end of the 8 h observation period (Fig. 2). TPR displayed early increases, with a maximum at 3 h, and remained elevated throughout the experiments. In these animals, there was a small reduction in MAP after 8 h, but this was not statistically significant as compared with the baseline or the sham-operated values (Table 2).

Administration of ETR-P1/fl peptide after CLP delayed and inhibited the reduction in CO, so that there was no significant CO decrease during the 8 h observation (Fig. 2). Additionally, ETR-P1/fl peptide treatment significantly reduced the CLP-induced increase in TPR during the experiments. The change in MAP did not differ from the corresponding value for the sham-operated or CLP-treated group throughout the observation period (Table 2).

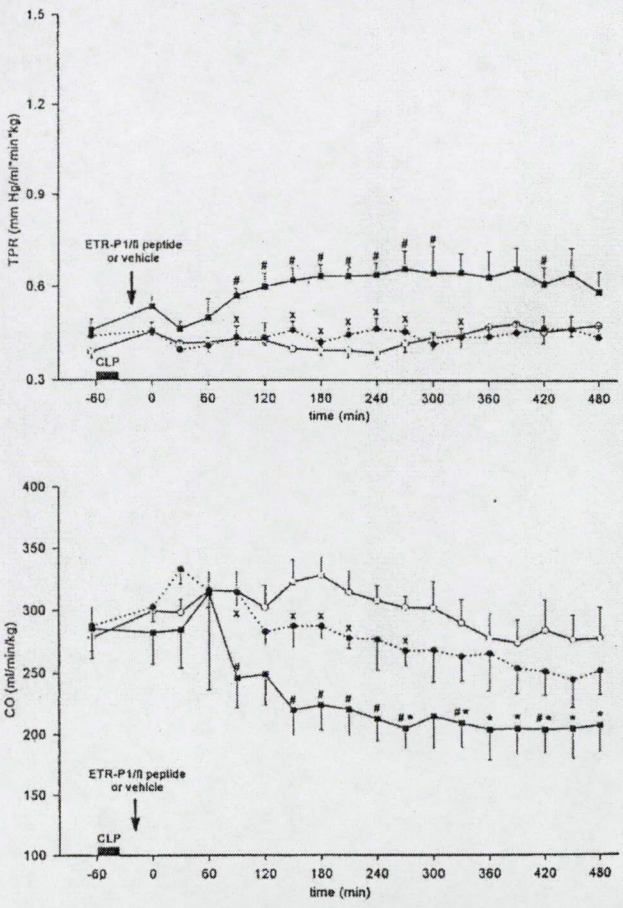

FIG. 2. **Changes in TPR (*upper panel*) and CO (*lower panel*) in sham-operated controls (○, n = 5), in 8 h CLP-treated animals (■, n = 5), and in rats that received ETR-P1/fl peptide treatment (●, n = 5) 10 min after CLP.** Values are means ± standard error. #$p < .05$ vs. control group; *$p < .05$ vs. baseline value (Kruskal-Wallis one-way analysis of variance on ranks and Friedman test).

DISCUSSION

The time course of septic metabolic, humoral, and hemodynamic responses is classically resolved into two parts (20). The hyperdynamic state is characterized by an early decrease in systemic resistance and mean blood pressure and an increase in

TABLE 2. **Effects of ETR-P1/f1 peptide treatment on hemodynamic changes in rats with cecal ligation and puncture (CLP)**

Treatment						
CLP	ETR-P1/f1 peptide	Measurement times	n	CO (mL/min/kg)	TPR (mmHg/mL·min·kg)	MAP (mmHg)
−	−	Baseline	6	300 ± 6.2	.46 ± .01	136 ± 4.3
−	−	90 min	6	316 ± 16.2	.43 ± .02	134 ± 3.8
−	−	180 min	6	329 ± 14.2	.40 ± .02	129 ± 4.3
−	−	480 min	6	279 ± 24.6	.48 ± .03	129 ± 4.5
+	−	Baseline	5	283 ± 25.4	.54 ± .03	148 ± 6.1
+	−	90 min	5	246 ± 24.3*	.57 ± .03*	139 ± 10.6
+	−	180 min	5	224 ± 20.3*	.63 ± .04*	140 ± 9.8
+	−	480 min	5	208 ± 22.1‡	.58 ± .07	118 ± 12.6
+	+	Baseline	6	303 ± 11	.46 ± .03	138 ± 5
+	+	90 min	6	315 ± 11.1§	.44 ± .04§	136 ± 7
+	+	180 min	6	288 ± 10.4§	.42 ± .01§	120 ± 3.6
+	+	480 min	6	253 ± 19.3	.44 ± .04	109 ± 8.8

Values expressed as means ± standard error.
*$p < 0.05$ vs. sham-operated group.
‡$p < 0.05$ vs. baseline value.
§$p < 0.05$ between ETR-P1/f1-treated and untreated CLP groups.

CO. During the hypodynamic phase, a moderate decrease in MAP is accompanied by a substantial fall in CO. The systemic vascular resistance increases, leading to a marked reduction in the perfusion of the intestine, kidneys, and skin, while the blood flow is redistributed to the vital organs (21). The results of the present study show that ET-1 causes hemodynamic changes similar to those observed during hypodynamic sepsis, and that some of these effects can be influenced significantly by treatment with an ET receptor antagonist.

First, we compared the abilities of ET-1 and ET receptor antagonists to influence the systemic hemodynamics in normal rats. The ET_B receptor antagonist IRL-1038 alone had no direct effects on any of the systemic parameters measured, but the ET_A receptor antagonist BQ-610 and ETR-P1/fl peptide induced a transient fall in MAP, implicating a role for ET-1 in the maintenance of vascular tone. This is in agreement with the findings of Haynes et al. (22) in humans, but it may contrast with what is found in other studies in normotensive rats (23, 24). A possible explanation is that BQ-610 is 30 times more potent than BQ-123, a widely used ET_A receptor antagonist (16). Additionally, we used the novel antisense-homology box-derived ET receptor antagonist ETR-P1/fl peptide, which binds to distinct regions of the ET_A receptor (15). Briefly, ETR-P1/fl peptide is an amphiphilic peptide fragment of the human ET_A receptor responsible for the maintenance of protein shape. It has been demonstrated that the peptide significantly reduces the constrictor effect of ET-1 in isolated vessels and actively inhibits the ET-1-induced Ca^{2+} influx *in vitro* (15).

There is good evidence that plasma levels of ET-1 are significantly elevated in experimental animals and in humans with sepsis and endotoxemia (11–14). In our study, we administered ET-1 to establish a pattern for comparison with the sequence of CLP-induced circulatory changes. The increase in MAP after ET-1 was accompanied by a rise in TPR, despite a fall in CO. The decrease in CO was most probably due to a reduction in stroke volume. These effects of ET-1 are possibly mediated by ET_A receptors present in the circulation, as administration of the specific ET_A receptor antagonist BQ-610 and ETR-P1/fl peptide significantly inhibited both the ET-1-induced increases in TPR and MAP and the decrease in CO. However, it should be noted that selective blockade of the ET_B receptors with IRL-1038 was partially effective in reducing the ET-1-induced changes. This suggests that the residual responses to ET-1 in the presence of ETR-P1/fl peptide may be mediated by unblocked ET_B receptors, and consequently that ETR-P1/fl peptide is a nonselective inhibitor of ET receptors, i.e., it has dual ET_A and ET_B receptor antagonist properties. This explanation seems plausible, as the ETR-P1/fl peptide exhibits a close amino acid sequence similarity to an antisense-homology box present in the ET_B receptor (L. Baranyi, unpublished observation). It may be assumed that the proficiency of ET-1 receptor antagonist compounds in inhibiting ET-1-induced central hemodynamic effects is related to their capacity to antagonize an activation of vasoconstriction-mediating ET receptors on vascular smooth muscle cells (e.g., ET_A and ET_{B2} receptors).

The hemodynamic changes of sepsis and endotoxemia involve several vasodilator and vasoconstrictor factors, but the activated endothelium clearly participates in and regulates the process (25). This is achieved by the production of a variety of vasoactive mediators, including ET-1. ET-1 release is stimulated *in vitro* by tumor necrosis factor α and endotoxin (26, 27). The tissue expression of ET-1 mRNA is significantly increased 6 h after the *in vivo* injection of endotoxin, and the ET-1 level reaches its maximum 8 h after CLP in rats (14, 28).

We aimed to investigate sepsis-induced circulatory changes in an acute model that mimics human sepsis more closely than endotoxin infusion. The 8 h CLP resulted in a septic hemodynamic state similar to that observed in patients during hypodynamic sepsis. A transient, initial hyperdynamic response was followed by a simultaneous increase in systemic resistance and a decrease in CO. The MAP remained stable.

Our main objective was to determine whether ET-1 participates in the circulatory alterations during hypodynamic sepsis, and the systemic hemodynamic changes that we recorded following 8 h of intraabdominal sepsis, with or without ET

receptor antagonist treatment, are in agreement with this hypothesis. Peripheral vascular resistance was significantly decreased after ETR-P1/fl peptide administration, suggesting a role for ET-1 in the hypodynamic circulatory reaction with respect to central tension regulation. However, it is still unclear whether ET-1 is a compensatory or detrimental factor of the septic hemodynamic derangement, because at least two possibilities should be considered. First, it should be noted that, among many other modes of action, ET-1 decreases CO and increases peripheral vascular resistance, and hence, tissue damage may be due to the maintenance of impaired peripheral tissue perfusion. This ongoing process can be blocked by the ET_A receptor antagonist ETR-P1/fl peptide. On the other hand, the ET-1-induced increase in peripheral resistance may be an important compensatory factor in maintaining circulatory support, so that antagonism of the vasoconstrictor ET receptors might be detrimental in the long run (29, 30).

