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An orally active non-selective endothelin receptor antagonist, bosentan, markedly reduces injury in a rat model of colitis

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Abstract

Activation of endothelial cells by vasoactive mediators, such as endothelins, may be an early, strategically important step in the initiation of inflammation in the intestine. In view of recent evidence that inflammatory bowel disease is associated with elevated intestinal concentrations of endothelins and upregulated expression of endothelin receptors on vascular endothelium in intestine, endothelins may become therapeutic targets in inflammatory bowel disease. The recent availability of an orally active, mixed endothelin receptor antagonist, bosentan, allowed us to examine the role of endothelins in a rat model of colitis. Colitis was induced by intra-rectal administration of trinitrobenzene sulphonic acid. In each treatment group, rats were treated with bosentan (10–60 mg/kg p.o.) 24 and 2 h prior to (pre-dose) or 1 h after the induction (post-induction) of colitis and all animals were treated every 24 h thereafter for 5 days. On day 6, stool consistency and the presence of adhesions in the peritoneal cavity were accessed. Colonic tissue samples were removed for determination of macroscopic and microscopic tissue injury, and myeloperoxidase activity. Colitis was typified by tissue ulceration in the distal colon and a corresponding 35-fold increase in myeloperoxidase activity compared to non-inflamed controls. Daily treatment with bosentan dose-dependently reduced colonic damage and myeloperoxidase activity when bosentan was given prior to induction of colitis. In the pre-dose group, the greatest beneficial effect of bosentan was observed at 60 mg/kg; colonic damage and granulocyte infiltration were attenuated by $> 80\%$. A partial therapeutic effect of bosentan was also observed at 60 mg/kg when the pre-treatment regimen was excluded. These findings demonstrate that an orally active, mixed endothelin receptor antagonist has marked protective and therapeutic effects in an animal model of colitis.

Keywords: Endothelin; Intestinal inflammation; Inflammatory bowel disease; Bosentan

1. Introduction

Considering that the 21 amino acid peptides referred to collectively as endothelins and individually as endothelin-1, endothelin-2 and endothelin-3 possess potent vasoconstrictor and mitogenic properties (Yanagisawa et al., 1988; Inoue et al., 1989), it is plausible that endothelins contribute to the pronounced vasoconstriction, congestion and neovascularization observed in inflammatory diseases of the gastrointestinal tract. Elevated intestinal tissue levels of endothelin-1 have been detected in ulcerative colitis and Crohn's disease (Murch et al., 1992). Immunohistochemical analysis of endothelin-1 immunoreactivity in tissue samples from patients with ulcerative colitis revealed that endothelin-1 immunoreactivity in polymorphonuclear leucocytes in the lamina propria was elevated when compared to non-inflamed control samples. In biopsies from patients with Crohn's disease, endothelin-1-positive cells were more prevalent in the submucosa than in the lamina propria of the intestine, most notably in peri-vascular aggregates of macrophages (Murch et al., 1992). Endothelin-1 is also produced by endothelial cells, vascular smooth muscle cells, adrenal medullary cells, leucocytes and macrophages (Miller et al., 1993). In a preliminary report, Hudson et al. (1994) demonstrated that endothelin-1 receptor density in the intestine of patients with Crohn's disease, most notably in the villi, intestinal smooth muscle and submucosal vessels of the intestine was also increased.

From animal studies, endothelin-1 has numerous pathophysiological effects in the stomach. For example, intra-arterial infusion of endothelin-1 caused haemorrhagic dam-

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age to the gastric mucosa (Whittle and Esplugues, 1988; Wallace et al., 1989). Endogenous endothelin-1 plays a prominent role in ischemia-reperfusion- (Michida et al., 1994), non-steroidal anti-inflammatory drug- (Katajima et al., 1993) and ethanol- (Masuda et al., 1993) induced gastric injury in the rat. Unfortunately, little is known about the pathological role of endothelins during prolonged intestinal inflammation. Specific actions of endothelins in the gastrointestinal tract have been elucidated on the basis of the distribution of the endothelin receptors, endothelin ET_A receptor and ET_B receptor. The endothelin ET_A receptor is widely distributed on vascular smooth muscle cells (Hirata et al., 1988), it has a high affinity for endothelin-1 and it mediates a major part of the vasoconstrictor effects of endothelin-1 and endothelin-2. The endothelin ET_B receptor is expressed predominately on endothelial cells, but it has also been localized on intestinal smooth muscle cells (Kitsukawa et al., 1994) and enteric neural tissue (Inagaki et al., 1991). The endothelin ET_B receptor is linked to nitric oxide synthesis in endothelial cells, thus its activation by endothelin-1, endothelin-2 or endothelin-3 stimulates vasorelaxation but it is important to note that in some systems endothelin ET_B receptor activation leads to vasoconstriction (Clozel et al., 1992).

The protective effects of bosentan (RO 47-0203), an orally active mixed (i.e. endothelin ET_A and ET_B receptor) endothelin receptor antagonist (Clozel et al., 1994), were examined in a commonly used rat model of colitis induced by trinitrobenzene sulphonic acid in 50% ethanol. This model has some histological features analogous to Crohn's disease (Morris et al., 1989) and the major cellular contributor (about 80%) to the injury in this model is the granulocyte which gains access to the colonic tissue through post-capillary venules (Wallace et al., 1992). After oral administration, bosentan has a long duration of action (up to 24 h) and has no intrinsic agonist activity (Clozel et al., 1994). Thus, the purpose of this study was to examine the involvement of endothelins in the distal colonic injury associated with colitis in the rat.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (180–200 g; Charles River Laboratories, St. Constant, Quebec, Canada) were kept in filter-topped cages with free access to food and water. All rats were housed in a positive pressure room with controlled temperature and photoperiod for the duration of these studies. All experimental procedures described below were approved by the Animal Care Committee at McMaster University and were in accordance with guidelines of the Canadian Council on Animal Care. As previously described (Morris et al., 1989), distal colitis was induced by intra-colonic instillation of 30 mg of 2,4,6-trinitrobenzene sulphonic acid (Eastman Kodak, Rochester, NY, USA) dissolved in 0.25 ml of 50% ethanol (v/v). All rats were anaesthetized with Metafane (methoxyflurane; Janssen Pharmaceutica, Mississigua, ON, Canada) and trinitrobenzene sulphonic acid/ethanol was injected into the colon, 8 cm proximal to the anus with a PE 50 cannula. The cannula was left in place for 15 s to prevent immediate expulsion of the solution, then the rats were returned to their cages. The control (i.e. uninflamed) groups received 0.25 ml of normal saline (0.9%; w/v) in a similar manner.

