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Involvement of superoxide and xanthine oxidase in neutrophil-independent rat gastric damage induced by NO donors

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1 Nitric oxide (NO) and the superoxide anion can interact to form the cytotoxic moiety, peroxynitrite. The involvement and potential source of superoxide in the gastric mucosal damage induced by local infusion of NO donors, has now been investigated in the pentobarbitone-anaesthetized rat.

2 Local intra-arterial infusion of the NO donor, sodium nitroprusside (40 μg kg^{-1} min^{-1}) for 10 min induced macroscopically apparent gastric mucosal injury.

3 This mucosal damage was dose-dependently reduced by prior administration of a systemically acting form of superoxide dismutase conjugated with polyethylene glycol (500-2000 iu kg^{-1}, i.v.).

4 Likewise, the mucosal damage induced by nitroprusside was dose-dependently reduced by prior administration of the xanthine oxidase inhibitor, allopurinol (20-100 mg kg^{-1}, i.p. or 100 mg kg^{-1}, p.o.).

5 Pretreatment with allopurinol (100 mg kg^{-1}, i.p.) also reduced the mucosal injury induced by local intra-arterial infusion of the nitrosothiol, S-nitroso-*N*-acetyl-penicillamine (40 μg kg^{-1} min^{-1}), but not that induced by local infusion of endothelin-1 (5 pmol kg^{-1} min^{-1}), indicating specificity of action.

6 Prior administration (4h) of rabbit anti-rat neutrophil serum (0.4 ml kg^{-1}, i.p.), which reduced circulating neutrophils by 90%, did not significantly protect against mucosal injury induced by nitroprusside.

7 Intravenous administration of the platelet-activating factor receptor antagonists, WEB 2086 (1 mg kg^{-1}) or BN 52021 (10 mg kg^{-1}), or the thromboxane synthase inhibitor, OKY 15181 (25 mg kg^{-1}), did not modify mucosal damage induced by nitroprusside, showing lack of involvement of these neutrophil-derived mediators.

8 These findings indicate the involvement of superoxide in the injurious actions of the NO donors, implicating a cytotoxic role of peroxynitrite. Xanthine oxidase, but not neutrophils, appears to be a source of the superoxide.

Keywords: Nitric oxide; NO donors; nitrovasodilators; nitrosothiols; nitroprusside; S-nitroso-*N*-acetyl-penicillamine; superoxide dismutase; allopurino; neutrophils

Introduction

Nitric oxide (NO), formed from L-arginine by a constitutive NO synthase, has a key modulator function in the regulation of microvascular integrity in the gastro-intestinal tract (Boughton-Smith *et al.*, 1990; Hutcheson *et al.*, 1990; Whittle *et al.*, 1990; Kubes & Granger, 1991; Kubes & Granger, 1992; Whittle, 1993). By contrast, the excessive production of NO in the gut by the inducible isoform of NO synthase following challenge with endotoxin (Salter *et al.*, 1991) results in microvascular permeability in the stomach and intestine and in epithelial cytotoxicity (Boughton-Smith *et al.*, 1993a; Laszlo *et al.*, 1994a,b; Tepperman *et al.*, 1994).

NO derived from exogenous sources can likewise exert a dual action on the integrity of the gastric mucosa. Thus, intragastric application of NO donors such as glyceryl trinitrate, isoamyl nitrate or nitroprusside can protect against acute haemorrhagic mucosal injury provoked by topical irritants (MacNaughton *et al.*, 1989; Kitagawa *et al.*, 1990). Local intra-arterial infusion of glyceryl trinitrate, as well as low doses of the nitrosothiol, S-nitroso-*N*-acetyl penicillamine (SNAP) can also protect against gastric mucosal injury (Lopez-Belmonte *et al.*, 1993). By contrast, close-arterial infusion of 2-4 fold higher doses of SNAP or nitroprusside can themselves provoke extensive haemorrhagic mucosal damage (Lopez-Belmonte *et al.*, 1993; Whittle, 1994).