In our severe hypodynamic sepsis model, the decrease in CO was significantly attenuated by ETR-P1/fl peptide administration. Similarly, the changes in TPR demonstrated that inhibition of the ET_A receptors did not impair the global hemodynamic status in this phase of sepsis in the rat. Thus, it seems that the increased circulating ET-1 output under hypodynamic septic conditions is involved in global hemodynamic derangements via ET_A receptor activation. An ET_A predominance is most likely in the rat circulation, and the ET_A receptors and (to a lesser extent) the ET_B receptors mediate vasoconstriction and may ultimately contribute to a reduced peripheral organ perfusion in hypodynamic septic states. As ET-1 has a higher affinity for ET_A than for ET_B receptor subtypes, these results demonstrate that the ET_A receptor is more important in the production of these changes (31, 32). These data, and the observations of elevated plasma levels during sepsis and endotoxemia, suggest a therapeutic potential for ET_A receptor antagonists in these conditions.

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DYNAMIC IN VIVO OBSERVATION OF VILLUS MICROCIRCULATION DURING SMALL BOWEL AUTOTRANSPLANTATION: EFFECTS OF ENDOTHELIN-A RECEPTOR INHIBITION[1]

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Background. **The aims of this study were to characterize the structural and microcirculatory changes in single intestinal villi during ischemia and reperfusion and determine the site of action of endothelin (ET)-A receptor inhibition during compromised mucosal perfusion.**

Methods. **Small bowel autotransplantation was performed in anesthetized dogs. One group was treated with the ET-A receptor antagonist ETR-p1/fl peptide. The epithelial thickness and villus microcirculatory parameters were observed by orthogonal polarization spectral imaging; the leukocyte-endothelial cell interactions were quantified with fluorescence videomicroscopy.**

Results. **Sixty-minute cold ischemia and 240-min reperfusion induced a decrease in villus functional capillary density and leukocyte-endothelial cell interactions. The epithelial layer was gradually removed, but denuded villi were not observed. ET-A receptor inhibition reduced the leukocyte adherence and attenuated epithelial exfoliation and the decrease in villus functional capillary density.**

Conclusions. **ET-A receptor activation mediates microvascular dysfunction through precapillary blockades and leukocyte-endothelial cell interactions after cold ischemia and reperfusion in the canine small bowel.**

Although novel immunosuppression strategies have improved the results of small bowel transplantation (SBTX), serious nonimmunological complications limit the success of such therapy (*1*). The impaired capillary perfusion, leukocyte-endothelial cell interactions, and other microvascular reactions induced by ischemia-reperfusion usually do not cause systemic circulatory manifestations but could lead to severe tissue injury in the long run (*2*). In seeking to identify novel effective tissue protective agents, a number of authors have demonstrated that endothelin (ET)-1 peptide plays a crucial role in ischemia-reperfusion-induced damage of the intestinal circulation (*3–5*). ET-1 is the most powerful vasoconstrictor substance known to date, and the vasoconstrictive effects are mediated predominantly via the endothelin-A (ET-A) receptors present on the vascular smooth muscle cells (*6*). We have previously shown that the inhibition of ET-A receptors improves graft-perfusion, decreases granulocyte activation, and extravasation after intestinal ischemia-reperfusion (*4*). In the present experiments, we investigated the microvascular alterations that occur in single intestinal villi

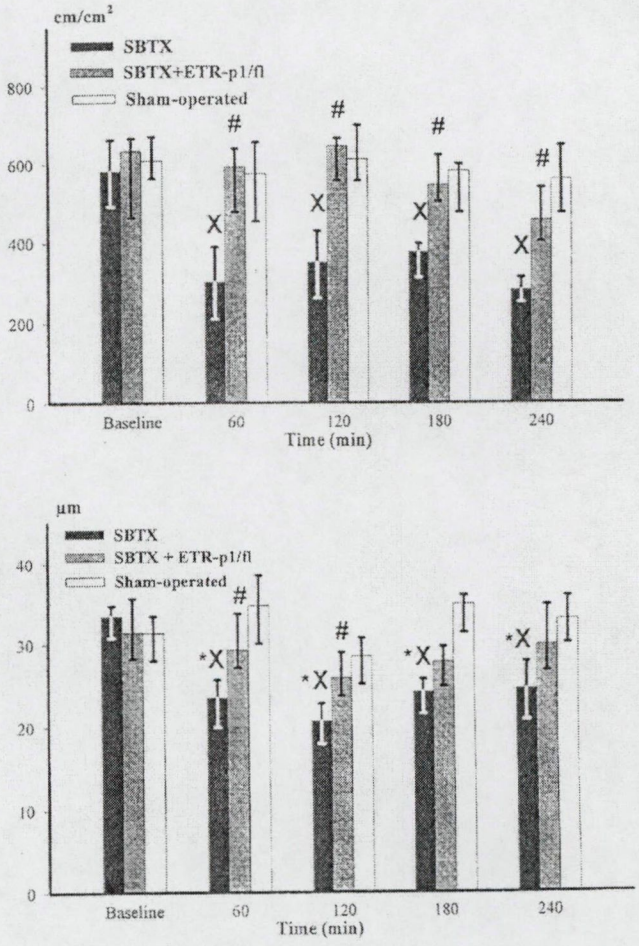

FIGURE 1. **Changes in functional capillary density (cm/cm²) (A) and in epithelial thickness (µm) (B) in the SBTX (black columns), ETR-p1/fl peptide-treated (gray columns), and sham-operated (white columns) groups. The tops of the columns show the median values. The capped bars indicate the 25th and 75th percentiles, respectively. *$P<0.05$ within groups vs. baseline values; $^{X}P<0.05$ between sham-operated and SBTX groups; $^{\#}P<0.05$ between SBTX and SBTX+ETR-p1/fl peptide-treated groups.**

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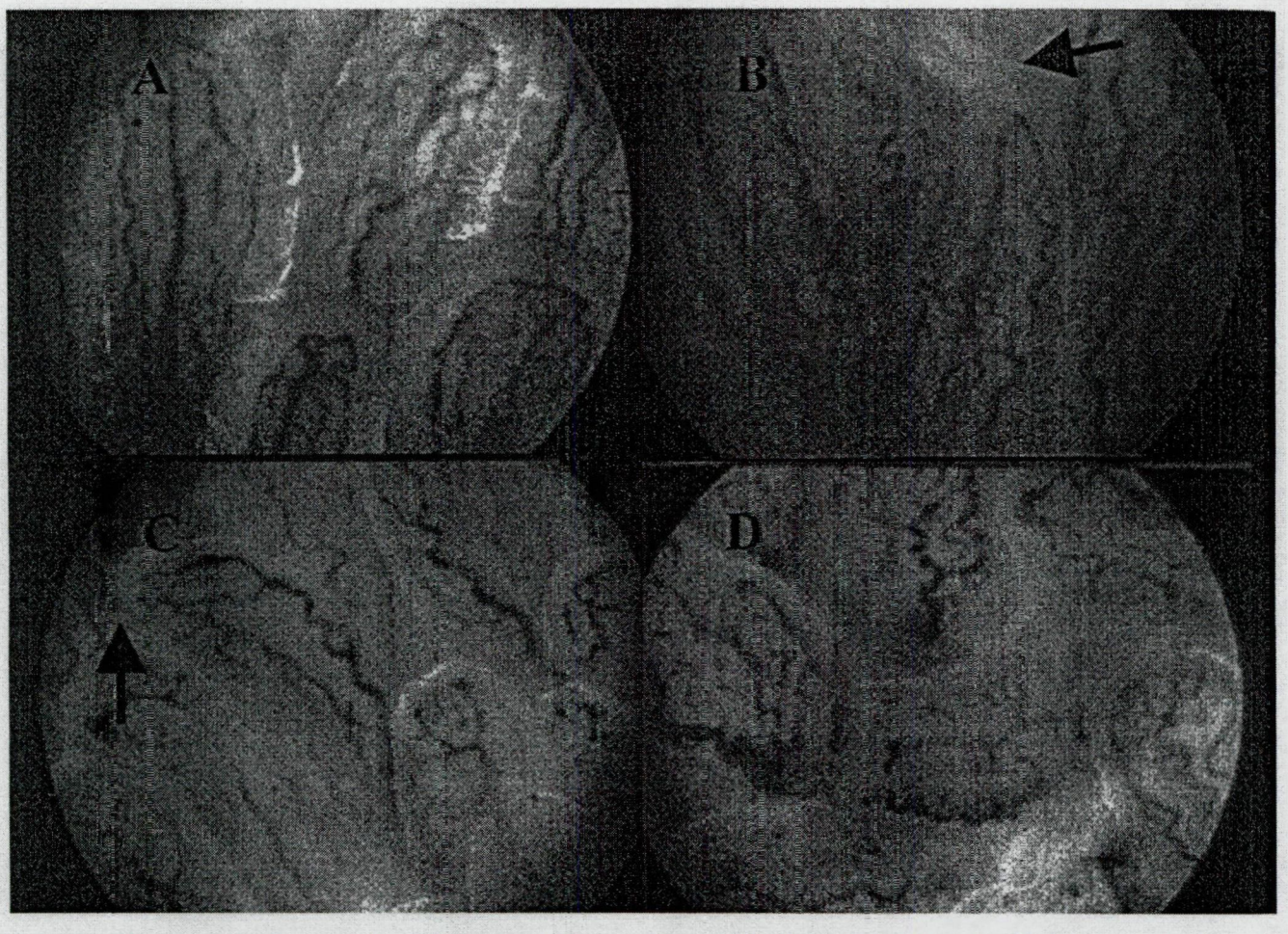

FIGURE 2. Micrographic views of the small intestinal mucosa after SBTX. (A) Canine intestinal villi showing typical normal microvascular architecture and no significant pathological lesions. Baseline conditions: the epithelium is essentially of normal thickness (OPS imaging, original magnification ×100). (B) The epithelial cells at the villus tip are elevated during reperfusion. A tapering column is formed, and the exfoliated original epithelial layer is removed. Several capillaries are not perfused (1-hr postreperfusion). (C) A new epithelium is closed above the villus stroma; "denuded" villi are not seen. (D) 2-hr postreperfusion: short, squat villi and negligible subepithelial lesions.

in a large-animal model of SBTX devoid of immune effects. Secondly, the microcirculatory effects of selective ET-A receptor inhibition were characterized.