2.2. Dosing protocol

At 24 and 2 h prior to induction of colitis, rats were treated with the oral preparation of bosentan (10, 30 or 60 mg/kg p.o.) suspended in 5% gum arabic (vehicle). Bosentan was administered twice prior to induction of colitis to ensure that adequate plasma levels of bosentan were present (Clozel et al., 1994). All rats were treated once daily thereafter for 5 days. To determine the efficacy of this compound after the initiation of inflammation, bosentan (10, 30 or 60 mg/kg p.o.) was administered 1 h after the induction of colitis and once daily for 5 days. In both treatment regimens, appropriate vehicle controls were administered in the same manner.

Uninflamed rats were also treated with bosentan (60 mg/kg p.o.) in a manner similar to that employed in the pre-dose groups receiving trinitrobenzene sulphonic acid in ethanol. These rats were monitored over the dosing period for any changes in fecal consistency.

2.3. Assessment of macroscopic damage

On day 6 following initiation of colitis, each rat was anaesthetized and killed by cervical dislocation, and the entire length of the colon was excised and opened longitudinally. All animals were killed on day 6 of colitis because this time point represents the transition point between the acute and chronic phases of inflammation in this model. At day 3, the acute inflammation in the distal colon, characterized by neutrophilic granulocyte infiltration, is maximal and by day 14 a pronounced lymphocytic/monocytic infiltrate is present most notably in the vicinity of ulcers (Morris et al., 1989). Colonic tissues were also removed from uninflamed rats that received bosentan alone. After noting the consistency of the luminal contents and the presence of adhesions in the peritoneal cavity, the entire length of the colon was cleaned and macroscopically visible damage was scored in a blinded fashion using a system described in detail by Wallace and Keenan (1990). Briefly, damage scores were based upon the extent of ulceration in the colon and the presence of other indices of tissue damage (i.e. thickening, discoloration and hyperaemia). The scoring scheme employed was as follows: 0, normal appearing colon; 3, ulceration with inflammation at one site, no thickening or hyperaemia; 5, damage extending

> 1 cm along colon, thickened and discoloured tissue, hyperaemia; 10, colonic damage extending > 5 cm along the length of the colon, severe thickening, hyperaemia and discoloration.

2.4. Colonic myeloperoxidase activity

Circular segments of colon (300–600 mg) were removed for the measurement of myeloperoxidase activity from an area approximately 4–6 cm proximal to the anus, corresponding to the area of major macroscopic injury. Samples were snap frozen in liquid nitrogen and stored at −70°C for a minimum of 24 h before myeloperoxidase was measured as previously described by Wallace and Keenan (1990). Myelperoxidase is a granule associated enzyme primarily found in neutrophils and its measurement has been commonly used as an index of granulocyte infiltration into intestinal tissue (Wallace and Keenan, 1990). Myeloperoxidase activity is reported here as units of myeloperoxidase per mg of wet tissue, where a unit of myeloperoxidase is defined as the quantity of enzyme able to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature (24°C).

2.5. Histology

All circular colonic samples for histological assessment were removed from a region immediately proximal to that taken for assay of colonic myeloperoxidase activity (see above). After fixing overnight in neutral-buffered formalin, each sample was processed routinely, with 4 μm thick cross-sections stained with haematoxylin and eosin. Tissues were assessed by an observer blinded to their treatment for extent of ulceration and inflammation.

2.6. Materials

Bosentan (Ro 47-0203; 4-tert-butyl-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide) was kindly provided by Dr. Martine Clozel of F. Hoffman-La Roche (Basel, Switzerland) and was prepared freshly each day. Unless otherwise stated, all reagents and chemicals were obtained from Sigma Chemical (St. Louis, MO, USA).

Table 1
Incidence of colonic-intestine adhesions and diarrhoea induced by trinitrobenzene sulphonic acid and 50% ethanol: effects of pretreatment and/or post-treatment dosing regimens with bosentan

Group	Dose (mg/kg)	Adhesions (%)	Diarrhoea (%)
Vehicle-control			
($n = 15$)	0	11 (73)	3 (20)
Pre-dose and post-induction treatment			
($n = 10$)	10	7 (70)	8 (80)
($n = 10$)	30	1 (10)	2 (20)
($n = 5$)	60	1 (20)	1 (20)
Post-induction treatment only			
($n = 5$)	10	5 (100)	5 (100)
($n = 5$)	30	3 (60)	3 (60)
($n = 5$)	60	2 (40)	2 (40)

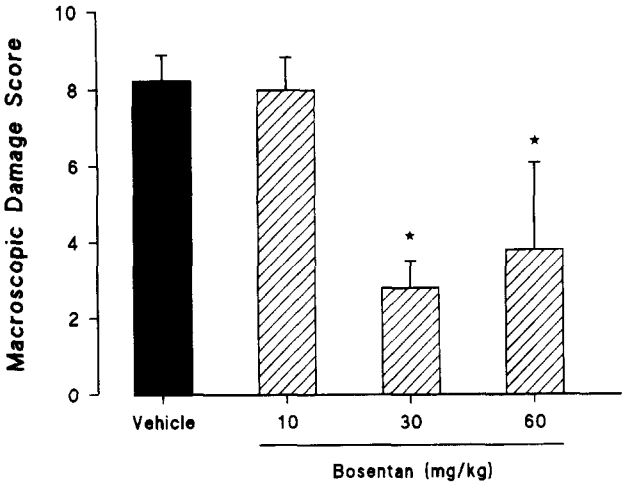

Fig. 1. Effects of bosentan (10–60 mg/kg p.o.) or vehicle (5% gum arabic) on macroscopic colonic damage scores 6 days after the induction of colitis. Bosentan treatment was given 24 and 2 h prior to induction of colitis and daily thereafter for 5 days. Colonic damage was determined by an observer blinded to the treatment using the scoring criteria outlined in Materials and methods. Each bar represents the mean and S.E.M. of 5–15 rats. Significant differences from the inflamed vehicle-control group: $^{*}P \leq 0.05$.