These acute injurious actions of high local intra-arterial concentration of NO may involve the production of the cytotoxic peroxynitrite moiety from NO and the superoxide anion, which can subsequently yield the reactive hydroxyl radical (Beckman *et al.*, 1990). Evidence for the ability of this factor to injure the gut comes from studies where direct application of a peroxynitrite-generating system to the rat colonic mucosa produces local tissue inflammation (Rachmilewitz *et al.*, 1993). Early studies have demonstrated the involvement of superoxide in the microvascular injury following ischaemia-reperfusion in the intestine (Parks *et al.*, 1982; Parks & Granger, 1983) and in the stomach (Itoh & Guth, 1985; Droy-Lefaix *et al.*, 1991). Furthermore, local infusion of a superoxide generating system can provoke gastric mucosal injury (Esplugues & Whittle, 1989). Endogeous superoxide may be generated from a number of cellular sources and enzymes. Thus, in studies of ischaemia-reperfusion injury in the intestine, the formation and release of superoxide is considered to be derived from local tissue as a result of activation of xanthine oxidase as well as from infiltrating neutrophils (Parks *et al.*, 1982; Morgan-Smith *et al.*, 1987; Nilsson *et al.*, 1994).

In the present study, the involvement of superoxide in the gastric mucosal injury provoked by NO donors in the pentobarbitone-anaesthetized rat, was evaluated by use of the systemically acting conjugate of superoxide dismutase (SOD) with polyethylene glycol (Boughton-Smith *et al.*, 1993b) which scavenges the superoxide anion. The specificity of this action was determined by evaluating its action on the gastric mucosal injury induced by local intravascular infusion of the ulcerogenic peptide, endothelin-1 (Whittle & Esplugues, 1989). To explore the involvement of neutrophils as a source of superoxide in NO donor-induced mucosal damage, rabbit anti-rat neutrophil serum was used to deplete circulating neutrophils. Further, since the release of the neutrophil-derived mediators, platelet activating factor (PAF) or thromboxane A_2 could also

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be involved in such tissue damage, the effects of the PAF-receptor antagonists, WEB 2086 and BN 52021 (Casals-Stenzal, 1987; Kolati *et al.*, 1991) or the thromboxane synthase inhibitor, OKY 1518 (Boughton-Smith *et al.*, 1989) were studied. The role of xanthine oxidase as a source of superoxide in the NO donor-induced rat mucosal injury was evaluated with the xanthine oxidase inhibitor, allopurinol, which has been shown to inhibit the oxygen-radical production and tissue injury associated with ischaemia-reperfusion in the stomach and intestine (Granger *et al.*, 1981; Itoh & Guth, 1985; Perry *et al.*, 1986; Nilsson *et al.*,1994).

Methods

Animal preparation

Male Wistar rats, weighing 200–250 g, were fasted overnight but allowed free access to water. The animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The abdomen was opened by a mild-line incision, the oesophagus and pylorus were ligated and 2 ml of 0.1 M hydrochloric acid (HCl) was instilled into the gastric lumen via a 25 g needle inserted through the forestomach. The left gastric artery was cannulated with tubing of 0.6 mm diameter (PE 10, Clay Adams, Parsippany, New Jersey, U.S.A.) as previously described (Esplugues & Whittle, 1989). Body temperature was monitored by a rectal thermometer and maintained at 36°C by a heating blanket.

Effect of NO donors on gastric mucosa

Nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$), SNAP (40 $\mu g\ kg^{-1}\ min^{-1}$) or isotonic saline was infused for 15 min through the left gastric artery at a rate of 13 $\mu l\ min^{-1}$. These doses of the NO donors were selected from previous dose-response studies as near-maximal for provocation of acute mucosal injury (Lopez-Belmonte *et al.*, 1993). After 20 min following termination of the infusion, the stomachs were removed for assessment of macroscopic or histological damage.

Effects of polyethylene glycol-SOD

The systemically acting conjugate of SOD and polyethylene glycol (SOD-PEG: 500–2000 iu kg^{-1}) or isotonic saline, was administered by an intravenous bolus injection, 15 min prior to the local intra-arterial infusion of nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$). The doses of SOD-PEG were taken from previous studies on its inhibitory action on the inflammatory response in the rat skin following systemic administration (Boughton-Smith *et al.*, 1993b).