Three separate series of experiments were performed on a total of 15 mongrel dogs under sodium pentobarbital anesthesia. The surgical procedure of jejunoileal orthotopic autotransplantation has been described previously (*4*). After the 60-min cold ischemia, reperfusion was induced and the microhemodynamic changes were observed for 240 min. Transplantation was performed in groups 1 (n=5) and 2 (n=5); in group 2 the animals were treated with the ET-A receptor antagonist ETR-p1/f1 peptide (Kurabo Ltd., Osaka, Japan) in a dose of 0.1 mg/kg at the beginning of revascularization. Group 3 (n=5) served as sham-operated control.

Leukocytes were stained in vivo with rhodamine-6G (Mw 479, Sigma, St. Louis, MO, 0.2%, 1 ml i.v.). Leukocyte-endothelial cell interactions in the postcapillary venules of the mesentery were monitored with a high-resolution Zeiss Axiotech Vario 100HD intravital fluorescence videomicroscope system. The microscopic images were analyzed off-line by frame-to-frame analysis of the videotaped images, including the observation of adherent and rolling leukocytes (*3*).

Orthogonal polarization spectral (OPS) imaging technique (Cytoscan A/R, Cytometrics, PA, USA) was used to monitor microvascular perfusion changes in the intestinal villi before harvesting and during the reperfusion phase. This novel intravital method utilizes polarized reflected light at 548 nm to visualize hemoglobin-containing structures, without the use of fluorescent dyes (*7*). The objective of the device was introduced into the intestinal lumen, and the functional capillary density (FCD), the red blood cell velocity (RBCV), the diameter of the central arterioles of the villi, and the thickness of the epithelial cell layer covering the villi were determined with a computer-assisted image analysis system (Pictron Ltd., Budapest, Hungary). All data were expressed as the means of five measurements at each time point.

Time-dependent differences within groups were analyzed with the Friedmann and Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method. In

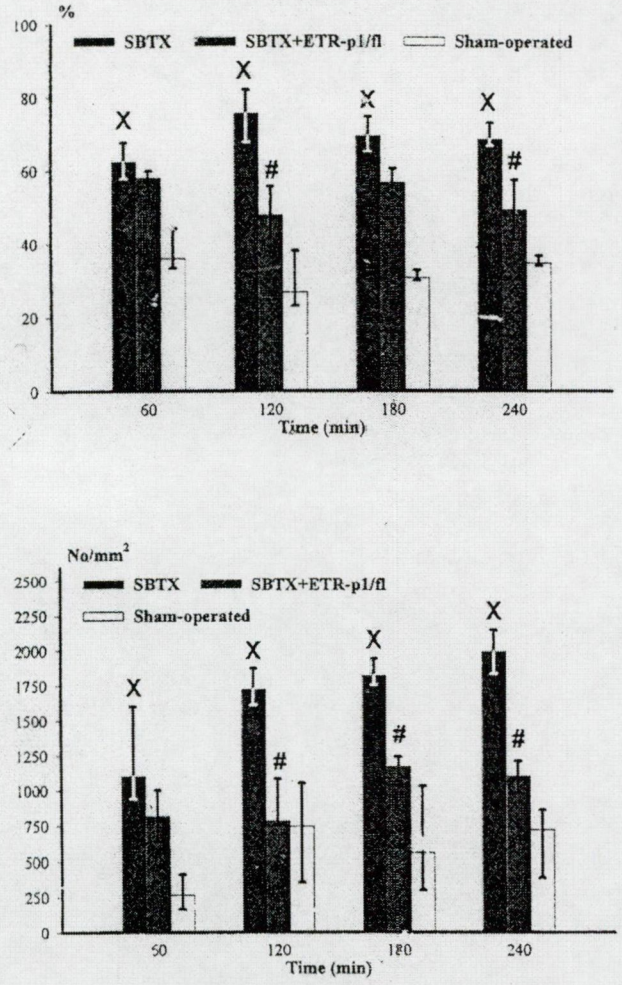

FIGURE 3. **Percentage of rolling leukocytes (A) and number of adherent leukocytes (B) in the mesenteric postcapillary venules in the SBTX (black columns), ETR-p1/fl peptide-treated (gray columns) and sham-operated (white columns) groups. The tops of the columns show the median values. The capped bars indicate the 25th and 75th percentiles, respectively. $^{x}P<0.05$ between sham-operated and SBTX groups; $^{\#}P<0.05$ between SBTX and SBTX+ETR-p1/fl peptide-treated groups.**

the figures, median values and 75th and 25th percentiles are given. P values<0.05 were considered significant.

The results show that the villus FCD was significantly reduced during reperfusion compared with the preharvesting values or with the FCD values of the sham-operated group. ET-A receptor inhibition significantly attenuated the decrease in FCD during the reperfusion phase (Fig. 1A).

The epithelial thickness remained unchanged in the sham-operated group, and there was a tendency toward an epithelial height-reduction in both transplanted groups. However, in the later phase the epithelial thickness was significantly higher in the ETR-p1/fl peptide-treated group (Fig. 1B).

RBCV decreased slightly, but significantly, during the reperfusion phase in both transplanted groups in the villus arterioles and venules. ETR-p1/fl treatment did not significantly influence this parameter. No difference was found between the diameters of the villus arterioles and venules (data not shown).

The epithelial cells at the villus tip started to elevate immediately after reperfusion. A tapering column was soon formed, and the exfoliated original epithelial layer was removed from the sand-glass-like villus. Meanwhile, a new epithelial layer was closed above the villus stroma, so that "denuded" villi were not seen. Finally, at approximately 2 hr postreperfusion, short, squat villi were present, without any signs of subepithelial damage (Fig. 2).

Transplantation was accompanied by a significant increase of the percentage of rolling leukocytes and number of adherent cells in the postcapillary mesenteric venules. Both primary and secondary leukocyte-endothelial cell interactions were significantly reduced after ET-A receptor blockade (Fig. 3).

It is recognized that maintenance of an adequate microvascular blood supply is critical for graft survival and function. There is also agreement regarding an increased rate of activation and accumulation of granulocytes within the ischemic-reperfused tissues. Previously, we reported decreased mesenteric blood flow and increased mesenteric vascular resistance during reperfusion in the same setting (*4*). In the present study, the microcirculatory pattern of the villus perfusion exhibited typical alterations during reperfusion, which might explain the increased macrocirculatory resistance. Ordinarily, the blood reaches the villus tip in a central arteriole, passes the capillaries, and returns through the postcapillary venules (*8*). Because no difference was found in central arteriolar and venular diameters and RBCV, the reduction in villus blood flow is not due to vasoconstriction in the central villus arterioles during reperfusion. The decreased FCD suggests that vasoconstriction developed at the level of the precapillary sphincters, i.e., a considerable proportion of the inflowing blood turned back into the venules without passing the capillaries.

In addition to precapillary vasoconstriction, other reperfusion-related factors can also contribute to the reduction of FCD. The no-reflow phenomenon may develop as a result of interstitial edema formation and compression of the capillaries, or it may be a result of intraluminal plug formation (*9*). In accordance with the latter, we observed increased leukocyte-endothelial cell interactions in the mesenteric postcapillary venules. In some of the vessels, especially those with smaller diameter, the adherent and rolling cells formed leukocyte plugs, leading to complete obstruction of the venule.

Although it is clear that the responses to ischemia-reperfusion involve a multiplicity of factors, our study confirms the instrumental role of ET-1 in these microcirculatory reactions. Interestingly, we found no difference in the diameters of the central arterioles and postcapillary venules in response to ET-A antagonism. However, ET-A receptor inhibitor treatment effectively attenuated the decrease in FCD in the intestinal villi, and these data suggest that endogenously liberated ET may be involved in the pathogenesis of capillary no-reflow. Depending on the localizations of the ET-A receptors, at least three possible mechanisms may be hypothesized. One possibility is a differential effect of ET on the precapillary sphincters, i.e., the outermost contractile elements of the circulatory system. Secondly, ET-A receptor-mediated precapillary vasoconstriction and the ensuing hypoxia could lead to perivascular edema. Indeed, it has been shown that ET-1 causes dose-dependent increases in vascu-

lar permeability through the activation of ET-A receptors as a consequence of the disruption of the endothelial barrier (*6*). Thirdly, there is evidence that ET-1 influences leukocyte activation in submucosal venules (*3*). The exact molecular mechanism of the pro-adhesive effect of ET-1 is still unclear. However, the development of firm leukocyte adhesion requires the expression of adhesion molecules, and antibodies against P-selectin were found to reduce the ET-induced leukocyte rolling in the rat (*10*).