2.7. Statistical analysis

All data are expressed as means ± S.E.M.; n refers to the number of rats. Statistical differences were calculated

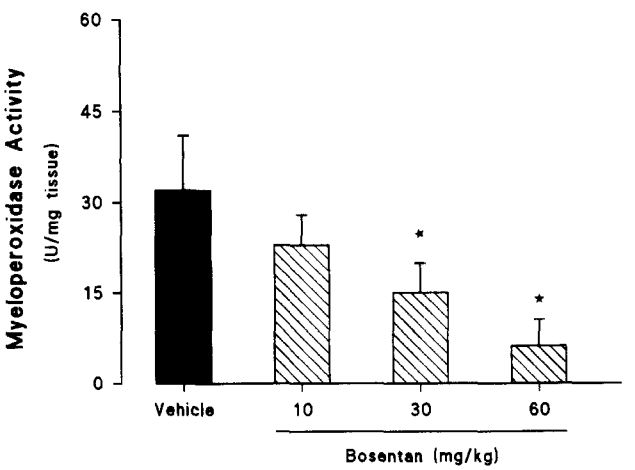

Fig. 2. Transmural myeloperoxidase activity in samples of distal colon removed from bosentan (10–60 mg/kg p.o.)- or vehicle-treated (5% gum arabic) rats on day 6 post-induction of colitis. Bosentan treatment was given 24 and 2 h prior to induction of colitis and daily thereafter for 5 days. Each bar represents the mean and S.E.M. of 5–15 rats. Significant differences from the inflamed vehicle-control group: $^{*}P \leq 0.05$.

using one-way analysis of variance, and multiple comparisons were performed using the Newman-Keuls multiple comparison test. An associated probability (P value) of $\leq 5\%$ was considered significant.

3. Results

3.1. Protective effects of bosentan in trinitrobenzene sulphonic acid-induced colitis

3.1.1. Attenuation of clinical and macroscopic injury

On day 6, the presence of multiple adhesions between the inflamed colon and small intestine and liquid stools (i.e. diarrhoea) was noted in rats gavaged with the vehicle (5% gum arabic solution) for bosentan (Table 1). Adhesions were noted in 11 out of 15 (73%) rats and diarrhoea was noted in 3 out of 15 (20%) rats killed 6 days after induction of colitis. Vehicle-control animals were gavaged twice with the vehicle at 24 h and 2 h before induction of colitis and each rat was subsequently gavaged daily for 5 days. Bosentan-treated rats received bosentan over the same time period. Using this treatment regimen, the lowest dose of bosentan (10 mg/kg p.o.) did not affect the incidence of adhesions (7 of 10 rats or 70%) between the affected colon and the small intestine, but the incidence of diarrhoea observed in 8 of 10 rats in this treatment group was markedly increased compared to that observed in the vehicle-control group (Table 1). Following treatment of rats with 30 mg/kg p.o. of bosentan, adhesions were observed in 1 rat of 10 examined at day 6. Diarrhoea was observed in 2 of the 10 animals (20%). Rats dosed with 60