Effect of allopurinol on NO donors damage

Allopurinol (20–100 mg kg^{-1}, i.p.) was administered 3 h prior to local intra-arterial infusion of nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$) or SNAP (40 $\mu g\ kg^{-1}\ min^{-1}$). In control rats, the vehicle for allopurinol was administered by i.p. injection. The doses of allopurinol were selected from a previous study on its use in ischaemic injury of the small intestine (Parks *et al.*, 1982). In a further series of experiments, allopurinol was administered orally (100 mg kg^{-1} for 2 days prior to study), using doses shown to inhibit gastric lesions produced by haemorrhagic shock (Yasue & Guth, 1988).

Effects of PAF-receptor antagonists and thromboxane synthase inhibitor

The PAF receptor antagonists, WEB 2086 (1 mg kg^{-1}) or BN 52021 (10 mg kg^{-1}), or the thromboxane synthase inhibitor, OKY 15181 (25 mg kg^{-1}) were administered as a bolus injection through a tail vein via a 25 g needle, 15 min before the local intra-arterial infusion of nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$), The doses of these agents were derived from previous dose-response studies on their actions on endotoxin-induced mucosal damage (Boughton-Smith *et al.*, 1989; Laszlo *et al.*, 1994c). Control rats received an intravenous bolus of isotonic saline, followed by the local intra-arterial infusion of nitroprusside or SNAP.

Effects of anti-rat neutrophil serum on mucosal injury

Rabbit anti-rat neutrophil serum (0.4 ml kg^{-1}, i.p.) was injected 4 h prior to local infusion of nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$) or SNAP (40 $\mu g\ kg^{-1}\ min^{-1}$). Control rats received isotonic saline (i.p.), followed by the local intra-arterial infusion of the NO donors. The dose of anti-neutrophil serum was derived from previous studies (Tepperman *et al.*, 1993). The reduction in the number of circulating neutrophils was determined by differential staining of a blood sample taken from the abdominal aorta of pentobarbitone-anaesthetized rats, 4 h after antibody injection, using a modified Wright-Giesma stain (Diff-Quick; Baxter Corp., Fl., U.S.A.).

In a series of control studies to demonstrate the efficacy of this anti-neutrophil serum in a model of gastric mucosal injury known to be neutrophil-dependent (Kvietys *et al.*, 1990), the effect of prior administration of anti-neutrophil serum (0.4 ml kg^{-1}, i.p.) was evaluated on the mucosal injury provoked by a 5 min intra-luminal exposure to acidified ethanol (2 ml of ethanol 40% in 0.1 M HCl). A control group of rats received saline (i.p.) followed by intraluminal instillation of acidified ethanol.

Effects of SOD-PEG or allopurinol on the mucosal injury induced by endothelin-1

To demonstrate the specificity of the protective actions, the effects of SOD-PEG (2000 iu kg^{-1}, i.v.) or allopurinol (100 mg kg^{-1}, i.p.), were evaluated on the mucosal injury induced by ET-1 (5 pmol $kg^{-1}\ min^{-1}$ in 0.1% bovine serum albumin in saline), which was infused for 15 min at a rate of 13 $\mu l\ min^{-1}$.

Assessment of mucosal damage

Twenty minutes after terminating the local intra-arterial infusion of the NO donors, the stomachs were removed and opened along the great curvature. They were either pinned, mucosal side up, to a wax block, immersed in neutral buffered formalin and photographed on colour transparency film, or a segment taken for histological evaluation. The extent of macroscopically visible damage was determined from the projected transparencies in a randomized manner via computerized planimetry, and expressed as the percentage of the total mucosal area. Macroscopic damage provoked by local infusion of ET-1, or intraluminal instillation of acidified ethanol was determined in a similar fashion, with tissue being taken 20 min after terminating the ET-1 infusion or immediately after the 5 min challenge with acidified ethanol.