In general terms, the microenvironment is usually inaccessible for clinical diagnosis. The OPS imaging technique enabled us to visualize the villus microcirculation and detect the progression of the morphological-structural changes. During the reperfusion phase, the epithelial layer on the tip of the villi was gradually removed from the villus, but we did not observe exposed, "denuded," or "amputated" villi, which are characteristically seen on light microscopy. In this respect, further comparative studies are needed to establish an in vivo scoring system similar to the histological scoring of intestinal biopsies. In the ET-A receptor antagonist-treated group, epithelial exfoliation was also present, but it was less severe, and the regeneration was also faster, as indicated by the significantly higher epithelium.

In conclusion, our experimental model allowed in vivo visualization of the intestinal villus microcirculation so that leukocyte-endothelial interactions and microvascular perfusion changes could be observed simultaneously. We demonstrated that SBTX considerably impair the microcirculation of the graft, as characterized by the reduced capillary perfusion of the villi and the leukocyte-endothelial cell interactions in the mesenteric venules. These changes led to distinctive morphological injury of the villi. The differences in vascular responses observed after ET receptor antagonist treatment could define the involvement of the ET-A receptor subtype in the intestinal microhemodynamic alterations in postischemic bowel injury.

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Endothelin-1 induces mucosal mast cell degranulation in the rat small intestine

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Abstract

The enhanced production of endothelial cell-derived vasoactive mediators and the activation of mast cells (MCs) have been implicated in the pathogenesis of mucosal damage during ischemia and reperfusion injuries. The first objective of our study was to define the *in vivo* relation between endothelin-1 (ET-1) and the MC system. Secondly, we determined whether pretreatment with ET receptor antagonists would attenuate MC responses to exogenous ET-1. In the first series of experiments, increasing doses of ET-1 (0.1, 1 and 3 nmol/kg i.v.) were administered to anesthetized rats. In the second series, the animals were pretreated with equimolar doses of the ET-A receptor antagonist BQ-610 or ETR-P1/fl peptide, and the ET-B receptor antagonist IRL-1038. Intestinal perfusion changes and macrohemodynamics were recorded, and the proportion of degranulated MCs was determined in ileal biopsies. The average mucosal thickness was recorded with an image analysis system. ET-1 induced dose-dependent alterations in the hemodynamic and morphological parameters and caused pronounced mucosal injury, with a significant reduction in villus height. The ratio of degranulated MCs was similar in all ET-treated groups (77%, 82% and 86%) to that observed in animals subjected to 15-min ischemia and 60-min reperfusion (85% degranulation). Pretreatment with BQ-610 and ETR-P1/fl peptide attenuated the ET-1 induced alterations in the hemodynamic parameters and decreased structural injury to the mucosa. ET-induced MC degranulation was significantly inhibited by the ET-A receptor antagonists, but not by IRL-1038. These results indicate that elevated levels of circulating ET-1 might induce intestinal mucosal tissue injury and MC degranulation via activation of ET-A receptors, and raise the possibility that ET-A receptor antagonist administration could exert a potentially beneficial effect through a mechanism other than the blockade of vasoconstriction in pathologies associated with an increased ET-1 release.

Keywords: Endothelin-1; ETR-P1/fl peptide; Endothelin-A receptor antagonist; Mast cell degranulation; Intestine; Mucosa

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Introduction

The endothelins (ETs) embrace a family of 21-amino acid vasoactive peptides produced by the endothelial cells. Among the three active isoforms (ET-1, ET-2 and ET-3), ET-1 is the most powerful endogenous vasoconstrictor substance known to date [1,2]. The vasoconstrictive effects of ET-1 are mediated predominantly via the endothelin-A (ET-A) receptors present on the vascular smooth muscle cells. The ET-B receptors mediate vasoconstriction ($ET\text{-}B_2$) and vasodilation ($ET\text{-}B_1$), but ET-1 has a higher affinity for ET-A than for ET-B receptor subtypes [3–5].

There is a growing body of evidence that, in addition to the separate role of ET-1 as a dominant vasoconstrictor, the peptide may also influence the biological activity of other cell types in the cardiovascular system. The effect of ET-1 on the gastrointestinal mast cells (MCs) is of special interest, since it may be an important component of the tissue response that occurs in the mucosa during inflammation or reoxygenation injuries. Intestinal MCs have both ET-A and ET-B receptors on their membrane surface, and this suggests a possible crosstalk between endothelial cell-derived humoral mediators and the MC system. This is supported by examinations revealing that ET-1 is involved in the pathogenesis of albumin-induced intestinal anaphylaxis via ET-A receptor activation [6,7].

Recent studies have demonstrated that the production of ET-1 increases significantly during experimental hypoxia and ischemia-reperfusion, which suggests that ET-1 may contribute to the circulatory changes in these conditions [8,9]. There is also evidence that intestinal ischemia-reperfusion leads to the discharge of a variety of MC-derived inflammatory compounds [10]. Moreover, previous studies have indicated severe mucosal morphological alterations in the small intestine in rats treated with exogenous ET-1 [11]. Although these data support the possibility that an upregulated ET-1 release may be associated with simultaneous MC activation and degranulation, there is no direct experimental evidence to allow these findings to be ascribed to the *in vivo* effects of ET-1.

The first series of our studies was directed to an examination of whether and how intestinal MCs respond to increasing doses of exogenously administered ET-1. Secondly, the hypothesis was evaluated that ET-1 receptor inhibition would attenuate ET-1-induced MC degranulation and accompanying tissue damage. To this end, the ET-1-induced microcirculatory effects were correlated to the indices of mucosal morphological changes and degrees of intestinal MC degranulation. We used ET-A receptor and ET-B receptor-selective antagonist treatments to investigate the roles of these receptor subtypes in mediating ET-1-induced intestinal MC activation. We present evidence that pharmacological inhibition of ET-A receptor activation reduces MC degranulation and significantly decreases ET-1-induced tissue injury in the rat small intestine.

Methods

Animals

The experiments described in this article were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. The study was approved by the Animal Welfare Committee of Szent-Györgyi Albert Medical University.

42 male Sprague-Dawley rats (weighing 200±20 g) were deprived of food but not water, for 12 h prior to the experiments. The animals were anesthetized with sodium pentobarbital

(60 mg/kg body weight i.p.) after premedication with atropine (0.1 mg/kg s.c.), and placed in a supine position on a heating pad for maintenance of the body temperature between 36 and 37°C. Tracheotomy was performed and the animals were mechanically ventilated with a rodent respirator (Harvard Apparatus, South Natick, MA, USA).

Polyethylene catheters (PE 50, ID 0.58 mm, Fa. Portex, Hythe, UK) were inserted into the left carotid artery and jugular vein for the recording of mean arterial pressure and the injection of test compounds, respectively. Throughout the experiment, the animals received an infusion of Ringer's lactate at a rate of 40 ml/kg/h. After a transverse laparotomy, a segment of the terminal ileum perfused by a single artery was selected. The marginal vessels were divided and ligated, and the intestinal segment with intact neurovascular connections was covered by plastic sheets.

Experimental protocol

After surgery, the animals were allowed to stabilize for 15 min and the cardiovascular parameters were then recorded to establish the baseline variables (0 min). The animals were randomly divided into groups. In the first series of experiments, dose responses to ET-1 (Alexis Corporation, Läufelfingen, Switzerland) were obtained. The animals were allotted into the following groups: group 1, sham-operated (n=6), group 2, 0.1 nmol/kg ET-1 (n=5), group 3, 1 nmol/kg ET-1 (n=5), group 4, 3 nmol/kg ET-1 (n=6). In these groups, 30 min after the end of baseline measurements, a solution of 0.1 ml ET-1 or vehicle was infused i.v. into the systemic circulation over 15 min. An additional group of animals (n=5) served as positive control. In this group, segmental intestinal ischemia was induced by a 15-min occlusion of the ileal artery.

In the second series, 3 nmol/kg ET-1 was administered in combination with ET receptor blockers. In these groups, the ET-A receptor antagonists ETR-P1/fl peptide (VLNLCALS-VDRYRAVASWRVI, Kurabo Ltd. Osaka, Japan, 0.3 μmol/kg), BQ-610 (0.3 μmol/kg), (homopiperidinyl-carbonyl-Leu-D-Trp(CHO)-D-Trp-OH, Alexis Corp., Läufelfingen, Switzerland), or the ET-B receptor antagonist IRL-1038 (Cys11-Cys15-endothelin-1(11-21), 0.3 μmol/kg) was infused i.v. for 30 min, followed by a 15-min of ET-1 infusion into the systemic circulation after the end of BQ-610, ETR-P1/fl peptide, or IRL-1038 pretreatment, respectively [12]. The circulatory changes were observed for 60 min. At the end of the observation period, a tissue sample was taken from the intestinal segment, and the animals were then killed with an overdose of pentobarbital.

Laser-Doppler measurements

Intestinal intramural blood flow of the segment was recorded with a laser-Doppler flow monitor (Moor Instruments, MBF 3D, Axminster, UK). Laser-Doppler flowmetry affords an estimate of the tissue perfusion, based on continuous measurement of the red blood cell (RBC) flux (the number of red blood cells × velocity). At each observation point, 3 measurements were performed over a 1 to 2-min period.

Histology

Intestinal biopsy samples for light microscopy were rapidly placed into ice-cold Carnoy's fixative and trimmed along the longitudinal axis. The fixed tissue was attached to a hard card-

board backing to ensure the optimal longitudinal direction of the section. The samples were embedded in paraffin, sectioned (6 μm) and stained with hematoxylin-eosin, acidic toluidine blue (pH 0.5) or alcian blue-safranin O (pH 0.4). An image analysis system system (IVM, Pictron Kft., Budapest, Hungary) was used to digitize the x and y coordinates of the sections. Three nonoverlapping fields were processed in each section and the height of a single villus was measured from its origin to the villus tip. In each field, an average of 10 consecutive measurements were used to calculate the average villus height. Mucosal damage was assessed according to the standard scale of Chiu et al. [13]. The grading was performed with the following criteria: grade 0, normal mucosa; grade 1, development of subepithelial Gruenhagen's space at the tip of the villus; grade 2, extension of the space with epithelial lifting; grade 3, massive epithelial lifting with a few denuded villi; grade 4, denuded villi with exposed capillaries; and grade 5, disintegration of the lamina propria, ulceration, and hemorrhage. MC stained positively were quantitated in the villi of an average of 20 villus-crypt units. The counting was performed in coded sections at × 400 optical magnification by one investigator. Loss of intracellular granules, stained material dispersed diffusely within the lamina propria was taken as evidence of mast cell degranulation.