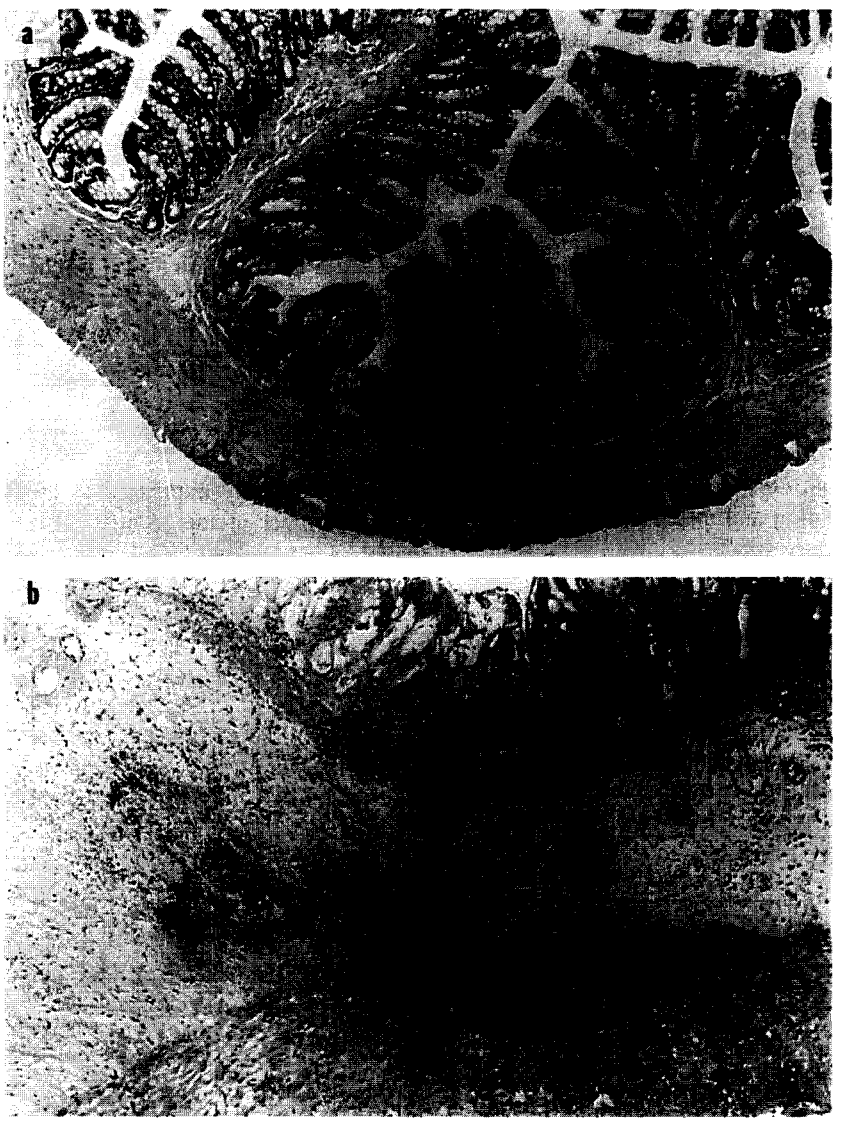

Fig. 3. Bosentan treatment was given 24 and 2 h prior to induction of colitis and daily thereafter for 5 days. Histological appearance of distal colon removed from uninflamed [panel A] and inflamed rats treated with vehicle [panel B]. Panel C demonstrates the effect of 30 mg/kg of bosentan and the protective effect of 60 mg/kg bosentan is depicted in panel D. Oral drug treatment was given 24 and 2 h prior to induction of colitis and daily thereafter for 5 days (see Materials and methods) (original magnification = 100 ×).

mg/kg of bosentan experienced a lower incidence of adhesions (1 of 5 rats or 20%) and a similar incidence of diarrhoea (1 of 5 rats or 20%) compared to vehicle-treated inflamed controls (Table 1). Damage scores in the vehicle-control and bosentan-treated colitic rats were calculated by macroscopic inspection of the colonic mucosa on day 6 post-trinitrobenzene sulphonic acid administration and these values are summarized in Fig. 1. Typically, injury and prominent ulcerations to the mucosa of the distal colon extended for 4–5 cm in vehicle-treated inflamed rats, corresponding to the extent of contact of the colonic mucosa with trinitrobenzene sulphonic acid and ethanol. Hyperaemia and pronounced transmural thickening of the colonic wall were also noted. The damage score in vehicle-treated colitic rats was 8 ± 0.5 (Fig. 1). Treatment of inflamed rats with 10 mg/kg of bosentan did not affect the degree of macroscopic damage, but distal colonic damage was significantly ($P \leq 0.05$) reduced in the 30 and 60 mg/kg treatment groups (Fig. 1). The greatest reduction in macroscopic damage was observed in rats treated with 30 mg/kg bosentan.

In rats that received bosentan (60 mg/kg p.o.) alone, there was no evidence of diarrhoea at any time during the treatment period (not shown).

3.1.2. Inhibition of granulocytic infiltration in the inflamed colon

This model of colitis is characterized by a marked granulocyte infiltration into the distal colon, which is reflected by elevations in transmural myeloperoxidase activity (Wallace and Keenan, 1990). In this study, myeloperoxidase activity was approximately 30-fold higher in vehicle control rats than in uninflamed rats (31.5 ± 9.2 vs. 1.3 ± 0.4 U/mg tissue). Further, treatment of uninflamed rats with 60 mg/kg bosentan had no effect on distal colonic myeloperoxidase values (not shown). Treat-

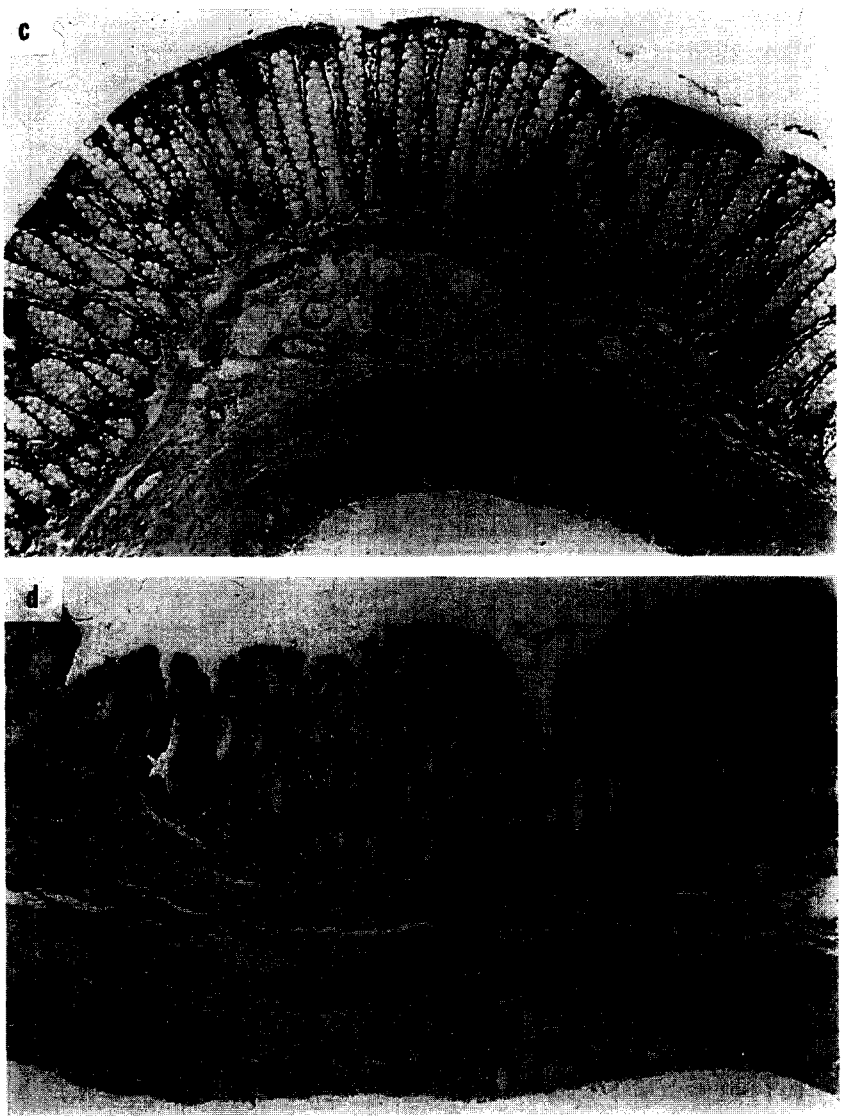

Fig. 3 (continued).