For histological confirmation of the effects on nitroprusside-induced mucosal damage, sections of mucosa were assessed by use of a histological score as described previously (Esplugues & Whittle, 1989). Two samples of the corpus mucosa were excised from comparable regions in each stomach and were processed by routine techniques before embedding in paraffin. Sections were stained with haematoxylin and eosin and examined under a light microscope, in a randomized fashion. The 1 cm length of each histological section was divided into 4 subsections. Each subsection was histologically assessed for epithelial cell damage (with a score 1); glandular disruption, vasocongestion or oedema in the upper mucosa (with a score of 2); haemorrhagic damage in the mid to lower mucosa (score of 3) and deep necrosis and ulceration (score of 4). Each subsection was evaluated on a cumulative basis (the maximum score being 4) and the overall mean score value was

taken as the histological index for that section. All determinations were performed in a randomized manner with the histological sections coded to eliminate observer bias.

Statistical analysis

All data are expressed as the means ± s.e.mean. Comparisons between groups of data were made by Student's *t* test for non-paired data. *P* values of less than 0.05 were taken as significant.

Drugs

Nitroprusside, superoxide dismutase-polyethylene glycol conjugate, allopurinol and endothelin-1 were purchased from Sigma Chemical Co. (Poole, Dorset) and rabbit anti-rat neutrophil serum from Accurate Chemical and Scientific Corporation (Westbury, N.Y., U.S.A.). Allopurinol was dissolved in equimolar solution of sodium hydroxide then diluted in normal saline. Endothelin-1 (human-porcine) was dissolved in sterile distilled water and kept frozen (−20°C) in aliquots. Samples were freshly diluted in 0.1% bovine serum albumin in saline when required. Nitroprusside and SNAP (synthesized in the Dept. Medicinal Chemistry, Wellcome Research Laboratories) were dissolved freshly in isotonic saline and kept on ice protected from the light. WEB 2086, BN 52021 and OKY 1581 were gifts from Boehringer Ingelheim KG (Germany), from IPSEN Industrie (France), and from ONO Pharmaceuticals (Japan) respectively. These drugs were prepared freshly for injection by dilution with isotonic saline or the vehicle supplied.

Results

Effects of SOD-PEG on gastric mucosal damage induced by NO donors

Local intra-arterial infusion of nitroprusside (40 $\mu g\ kg^{-1}\ min^{-1}$) for 15 min induced haemorrhage gastric mucosal damage, when assessed macroscopically 20 min after termination of the infusion (Figure 1). Intra-arterial infusion of the vehicle, isotonic saline, did not induce any significant mucosal damage (4 ± 2% of total mucosal area, $n=6$).

Bolus intravenous administration of SOD-PEG (500–2000 iu kg^{-1}) caused a significant dose-dependent reduction in the extent of damage induced by the local intra-arterial infusion of nitroprusside (Figure 1). With the highest dose of SOD-PEG (2000 iu kg^{-1}, i.v.), the area of mucosal exhibiting detectable mucosal injury following nitroprusside infusion was not significantly different from that observed under control conditions (Figure 1).

Effect of allopurinol on gastric mucosal damage induced by NO donors

Prior administration of allopurinol (20–100 mg kg^{-1}, i.p.) caused a significant dose-dependent reduction in the gastric mucosal damage induced by local infusion of nitroprusside (Figure 2). Likewise, oral pretreatment of allopurinol (100 mg kg^{-1} for 2 days) caused significant reduction in the extent of mucosal damage induced by nitroprusside (from 51 ± 4% to 23 ± 6% of total mucosal area, $n=13$ and 7 respectively; $P<0.01$).

The gastric damage induced by local infusion of SNAP (40 $\mu g\ kg^{-1}\ min^{-1}$) was also significantly inhibited by administration of allopurinol (100 mg kg^{-1}, i.p.), being reduced from 34 ± 4% to 20 ± 3% of total mucosal area ($n=5$ for each, $P<0.05$).

Effects of PAF receptor antagonists or a thromboxane synthase inhibitor

Pretreatment with the PAF receptor antagonists, WEB 2086 (1 mg kg^{-1}) or BN 52021 (10 mg kg^{-1}), or with the thromboxane synthase inhibitor, OXY 1581 (25 mg kg^{-1}), did not significantly reduce mucosal damage induced by nitroprusside (Table 1).