Statistics

Data analysis was performed with a statistical software package (SigmaStat for Windows. Jandel Scientific, Erkrath, Germany). The Friedman test followed by Dunnett's method was applied for multiple comparisons with a control. Differences between groups were analyzed with the Kruskal-Wallis one-way analysis of variance on ranks. p values <0.05 were considered significant. Mean values ± SD are given.

Results

The resting hemodynamic parameters were similar in each of the groups studied. In the first series of experiments, the increasing doses of ET-1 caused marked changes in intestinal hemodynamics. The intestinal RBC flux displayed a dose-dependent decrease when the MAP was kept elevated. The reduction in intestinal RBC flux was more pronounced in the 3 nmol/kg ET-1-treated animals as compared with those undergoing 0.1 nmol/kg ET-1 treatment (Table 1).

In the sham-operated group, the villus MC count was unchanged and no significant increase in degranulation was observed in biopsies taken at the end of the observation period. A significant, dose-dependent diminution of the villus height was induced by ET-1 infusion as compared to the control group. The shortening of the villi was statistically significant after the administration of 1 or 3 nmol/kg ET-1, and there was a significant difference in this parameter between the 0.1 and 3 nmol/kg ET-1-treated animals (Fig. 1, upper panel). Simultaneously, the mucosal alterations as assessed on the Chiu scale were statistically different from the control in the 1 and 3 nmol/kg ET-1-treated groups (Fig. 3, upper panel). The MC degranulation ratio exhibited a significant increase after the ET-1 treatment. The ET-1 infusions elevated the proportion of degranulated MCs almost twofold in each of the ET-1-treated groups (Fig. 1, lower panel).

The ET-A receptor antagonists ETR-P1/fl peptide and BQ-610 attenuated the ET-1-induced fall in RBC flow, (Table 2), the reduction of villus length (Fig. 2, upper panel) and

Table 1
Effect of ET-1 or ischemia-reperfusion (IR) on the mean arterial pressure (MAP) and the small intestinal microcirulation (RBC flux) in the rat

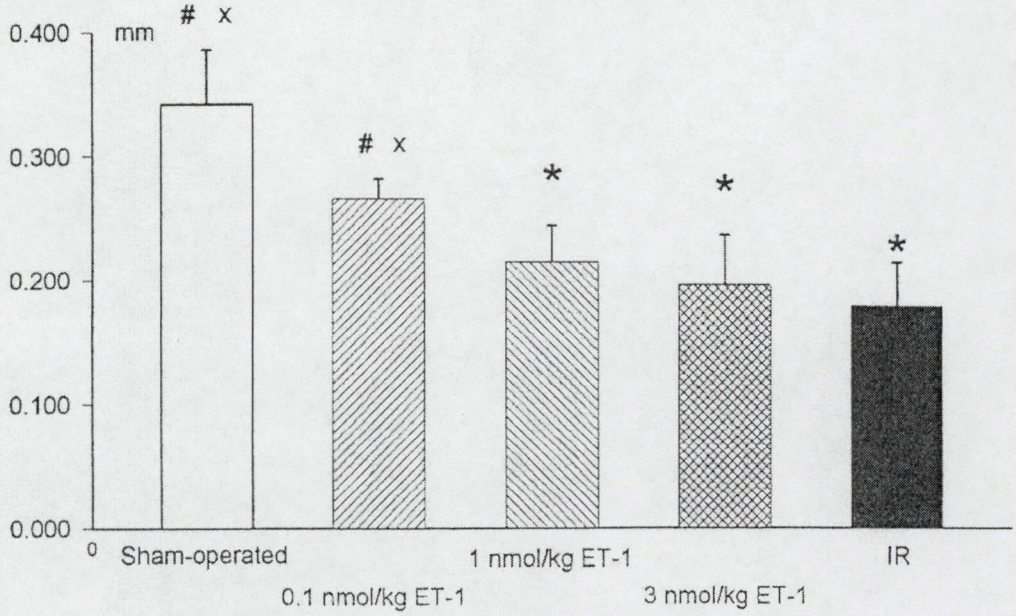
Treatment			*n*	*MAP*	*RBC flux*
IR	*ET-1 doses* (nmol/kg i.v.)			(mm Hg)	(RBC count x velocity)
-	-	Baseline	6	92±16	480±44
-	-	45 min	6	91±11	487±78 c, d
-	-	105 min	6	96±20	406±57 c, d
-	0.1	Baseline	5	102±7	416±73
-	0.1	45 min	5	105±12	379±62 c, d
-	0.1	105 min	5	107±15	345±86 a
-	1	Baseline	5	95±15	513±55
-	1	45 min	5	104±16	273±33 a, b
-	1	105 min	5	108±26	253±44 a, b
-	3	Baseline	6	93±12	540±44
-	3	45 min	6	110±18	207±23 a, b
-	3	105 min	6	88±16	235±78 a, b
+	-	Baseline	4	97±5	435±62
+	-	45 min	4	102±10	175±40 a, b
+	-	105 min	4	103±9	216±55 a, b

a: $p < 0.05$ vs. baseline; b: $p < 0.05$ vs. sham-operated group; c: $p < 0.05$ vs. 3.0 nmol/kg ET-1-treated group; d: $p < 0.05$ vs. IR group. Values expressed as means ± SD.

the ET-induced mucosal damage (Fig. 3, lower panel). Similarly, MC degranulation was significantly inhibited by the ET-A receptor antagonist pretreatment (Fig. 2, lower panel). ET-B receptor antagonist IRL-1038 peptide administration did not influence the 3 nmol/kg ET-1-induced local intestinal hemodynamic and morphological alterations.

Discussion

Impairment of splanchnic perfusion is a key event in the development of the acute circulatory response in systemic and local low-flow states, and the production of ET-1 increases significantly under these conditions [14]. Previous studies have revealed severe mucosal morphological alterations in the small intestine after exogenous ET-1 administration [11]. There



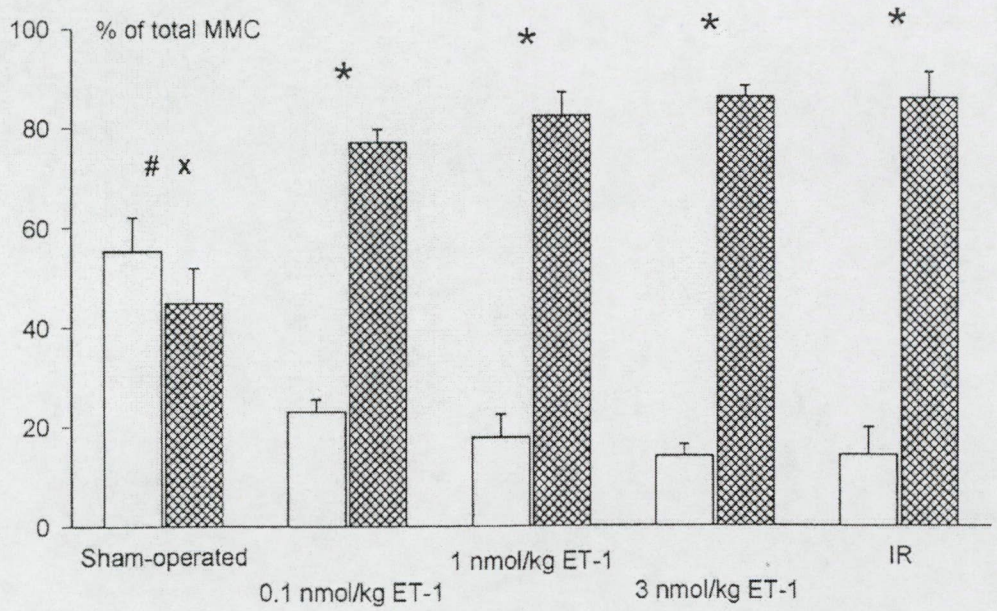


Fig. 1. Changes in small intestinal villus length (upper panel) and mucosal mast cell (MMC) degranulation (lower panel) in rats treated with 0.1 (n=5), 1 (n=5) or 3 (n=6) nmol/kg ET-1 or following 15-min ischemia-reperfusion (IR, n=4). In the lower panel, open bars represent the percentage of intact MMCs, while gray bars show the proportion of degranulated MMCs. Values are expressed as means ± SD. * $p<0.05$ vs. sham-operated group, $^{\#}p<0.05$ vs. 3 nmol/kg ET-1-treated group, $^{x}p<0.05$ vs. IR group. Kruskal-Wallis one-way analysis of variance on ranks.