ment of inflamed rats with 10 mg/kg of bosentan reduced distal colonic myeloperoxidase by approximately 30% compared to vehicle controls. Corresponding with the marked attenuation in macroscopic colonic injury, myeloperoxidase activity in colon taken from rats treated with 30 mg/kg was 50% lower ($P \leq 0.05$) than in vehicle controls (Fig. 2). The greatest inhibition of distal colonic myeloperoxidase activity was observed in rats receiving 60 mg/kg p.o. In these animals, myeloperoxidase activity was reduced approximately 4-fold compared to vehicle-treated colitic rats.

3.1.3. Microscopic improvement in distal colon

Histological assessment of distal colon from vehicle-control rats revealed severe transmural disruption of the normal architecture of the colon (Fig. 3, Panel A [uninflamed] vs. Panel B [colitis]). Ulceration, disrupted crypt architecture, a pronounced inflammatory infiltrate and marked transmural intestinal thickening were apparent in histological cross-sections of colon. Macrophages and lymphocytes were also evident in some regions of inflamed colon. Groups treated with bosentan at 30 and 60 mg/kg p.o. (Fig. 3, Panels C and D, respectively) had markedly less microscopic tissue injury.

3.2. Therapeutic effects of bosentan in trinitrobenzene sulphonic acid-induced colitis

3.2.1. Clinical and macroscopic assessment

To determine whether oral bosentan treatment would interrupt the inflammatory process, other groups of five rats received bosentan 1 h following the induction of colitis. All rats were gavaged once daily with vehicle or

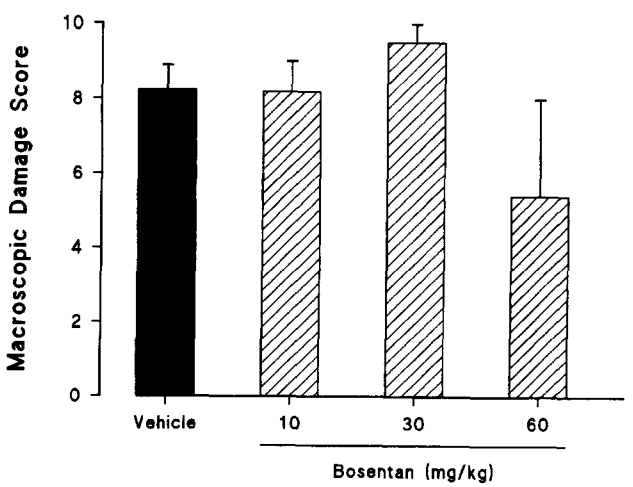

Fig. 4. Effects of bosentan (10–60 mg/kg p.o.) or vehicle (5% gum arabic) on macroscopic colonic damage scores 6 days after the induction of colitis. Oral bosentan treatment was started 1 h after the induction of colitis and daily thereafter for 5 days. Colonic damage was determined by an observer blinded to the treatment using the scoring criteria outlined in Materials and methods. Each bar represents the mean and S.E.M. of 5 rats.

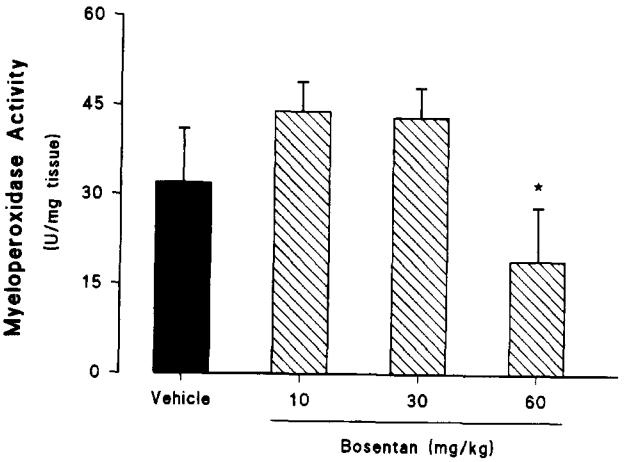

Fig. 5. Transmural myeloperoxidase activity in samples of distal colon removed from bosentan (10–60 mg/kg p.o.)- or vehicle-treated (5% gum arabic) rats on day 6 post-induction of colitis. Oral bosentan treatment was started 1 h after initiation of colitis and daily thereafter for 5 days. Each bar represents the mean and S.E.M. of 5 rats. Significant differences from the inflamed vehicle-control group: $^{*}P \leq 0.05$.

bosentan (10–60 mg/kg p.o.) for 5 days thereafter. On day 6, adhesions were observed in all rats in the 10 mg/kg bosentan treatment group, 3 of 5 rats in the 30 mg/kg group and 2 of 5 rats in the 60 mg/kg group (Table 1). The incidence of diarrhoea was identical to the incidence of adhesions and was significantly ($P \leq 0.05$) increased in this treatment group compared to inflamed vehicle controls. The incidence of adhesions was reduced in the 30 mg/kg treatment group, but the incidence of diarrhoea was markedly elevated in this group compared to the vehicle-treated inflamed group. In rats receiving 60 mg/kg p.o. bosentan, there was a further decrease in the incidence of colon-to-intestine adhesions, but the incidence of diarrhoea was still double that observed in the control group (Table 1). A therapeutic effect by bosentan at the macroscopic level was observed in the 60 mg/kg p.o. bosentan-treated group, however, while the damage scores were lower in the distal colon, there was not a statistically significant difference between this group and the other treatment groups (Fig. 4).