Effects of anti-neutrophil serum

Pretreatment (4h) with rat anti-neutrophil serum (0.4 ml kg^{-1}, i.p.), which reduced the circulating neutrophil count by 90 ± 5% ($n=6$), did not reduce the mucosal damage induced by concurrent local infusion of nitroprusside (Figure 3). Likewise, the damage induced by SNAP (40 $\mu g\ kg^{-1}\ min^{-1}$) was not significantly reduced by pretreatment with anti-neutrophil serum (35 ± 8 and 44 ± 7% of total mucosal area, with and without anti-neutrophil pretreatment respectively; $n=5$ for each).

By contrast, in a control study, pretreatment with anti-neutrophil serum substantially reduced the gastric mucosal damage induced by intra-luminal challenge with acidified ethanol (2 ml ethanol 40% in 0.1 M HCl) as shown in Figure 3.

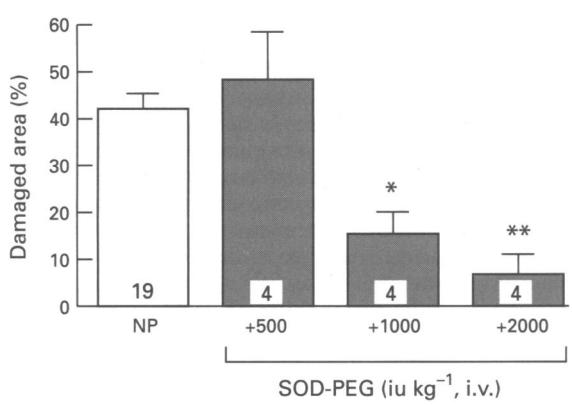

Figure 1 Effects of bolus i.v. pretreatment (15 min) of polyethylene glycol conjugated with superoxide dismutase (SOD-PEG; 500–2000 iu kg^{-1}) on the gastric mucosal damage induced by local intra-arterial infusion of nitroprusside (NP, 40 $\mu g\ kg^{-1}\ min^{-1}$ for 15 min). Results, expressed as % of the mucosal area shown macroscopic damage, are shown as mean ± s.e.mean of *n* (number in columns) values, where significant difference from the control group (nitroprusside alone) is shown as $*P<0.01$, $**P<0.001$.

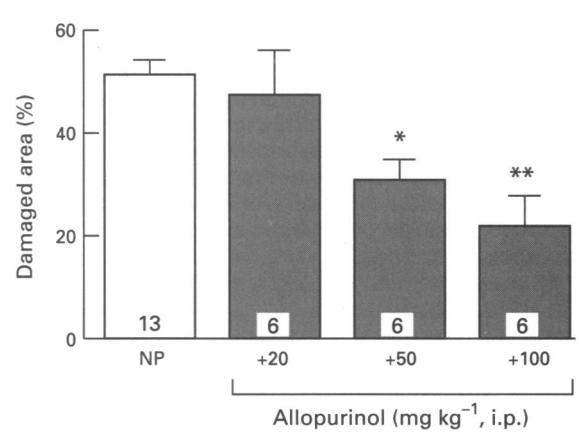

Figure 2 Effects of administration of allopurinol (20–100 mg kg^{-1}) on rat gastric mucosal damage induced by local intra-arterial infusion (15 min) of nitroprusside (NP, 40 $\mu g\ kg^{-1}\ min^{-1}$). Results, expressed as % of the mucosal area exhibiting macroscopic injury, are shown as the mean ± s.e.mean, of *n* (number in column) values, where significant differences from the control group (nitroprusside alone) is shown as $*P<0.05$, $**P<0.01$.

Table 1 Lack of effect of PAF receptor antagonists, WEB 2086 (1 mg kg^{-1}, i.v.) or BN 52021 (10 mg kg^{-1}, i.v.), and the thromboxane synthase inhibitor, OKY 15181 (25 mg kg^{-1}, i.v.) on gastric muscosal damage induced by local intra-arterial local infusion of sodium nitroprusside (NP, 40 μg kg^{-1} min^{-1} for 15 min)

Treatment	*% damage*	n
NP	38±4	(21)
NP+WEB 2086 (1 mg kg^{-1})	43±5	(7)
NP+BN 52021 (10 mg kg^{-1})	53±5	(4)
NP+OKY 15181 (25 mg kg^{-1})	40±4	(4)

Results are shown as % area of the rat gastric mucosa exhibiting macroscopic damage, and are expressed as mean±s.e. mean of (*n*) animals.