Table 2
Effects of ET receptor antagonist pretreatment on the 3.0 nmol/kg ET-1-induced changes in the mean arterial pressure (MAP) and the microciruclation of the rat small intestine (RBC flux)

Treatment			*n*	*MAP*	*RBC flux*
ET receptor Antagonist	*ET-1* (3 nmol/kg i.v.)			(mm Hg)	(RBC count x velocity)
-	-	Baseline	6	92±16	480±44
-	-	45 min	6	91±11	487±78 c
-	-	105 min	6	96±20	406±57 c
ETR-P1/fl	+	Baseline	5	98±15	435±40
ETR-P1/fl	+	45 min	5	101±14	430±117
ETR-P1/fl	+	105 min	5	98±22	431±105
BQ-610	+	Baseline	5	103±15	505±174
BQ-610	+	45 min	5	107±20	436±100
BQ-610	+	105 min	5	95±27	519±170 c
IRL-1038	+	Baseline	6	105±25	513±92
IRL-1038	+	45 min	6	123±23	318±50
IRL-1038	+	105 min	6	105±25	306±76 a
-	+	Baseline	6	93±12	540±44
-	+	45 min	6	110±18	207±23 a, b
-	+	105 min	6	88±16	235±78 a, b

a: $p < 0.05$ vs. baseline; b: $p < 0.05$ vs. sham-operated group; c: $p < 0.05$ vs. 3.0 nmol/kg ET-1-treated group. Values expressed as means ± SD.

is also evidence that even a short period of intestinal arterial occlusion leads to structural damage to the mucosal layer and triggers the discharge of a variety of MC-derived inflammatory mediators into the mesenteric circulation [10,15,16]. It has further been demonstrated that ET-1 may liberate histamine and leukotriene C4 from bone marrow-derived MCs through the activation of ET-A receptors [17].

Various data suggest a multiple connection between endothelial cell-derived vasoactive mediators and the activation of intestinal MCs. It has been demonstrated that inhibition of nitric oxide (NO) synthesis leads to MC degranulation and a rise in microvascular permeability [18]. It has also been found that NO may decrease the intestinal permeability secondary to the inhibition of MC activation [19]. The major finding of the present study is that the vaso-

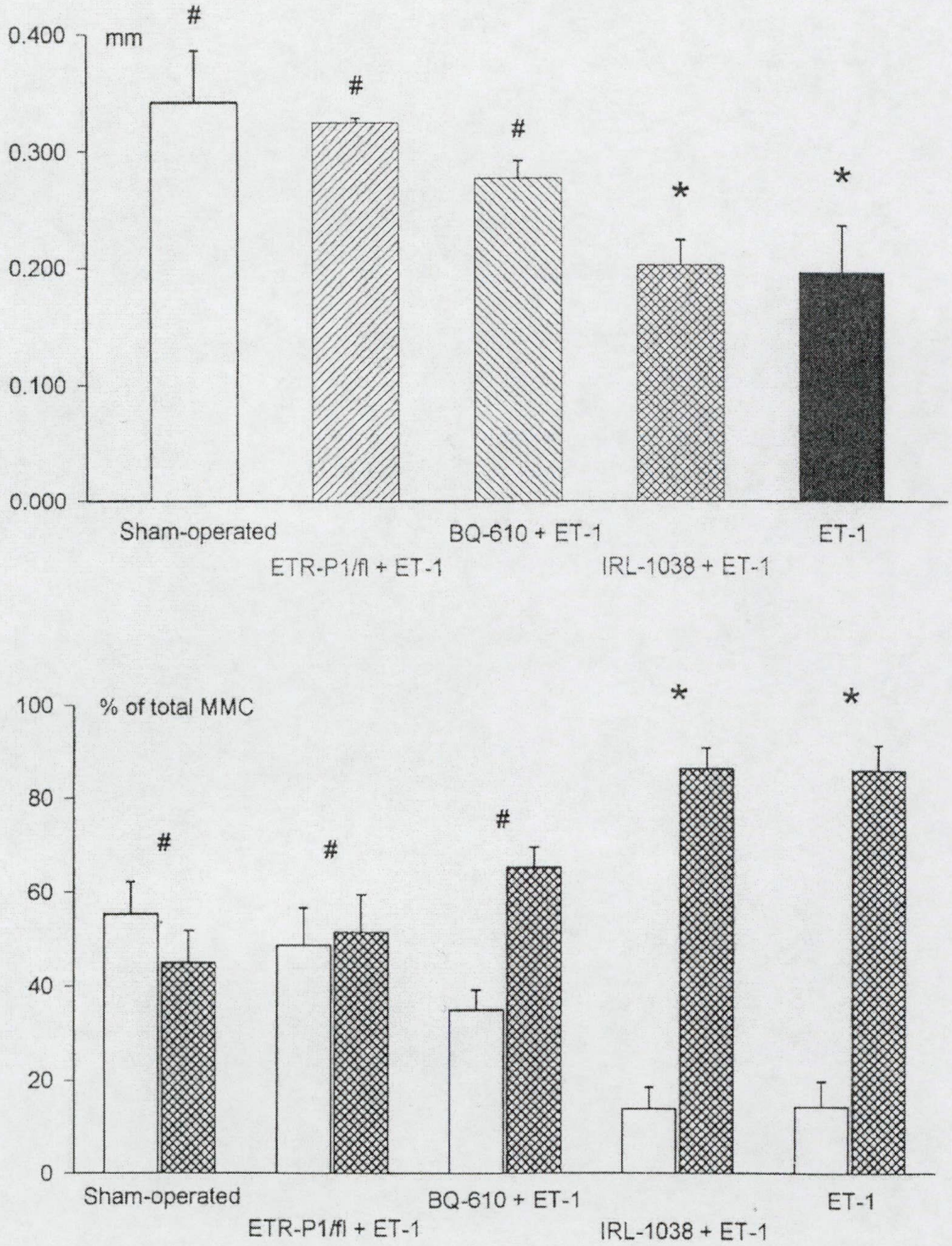


Fig. 2. Changes in small intestinal villus length (upper panel) and mucosal mast cell (MMC) degranulation (lower panel) in rats treated with 3 nmol/kg ET-1 (n=6) and in animals that received ET receptor antagonist pretreatment. ETR-P1/fl (n=5), BQ-610 (n=5) or IRL-1038 (n=6) was administered in a 300 nM/kg dose. In the lower panel, open bars represent the percentage of intact MMCs, while gray bars show the proportion of degranulated MMCs. Values are expressed as means ± SD. * $p<0.05$ vs. sham-operated group, # $p<0.05$ vs. 3 nmol/kg ET-1-treated group. Kruskal-Wallis one-way analysis of variance on ranks.

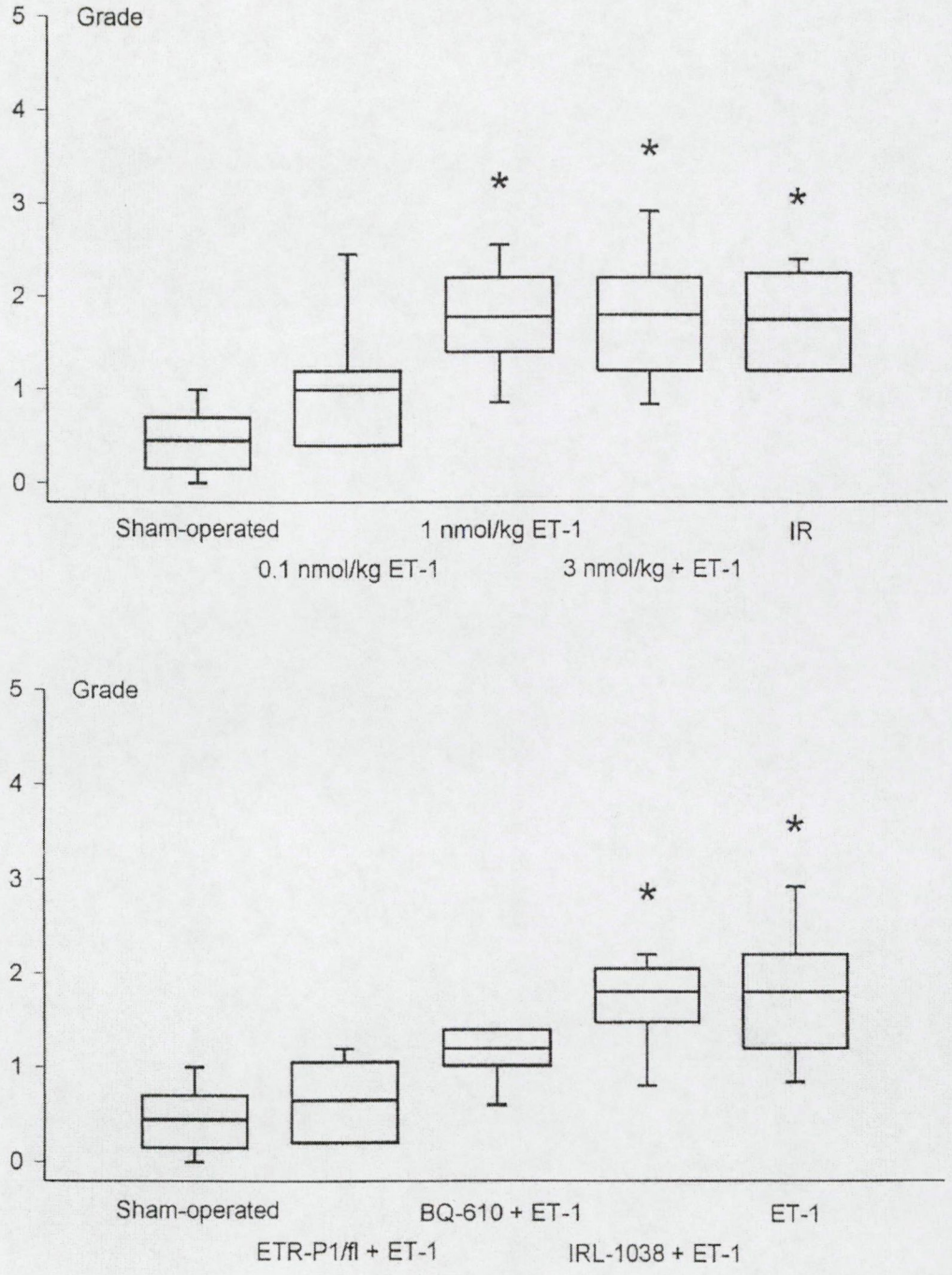


Fig. 3. Grading of mucosal damage according to the standard scale of Chiu. The upper panel represents the mucosal corruption induced by 0.1 (n=5), 1 (n=5) or 3 (n=6) nmol/kg ET-1 infusion or 15-min ischemia (n=4), respectively. In the lower panel data on pretreatment with ETR-P1/fl (n=5), BQ-610 (n=5) or IRL-1038 (n=6) are shown. Horizontal lines of boxes present the first, median and third quartiles of data. Whiskers show the 10th and 90th percentiles. * $p<0.05$ vs. sham-operated group (n=6). Kruskal-Wallis one-way analysis of variance on ranks.