3.2.2. Granulocytic infiltration in the inflamed colon

Transmural distal colonic myeloperoxidase activity in the 10 and 30 mg/kg p.o. bosentan treatment groups was elevated above those values measured in vehicle-control rats (Fig. 5). However, treatment of colitic rats with 60 mg/kg p.o. bosentan significantly reduced myeloperoxidase activity in the distal colon.

3.2.3. Microscopic assessment

Microscopic assessment of distal colonic tissue confirmed that blockade of endothelin ET_A and ET_B receptors following the induction of TNB colitis with a 60 mg/kg bosentan treatment did attenuate tissue injury in the distal colon (not shown).

4. Discussion

Recently, Clozel et al. (1994) characterized a new potent orally active non-peptide ET receptor antagonist that has little effect on baseline arterial pressure. This compound is unique because it binds endothelin ET_A and ET_B receptors, as demonstrated using in vitro and in vivo systems. Further, the fact that bosentan is a mixed receptor antagonist is important since selective endothelin receptor antagonists, such as BQ-123 (endothelin ET_A receptor), have had limited therapeutic benefit in animal models of endothelin-induced injury in the gastrointestinal tract (Michida et al., 1994) and in the lung (Filep et al., 1995). In these systems, the blockade of endothelin ET_A and ET_B receptors appears to be required for the total inhibition of endothelin-1-induced vasoconstriction. From binding and functional studies, bosentan was shown to have a higher affinity for endothelin ET_A receptor than for endothelin ET_B receptor and a single oral dose of bosentan (30 mg/kg) inhibited the pressor effect of exogenous endothelin-1 by 16% at 24 h post-administration. The efficacy of bosentan in preventing endothelin-induced injury and dysfunction has now been tested in various animal models. For example, Lasaratos et al. (1995) reported that a 1 h pre-treatment with bosentan at a dose of 30 mg/kg antagonized the vasodilator, vasoconstrictor and ulcerogenic effects of endothelin-1 in the rat stomach. While it had no effect on arterial blood pressure in normotensive rats (Clozel et al., 1994), oral bosentan markedly reduced the elevations in arterial blood pressure and vascular hypertrophy in a model of hypertension in rats (Li et al., 1994). Bosentan also significantly attenuated the elevated mean arterial blood pressure in rats with chronic heart failure due to its prevention of the vasoconstrictive effects of endothelins (Teerlink et al., 1994). More recently, Stevens and Tomlinson (1995) demonstrated that a 5–6 week treatment of endothelin receptor antagonism with oral bosentan reversed, in part, the peripheral nerve dysfunction in experimental diabetes in rats.

The availability of an oral antagonist of endothelin receptors permitted an examination of the role of endothelins in the initiation and maintenance of experimental colonic injury. We chose the trinitrobenzene sulphonic acid in 50% ethanol colitis to evaluate the effects of endothelin antagonism on colonic inflammation since it is a well-characterized model that exhibits many of the histological characteristics of Crohn's disease. Yamada et al. (1992) have shown that this compound has direct cytotoxic effects on the colonic epithelium barrier. Disruption of this barrier permits the entry of luminal products which, in turn, incites the upregulation of local immune cells in the lamina propria. There is also a chronic immune component to this model that involves activation of macrophages and/or infiltrating monocytes in the lamina propria by the trinitrobenzene sulphonic acid hapten. Margination and extravasation of circulating granulocytes across the activated vascular endothelium into the distal colon contributes markedly (i.e. > 80%) to the acute and chronic injury in this model of inflammatory bowel disease. Wallace et al. (1992) have demonstrated that pre-treatment of rabbits with a monoclonal antibody directed against CD18 on leucocytes markedly attenuated granulocytic infiltration into the inflamed colon. In the present study, we hypothesized that antagonism of endothelin receptors with bosentan would provide protection to the distal colon in a rat model of colitis. Our hypothesis was validated by demonstrating, for the first time, the therapeutic efficacy of bosentan in this model. When given twice prior to induction of colitis and daily thereafter for 6 days, orally administered bosentan markedly reduced the incidence of adhesions between the colon and small intestine, the incidence of diarrhoea, transmural myeloperoxidase activity and microscopic injury to the distal colon. Sparing of colonic tissue from injury by bosentan treatment was dose-dependent with the maximal beneficial effect was observed at a dose of 60 mg/kg p.o. However, without the bosentan pre-treatment regimen, attenuation of the colonic injury and inflammation was observed at the 60 mg/kg p.o. dose in this model of colitis. These last findings suggest that bosentan probably acts more effectively in a protective fashion rather than through an acceleration of tissue healing (i.e. therapeutic effect) in this colitis model. Taken together, these findings suggest that endothelins, acting through endothelin ET_A and/or ET_B receptors, are pivotal to the initiation and, to a lesser degree, the maintenance of mucosal injury in this colitis model.

In the present study, bosentan dose-dependently reduced tissue injury and granulocyte infiltration into the inflamed colon, possibly through one or the combination of many mechanisms. First, bosentan may provide protection to the colon simply through its direct prevention of granulocyte adherence to the vascular endothelium and subsequent infiltration into the inflamed site. Second, as a potent inhibitor of endothelin-induced vasoconstriction (Clozel et al., 1994), bosentan may exert its protective effect through prevention of microcirculatory disturbances in the distal colon that result following injury (Wallace, 1994). Third, endothelin antagonism by bosentan may indirectly prevent tissue injury through its modulatory effect on endothelin-induced synthesis and release of other causative vasoactive factors of colitis. Numerous pro-inflammatory mediators contribute to either the initiation and/or maintenance of colonic injury in this model of experimental colitis. For example, interleukin-1β is critical for the initial leucocyte margination and extravasation whereas leukotrienes, platelet-activating factor and free radicals (i.e. superoxide, nitric oxide and peroxynitrite) play a prominent role in the maintenance of prolonged inflammation and tissue injury in this model (Wallace, 1994). Since endothelin-1 enhances superoxide generation and platelet-activating factor synthesis by activated leucocytes (Miller et al., 1993), the vasodilation and mucosal

hyperaemia should also be considered as a potential mechanism of tissue damage. Endothelins also stimulate nitric oxide synthesis following activation of the endothelin ET_B receptor (Whittle and Esplugues, 1988). Increased nitric oxide synthesis during TNB colitis is deleterious to the colon as we have demonstrated recently; inhibition of the synthesis of this free radical by an orally active nitric oxide synthase inhibitor reduced significantly most of the colonic injury in this model (Hogaboam et al., 1995). Thus, bosentan may provide its protective and therapeutic effects in this model through its prevention of endothelin-mediated vasoconstriction or inhibition of endothelin-mediated inflammatory mediator synthesis.

A 'side-effect' of bosentan treatment (particularly at the dose 10 mg/kg p.o.) in the inflamed rats was the exacerbation of diarrhoea observed in this model. However, bosentan administered alone to rats did not provoke diarrhoea. The explanation for these findings is not presently clear, but it may be related to emerging evidence that endothelin-1 may function as a neurotransmitter and as such may modulate intestinal secretion and/or motility during inflammation. Others have shown that endothelin binding sites in the rat colon are localized in the mucosal layer, in the myenteric plexus and on blood vessel walls (Takahashi et al., 1990). Interestingly, endothelins are not only produced by vascular endothelial cells, but also by mucosal epithelial cells and enteric nerves in the rat colon (Takahashi et al., 1990). Further, endothelin-1 and endothelin-3 are potent stimulators of ion secretion in the rabbit ileum (Brown and Smith, 1991) and in the rat colon (Kiyohara et al., 1993). It is plausible then that oral bosentan treatment altered some, as yet undescribed, regulatory role of endothelins on ion transport in the inflamed colon; these studies are ongoing. In addition, our preliminary studies of colonic contractility suggested that oral bosentan treatment markedly augments the contractile response of the distal colon to various agonists under non-inflamed and inflamed conditions (unpublished observations). These findings imply that endothelins play an important role in regulating intestinal smooth muscle contractility in the colon. Studies have been initiated to explore this possibility.

Bosentan or related antagonists may have potential benefit in the treatment of disorders associated with prolonged inflammation, such as inflammatory bowel disease. Since the microvasculature is extremely sensitive to the constrictor effects of endothelins, increased expression of endothelin receptors and/or increased production of endothelins may explain alterations in the microvasculature, particularly in Crohn's disease. The mitogenic properties of endothelins may explain the neovascularization described in Crohn's disease while the disordered and disrupted vasculature in Crohn's disease is postulated to result from a macrophage/endothelial cell interaction leading to vascular obstruction due to granuloma formation (Wakefield et al., 1989, 1991). In Crohn's disease, endothelin-1-immunoreactive cells were prominent in the submucosa, particularly macrophages situated around blood vessels in this region of the intestine (Murch et al., 1992). In a preliminary study, Hudson et al. (1994) demonstrated that [^{125}I]endothelin-1 binding sites (putative receptors) were elevated significantly, as compared to normal, in villi and submucosal vessels in intestine from Crohn's patients. Considering that endothelin is released by T lymphocyte- or lipopolysaccharide-activated perivascular macrophages or polymorphonuclear leucocytes (Sessa et al., 1991), these findings may explain the focal ischemia and vascular occlusion in Crohn's disease. It was also suggested that since free radical synthesis is markedly elevated, ischemia-reperfusion injury best describes the histopathology characteristic to Crohn's disease and the contrasting concomitant vasoconstrictor and vasodilator actions of endothelins may precipitate this event in the intestine. Other macrophage-derived mediators, such as tumour necrosis factor α and leukotrienes, possibly exacerbate the effects of endothelins in Crohn's disease. In ulcerative colitis, lipopolysaccharide-activated macrophages or infiltrating leucocytes in the lamina propria may be responsible for excessive endothelin-1 synthesis.

In conclusion, the results from this study demonstrate that endothelins are important for the initiation of focal injury in an experimental model of colitis. Using bosentan or other related compounds, further study of the role of endothelins in intestinal dysfunction during inflammation is now possible.

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References

Brown, M.A. and P.L. Smith, 1991, Endothelin: a potent stimulator of intestinal ion secretion in vitro, Reg. Peptides 36, 1.

Clozel, M., V. Breu, G.A. Gray, B. Kalina, B.-M. Loffler, K. Burri, J.-M. Cassal, G. Hirth, M. Muller, W. Niedhart and H. Ramuz, 1994, Pharmacological characterization of bosentan, a new potent orally active non-peptide endothelin receptor antagonist, J. Pharmacol. Exp. Ther. 270, 228.

Clozel, M., G.A. Gray, V. Breu, B.-M. Loffler and R. Osterwalder, 1992, The endothelin ET_B receptor mediates both vasodilation and vasoconstriction in vivo, Bochem. Biophys. Res. Commun. 186, 867.

Filep, J.G., A. Foiurnier and E. Foldes-Filep, 1995, Acute pro-inflammatory actions of endothelin-1 in the guinea-pig lung: involvement of ET_A and ET_B receptors, Br. J. Pharmacol. 115, 227 (Abstract).

Hirata, Y., H. Yoshimi, S. Takata, T.X. Watanabe, K. Kumagai, K. Nakajima and S. Sakakibara, 1988, Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 154, 868.

Hogaboam, C.M., K. Jacobson, S.M. Collins and M.G. Blennerhassett, 1995, The selective beneficial effects of nitric oxide inhibition in experimental colitis, Am. J. Physiol. Gastrointest. Liver Physiol. 268, G673.

Hudson, M., M.R. Dashwood, R.E. Pounder and A.J. Wakefield, 1994, ^{125}I endothelin-1 binding in normal human intestine and inflammatory bowel disease, Gastroenterology 108, A2583 (Abstract).