constrictor mediator ET-1 could be involved in the mechanism of induction of MC responses in the small intestinal mucosa. The results show that ET-1 exerts significant effects on MC degranulation, and concomitantly induces significant intestinal microcirculatory disturbances and mucosal damage via the ET-A receptors. ET-1 undergoes mainly abluminar release and the half-life of the peptide in the circulation is very short as a consequence of the effective eliminating mechanisms. However, the ET-1 plasma level usually rises 2–5-fold over the baseline in ischemic conditions [9,20], and other observations indicate that the intestinal endothelial NO synthase activity is decreased after arterial occlusion [21]. It is possible, therefore, that a shift in the equilibrium between endothelial cell-derived vasoconstrictor and vasodilator factors has a significant influence on mucosal MC responses.

We used ET receptor antagonist pretreatments to separate the ET-receptor subtype-mediated effects of ET-1 in MC degranulation, and these changes were compared with those observed in the small intestine after a standardized ischemia-reperfusion challenge. The present results confirm that ET-1 may contribute to MC degranulation *in vivo* through the activation of ET-A receptors.

Depending on the localizations of the ET-A receptors, at least three possible mechanisms may be hypothesized. One possibility might be a direct effect of ET on MCs. This notion is supported by the data of Liu et al. [22] demonstrating ET-A receptor expression on the surface of MCs in the rat. A direct interaction between MC degranulating peptide (MDP) and G-proteins in MCs [23,24] has also been reported. With regard to the very close structural similarities between ET-1 and MDP [25], a similar connection between ET-1 and G-proteins in MCs might be suggested.

On the other hand, the profound ET-A receptor-mediated microvascular vasoconstriction and the ensuing ischemic injury could be another plausible explanation for the MC degranulation. The structural injury of the small intestinal mucosa may be directly connected with the hemodynamic consequences of ET administration. The mucosal lesions were similar to those described in animal models of intestinal ischemia-reperfusion or after nanomolar doses of exogenous ET-1 [11]. In this case, tissue hypoxia, or oxygen-derived free radicals generated during local ischemia-reperfusion injuries could also be MC-degranulating factors.

Another explanation may be provided by the altered osmolarity of the intestinal mucosa as a result of localized perivascular edema. The fragility of the MC membranes to osmotic stress has been demonstrated. Intraperitoneal injection of hypotonic salt solutions results in MC membrane destruction, without induction of the osmotic disruption of other cell types [26]. Indeed, Filep et al. have shown that ET-1 causes dose-dependent increases in vascular permeability through the activation of ET-A receptors as a consequence of the disruption of the endothelial barrier [27]. Following the decrease in arterial inflow, the declining energy supply for active membrane transport processes and the lack of removal of metabolites may be accompanied by a rapid fluid movement from the vascular lumen to the lamina propria. An acute circulatory breakdown may therefore rapidly cause perivascular edema, leading to MC degranulation.

The ET-1-induced structural damage was characterized by a significant shortening of the villus height, a loss of villus epithelium, and the invasion of inflammatory cells. Similar to samples from the ischemic-reperfused ischemic tissue, there was a clear tendency in each case toward a villus height reduction. ET-A receptor antagonism by BQ-610 or ETR-P1/fl

peptide was effective in reducing the morphological signs of tissue injury. In a previous study, ET-A receptor antagonist pretreatment significantly diminished the postocclusive damage to the intestinal mucosa [28]. Our results demonstrate that ET-A receptor antagonism may have additional beneficial activity through the inhibition of MC reactions during intestinal pathologies.

In conclusion, exogenous ET-1 infusion significantly affects the perfusion characteristics of the small bowel and enhances degranulation of the intestinal MCs by an ET-A receptor-dependent mechanism. Recent data indicate that MCs and ET-1 may both be involved in the mechanism of endothelial cell-leukocyte interactions and neutrophil sequestration after ischemia [12,29]. The MCs are located in the vicinity of the submucosal collecting venules, which are primary targets of leukocyte-endothelial interactions during mucosal pathologies. Our data suggest that an important connection exists between endothelial cell-derived humoral mediators and the perivascular MC system. If ET-1 acts as an amplifier of the process of leukocyte activation, any alteration in this mechanism could have important consequences in local tissue responses.

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Endothelin-1 induces mucosal mast cell degranulation and tissue injury via ET_A receptors

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A B S T R A C T

The effects of endothelin-1 (ET-1) on mucosal mast cells are of special interest, since they may be an important component of the tissue response that occurs during ischaemic preconditioning or ischaemia/re-oxygenation injuries. Increasing doses of ET-1 were administered intravenously to anaesthetized rats. In a second series of experiments, animals were pretreated with the ET_A receptor antagonists BQ-610 or ETR-P1/f1 peptide, or with the ET_B receptor antagonist IRL-1038. Intestinal perfusion changes were recorded, and the proportion of degranulated mast cells and the degree of mucosal damage were determined in ileal biopsies. ET-1 induced dose-dependent alterations in the haemodynamic and morphological parameters, and caused significant mast cell degranulation. These changes were inhibited significantly by pretreatment with the ET_A receptor antagonists, but not with the ET_B receptor antagonist. We conclude that a cross-talk exists between endothelial cell-derived humoral mediators and the intestinal mast cell system.

INTRODUCTION

The mucosal mast cells (MCs) of the gastrointestinal tract are a unique cellular source of both preformed and *de novo* synthesized mediators. They are located mainly around postcapillary venules, from where they can influence local tissue reactions [1]. Intestinal MCs have both ET_A and ET_B receptors on their membrane surface, and this suggests a possible cross-talk between endothelial cell-derived humoral mediators and the MC system [2,3].

One of the early events in low-flow conditions is the production of activators of secondary circulatory responses. There is a growing body of evidence that, in addition to the role of endothelin-1 (ET-1) as a dominant vasoconstrictor [4], this peptide may also influence the biological activity of other cell types in the cardiovascular system, including polymorphonuclear leucocytes [5]. The effect of ET-1 on MCs is of special interest, since it may also be an important component of the tissue response that occurs in the mucosa during inflammation or ischaemia/reperfusion injuries.

Our studies were directed towards an examination of whether and how intestinal MCs respond to increasing doses of exogenously administered ET-1. To this end, ET-1-induced mucosal morphological changes were correlated with the degree of MC degranulation. Secondly, we used ET_A- and ET_B-receptor-selective antagonists to investigate the roles of these receptor subtypes in mediating ET-1-induced intestinal MC activation.

MATERIALS AND METHODS

Animals

The experiments were performed in accordance with U.S. National Institutes of Health guidelines on the use

Key words: ET_A receptor, ETR-P1/f1 peptide, mucosal damage, rat, small intestine.
Abbreviations: ET-1, endothelin-1; MC, mast cell.
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of experimental animals. A total of 42 male Sprague–Dawley rats (body weight 200±20 g) were deprived of food, but not water, for 12 h prior to the experiments. The animals were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal). The left carotid artery and jugular vein were cannulated for the recording of mean arterial pressure and the injection of test compounds respectively. Throughout the experiment, the animals received an infusion of Ringer's lactate at a rate of 40 ml · kg^{-1} · h^{-1}. After a transverse laparotomy, a segment of the terminal ileum perfused by a single artery was selected. The marginal vessels were divided and ligated, and the intestinal segment with intact neurovascular connections was covered by plastic sheets.

Experimental protocol

In the first series of experiments, dose responses to ET-1 (Alexis Corp., Läufelfingen, Switzerland) were obtained. The animals were randomly allotted into the following groups: group 1, sham-operated ($n = 6$); group 2, 0.1 nmol/kg ET-1 ($n = 5$); group 3, 1 nmol/kg ET-1 ($n = 5$); group 4, 3 nmol/kg ET-1 ($n = 6$). At 30 min after the end of baseline measurements, a solution of 0.1 ml of ET-1 or vehicle was infused intravenously into the systemic circulation over 15 min. In an additional group of animals ($n = 5$) segmental intestinal ischaemia was induced by a 15-min occlusion of the ileal artery.

In the second series of experiments, an ET_A receptor antagonist {ETR-P1/fl peptide (Kurabo Ltd, Osaka, Japan) or BQ-610 [homopiperidinyl-carbonyl-Leu-D-Trp(CHO)-D-Trp-OH, Alexis Corp.]}, or the ET_B receptor antagonist IRL-1038 {[Cys^{11},Cys^{15}]endothelin-1-(11–21)}, was infused intravenously (0.3 μmol/kg) for 30 min, followed by a 15-min infusion of ET-1 into the systemic circulation after the end of BQ-610, ETR-P1/fl peptide or IRL-1038 pretreatment. The circulatory changes were observed for 60 min, and at the end of the observation period a tissue sample was taken from the intestinal segment.

Histology

Intestinal biopsy samples were placed into Carnoy's fixative and trimmed along the longitudinal axis. The samples were embedded in paraffin, sectioned (6 μm) and stained with haematoxylin/eosin and Alcian Blue/safranin O (pH 0.4). An image analysis system (IVM; Pictron Kft., Budapest, Hungary) was used to digitize the x and y coordinates of the sections. Three non-overlapping fields were processed in each section, and the average height of a single villus was measured from its origin to the villus tip. Mucosal damage was assessed according to the standard scale of Chiu et al. [5a]. The grading was performed with the following criteria: grade 0, normal mucosa; grade 1, development of subepithelial space at the tip of the villus; grade 2, extension of the space with epithelial lifting; grade 3, massive epithelial lifting; grade 4, denuded villi; grade 5, disintegration of the lamina propria. Positively stained MCs were quantified in the villi of an average of 20 villus–crypt units. Counting was performed in coded sections at ×400 optical magnification by one investigator. Loss of intracellular granules, with stained material dispersed diffusely within the lamina propria, was taken as evidence of MC degranulation.

Statistics

The Friedman test followed by Dunnett's method was applied for multiple comparisons with a control. Differences between groups were analysed by Kruskal–Wallis one-way ANOVA on ranks. P values of < 0.05 were considered significant. Mean values ± S.D. are given.

RESULTS

The resting haemodynamic parameters were similar in each of the groups studied (results not shown). In the sham-operated group, the villus MC count was unchanged, and no significant increase in degranulation was observed in biopsies taken at the end of the observation period. A significant, dose-dependent diminution of villus height was induced by ET-1 infusion as compared

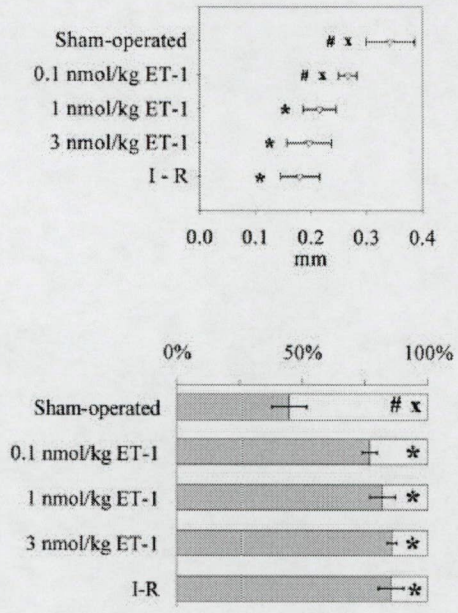

Figure 1 ET-1-induced MC degranulation

Upper panel: changes in intestinal villus length in rats treated with 0.1, 1 or 3 nmol/kg ET-1, or following 15 min of ischaemia/60 min of reperfusion (I-R). Significance of differences: $^*P < 0.05$ compared with sham-operated group; $\#P < 0.05$ compared with 3 nmol/kg ET-1 group; $\times P < 0.05$ compared with I-R group. Lower panel: changes in mucosal MC degranulation as a percentage of intact MCs. Values are expressed as means ± S.D.

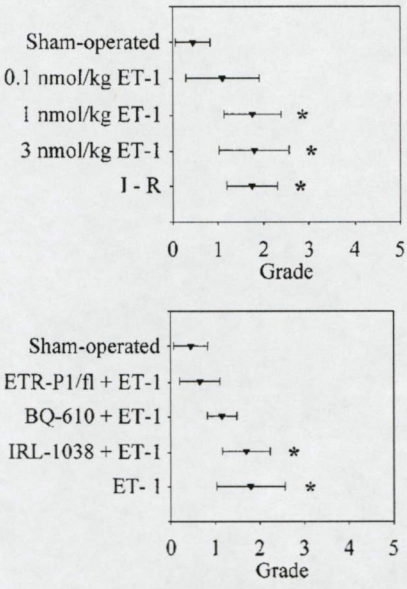

Figure 2 ET-1-induced mucosal damage

Upper panel: grading of mucosal damage after infusion of 0.1, 1 or 3 nmol/kg ET-1, or after 15 min of ischaemia/60 min of reperfusion (I-R). Significance of differences: $^{*}P < 0.05$ compared with sham-operated group. Lower panel: mucosal damage in rats that received pretreatment with an ET receptor antagonist.

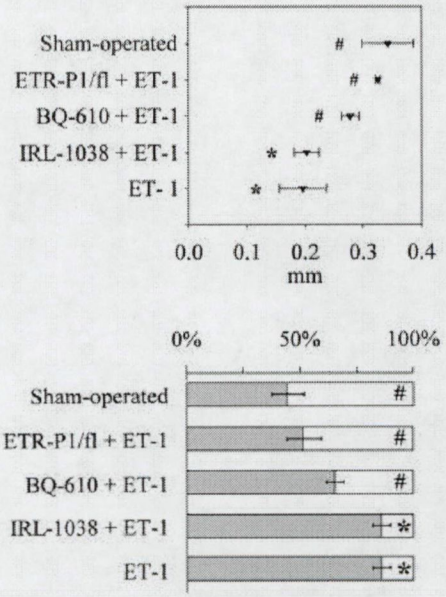

Figure 3 Effects of pretreatment with ET receptor antagonists

Upper panel: changes in small intestinal villus length in animals that pretreated with an ET receptor antagonist. ETR-P1/fl, BQ-610 or IRL-1038 was administered at a dose of 300 nmol/kg. Lower panel: changes in small intestinal mucosal MC degranulation, as a percentage of intact MCs, in rats treated with 3 nmol/kg ET-1 following ET receptor antagonist pretreatment. Significance of differences: $^{*}P < 0.05$ compared with sham-operated group; $\#P < 0.05$ compared with 3 nmol/kg ET-1 group (no ET receptor antagonist pretreatment).

with the control group. The shortening of the villi was statistically significant after the administration of 1 or 3 nmol/kg ET-1, and there was a significant difference in this parameter between the 0.1 and 3 nmol/kg ET-1 groups (Figure 1, upper panel). The MC degranulation ratio exhibited a significant increase after ET-1 treatment. The ET-1 infusions elevated the proportion of degranulated MCs almost 2-fold in each of the ET-1-treated groups (Figure 1, lower panel). Simultaneously, mucosal alterations, as assessed on the Chiu scale, were statistically different from the control in the 1 and 3 nmol/kg ET-1 groups (Figure 2).

The ET_A receptor antagonists ETR-P1/fl peptide and BQ-610 attenuated the ET-induced villus shortening and mucosal damage (Figure 3, upper panel). Similarly, MC degranulation was significantly inhibited by pretreatment with the ET_A receptor antagonists (Figure 3, lower panel). Administration of the ET_B receptor antagonist IRL-1038 did not influence the MC degranulation and morphological alterations induced by 3 nmol/kg ET-1.

DISCUSSION

Previous studies have revealed that even a short period of intestinal arterial occlusion leads to structural damage to the mucosal layer and triggers the discharge of a variety of MC-derived inflammatory mediators into the mesenteric circulation [1]. The major finding of the present study is that the vasoconstrictor mediator ET-1 induces intestinal mucosal damage, and concomitantly exerts significant effects on MC degranulation via ET_A receptors. Pretreatment with ET_A receptor antagonists was effective in reducing the morphological signs of ET-1-induced structural damage.

Depending on the localization of the ET_A receptors, at least three possible mechanisms may be hypothesized to account for the observed results. (1) One possibility is a direct effect of ET-1 on MCs. This notion is supported by data demonstrating ET_A receptor expression on the surface of MCs in the rat [2,3]. A direct interaction between MC-degranulating peptide and G-proteins in MCs has also been reported [6]. Given the very close structural similarities between ET-1 and MC-degranulating peptide, a similar interaction between ET-1 and G-proteins in MCs might be suggested. (2) The profound ET_A-receptor-mediated microvascular vasoconstriction and the ensuing ischaemic injury could be another plausible explanation for the observed MC degranulation. In this case, structural injury to the small intestinal mucosa may be connected directly to the

haemodynamic consequences of ET administration. The mucosal lesions observed in the present study were similar to those described in animal models of intestinal ischaemia/reperfusion or after administration of nanomolar doses of exogenous ET-1 [7]. In this case, tissue hypoxia, or oxygen-derived free radicals generated during local ischaemia/reperfusion injury, could also be MC-degranulating factors. (3) Another explanation may be provided by the alteration to the osmolarity of the intestinal mucosa that occurs as a result of localized perivascular oedema. The fragility of the MC membranes under conditions of osmotic stress has been demonstrated. Indeed, Filep et al. [8] have shown that ET-1 causes dose-dependent increases in vascular permeability through the activation of ET_A receptors as a consequence of the disruption of the endothelial barrier. Following the decrease in arterial inflow, the declining energy supply for active membrane transport processes and the lack of removal of metabolites may be accompanied by rapid fluid movement from the vascular lumen to the lamina propria. An acute circulatory breakdown may therefore rapidly cause perivascular oedema, leading to MC degranulation.

In conclusion, infusion of exogenous ET-1 significantly enhanced degranulation of intestinal MCs by an ET_A-receptor-dependent mechanism. Previous data indicate that MCs and ET-1 may both be involved in the mechanisms of endothelial-cell–leucocyte interactions and the sequestration of polymorphonuclear leucocytes after ischaemia [5,7,9]. Our results demonstrate that ET_A receptor antagonism may have additional beneficial activity through the inhibition of MC reactions during intestinal pathologies. Similarly, these data suggest that an important interaction exists between endothelial cell-derived humoral mediators and the perivascular MC system. If ET-1 acts as an amplifier of the process of leucocyte activation, any alteration in this mechanism could have important consequences for local tissue responses.

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