Inagaki, H., A.E. Bishop, C. Escrig, J. Wharton, T.G. Allen-Mersh and J.M. Polak, 1991, Localization of endothelin-like immunoreactivity and endothelin binding sites in human colon, Gastroenterology 101, 47.

Inoue, A., M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto and T. Masaki, 1989, The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes, Proc. Natl. Acad. Sci. USA 86, 2863.

Katajima, T., T. Yamaguchi, K. Tani, Y. Kubota, M. Okuhira, K. Inoue and H. Yamada, 1993, Role of endothelin and platelet-activating factor in indomethacin-induced gastric mucosal injury in rats, Digestion 54, 156.

Kitsukawa, Y., Z.-F. Gu, P. Hildebrand and R.T. Jensen, 1994, Gastric smooth muscle cells possess two classes of endothelin receptors but only one alters contraction, Am. J. Physiol. Gastrointest. Liver Physiol. 266, G713.

Kiyohara, T., M. Okuno, T. Nakanishi, Y. Shinomura and Y. Matsuzawa, 1993, Effect of endothelin-1 on ion transport in isolated rat colon, Gastroenterology 104, 1328.

Lasaratos, S., A. Nakahara, K. Goto and H. Fukutomi, 1995, Bosentan antagonizes the effects of endothelin-1 on rat gastric blood flow and mucosal integrity, Life Sci. 56, 195.

Li, J.S., R. Lariviere and E.L. Schiffrin, 1994, Effect of a nonselective endothelin antagonist on vascular remodeling in deoxycorticosterone acetate-salt hypertensive rats. Evidence for a role of endothelin in vascular hypertrophy, Hypertension 24, 183.

Masuda, E., S. Kawano, K. Nagano, S. Tsuji, Y. Takei, N. Hayashi, M. Tsujii, M. Oshita, T. Michada, I. Kobayashi, H.B. Peng, H. Fusamoto and T. Kamada, 1993, Role of endogenous endothelin in the pathogenesis of ethanol-induced gastric mucosal injury in rats, Am. J. Physiol. 265, G474.

Michida, T., S. Kawano, E. Masuda, I. Kobayashi, Y. Nishimura, M. Tsujii, N. Hayashi, Y. Takei, S. Tsuji, K. Nagano, H. Fusamoto and T. Kamada, 1994, Role of endothelin-1 in haemorrhagic shock-induced gastric mucosal injury in rats, Gastroenterology 106, 988.

Miller, R.C., J.T. Pelton and J.P. Huggins, 1993, Endothelins – from receptors to medicine, TIPS 14, 54.

Morris, G.P., P.L. Beck, M.S. Herridge, W.T. Depew, M.R. Szewczuk and J.L. Wallace, 1989, Hapten-induced model of chronic inflammation and ulceration in the rat colon, Gastroenterology 96, 795.

Murch, S.H., C.P. Braegger, W.C. Sessa and T.T. MacDonald, 1992, High endothelin-1 immunoreactivity in Crohn's disease and ulcerative colitis, Lancet 339, 381.

Sessa, W.C., S. Kaw, M. Hecker and J.R. Vane, 1991, The biosynthesis of endothelin-1 by human polymorphonuclear leucocytes, Bochem. Biophys. Res. Commun. 174, 613.

Stevens, E.J. and D.R. Tomlinson, 1995, Effects of endothelin receptor antagonism with bosentan on peripheral nerve function in experimental diabetes, Br. J. Pharmacol. 115, 373.

Takahashi, K., P.M. Jones, S.M. Kanse, H.-C. Lam, R.A. Spokes, M.A. Ghatel and S.R. Bloom, 1990, Endothelin in the gastrointestinal tract. Presence of endothelin-like immunoreactivity, endothelin-1 mRNA, endothelin receptors and pharmacological effect, Gastroenterology 99, 1660.

Teerlink, J.R., V. Breu, U. Sprecher, M. Clozel and J.P. Clozel, 1994, Potent vasoconstriction mediated by endothelin ET_B receptors in canine coronary arteries, Circ. Res. 74, 105.

Wakefield, A.J., E.A. Sankey, A.P. Dhillon, A.M. Sawyerr, L. More, R. Sim, M. Pittilo, P.M. Rowles, M. Hudson, A.A.M. Lewis and R.E. Pounder, 1991, Granulomatous vasculitis in Crohn's disease, Gastroenterology 100, 1279.

Wakefield, A.J., A.M. Sawyer, A.P. Dhillon, A.P. Pittilo, P.M. Rowles, A.A.M. Lewis and R.E. Pounder, 1989, Pathogenesis of Crohn's disease: multifocal gastrointestinal infarction, Lancet 1, 1057.

Wallace, J.L., 1994, Novel targets for anti-inflammatory therapy in inflammatory bowel disease, in: Inflammatory Bowel Disease. Basic Research, Clinical Implications and Trends in Therapy, eds. L.R. Sutherland, S.M. Collins, F. Martin, R.S. McLeod, S.R. Targan, J.L. Wallace and C.N. Williams (Kluwer Academic Publishers, London) p. 374.

Wallace, J.L., G. Cirino, G. DeNucci, W. McKnight and W.K. MacNaughton, 1989, Endothelin has potent ulcerogenic and vasoconstrictor actions in the stomach, Am. J. Physiol. 256, G661.

Wallace, J.L., A. Higa, G.W. McKnight and D.E. MacIntyre, 1992, Prevention and reversal of experimental colitis by a monoclonal antibody which inhibits leucocyte adherence, Inflammation 16, 343.

Wallace, J.L. and C.M. Keenan, 1990, An orally active inhibitor of leukotriene synthesis accelerates healing in a rat model of colitis, Am. J. Physiol. 258, G527.

Whittle, B.J.R. and J.V. Esplugues, 1988, Induction of rat gastric damage by the endothelium-derived peptide, endothelin, Br. J. Pharmacol. 95, 1011.

Yamada, T., S. Marshall, R.D. Specian and M.B. Grisham, 1992, A comparative analysis of two models of colitis in rats, Gastroenterology 102, 1524.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature (London) 332, 411.