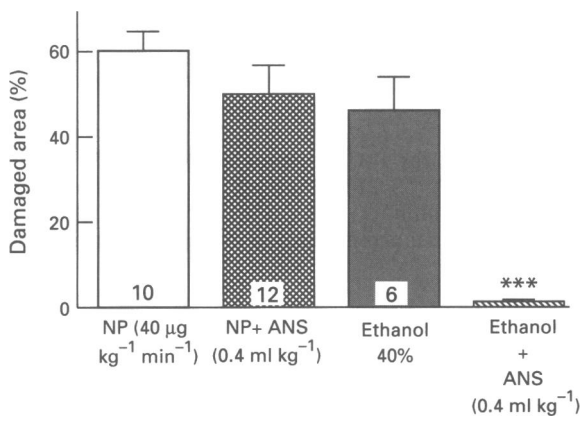

Figure 3 Effects of pretreatment with anti-rat neutrophil serum (ANS; 0.4 ml kg^{-1}, i.p.) on gastric mucosal damage induced by local intra-arterial infusion of nitroprusside (NP, 40 μg kg^{-1} min^{-1} for 15 min) or by a 5 min intraluminal challenge with acidified ethanol (40% in 0.1 M HCl). Results, expressed as % of the mucosal area exhibiting macroscopic damage are shown as mean±s.e.mean of *n* (number in column) values, where significant difference from corresponding control value is shown by ***$P<0.001$.

Effects of SOD-PEG or allopurinol on endothelin-1 induced damage

Close-arterial infusion of endothelin-1 (5 pmol kg^{-1} min^{-1} for 15 min) induced haemorrhagic injury of the mucosa, involving 38±5% ($n=10$) of total gastric area when assessed 20 min later. Concomitant administration SOD-PEG (2000 iu kg^{-1}, i.v.) or administration of allopurinol (100 mg kg^{-1}, i.p.) did not significantly alter the extent of mucosal damage, being 42±5% ($n=7$) and 28±3% of total mucosal area ($n=4$), respectively.

Histological assessment of gastric damage

The damage induced by local intra-arterial infusion of nitroprusside was histologically characterized by severe damage to the epithelium, disruption of the glandular structure, focal areas of haemorrhage and, in some cases, necrosis in the deeper parts of the mucosa. Pretreatment with SOD-PEG (2000 iu kg^{-1}, i.v.) or allopurinol (100 mg kg^{-1}, i.p.) reduced the levels of histologically assessed damage to values similar to that of the controls (Table 2). By contrast, pretreatment with anti-neutrophil serum (0.4 ml kg^{-1}, i.p.) did not modify the degree of histological damage (Table 2).

Table 2 Effects of prior administration of superoxide dimutase conjugated with polyethylene glycol (SOD-PEG: 2000 iu kg^{-1}, i.v.), allopurinol (100 mg kg^{-1}, i.p.) or rabbit anti-rat neutrophil serum (ANS, 0.4 ml kg^{-1}, i.p.) on the damage to the rat gastric mucosa following local intra-arterial infusion of sodium nitroprusside (NP, 40 μg kg^{-1} min^{-1} for 15 min), as determined histologically

Treatment	*Histological index*	(n)
Saline	0.7±0.3	4
NP	2.4±0.2*	14
NP+SOD-PEG (2000 iu kg^{-1})	0.6±0.4†	4
NP+allopurinol (100 mg kg^{-1})	0.2±0.1††	4
NP+ABS (0.4 ml kg^{-1})	1.9±0.2	7

Results are expressed as a histological index (scale, 0–4) and shown as mena±s.e.mean of (*n*) animals, where statistical difference from control is given as *$P<0.01$ and from the nitroprusside group as †$P<0.05$, ††$P<0.01$.

Discussion

The present study indicates that the acute gastric mucosal injury provoked by local intra-arterial infusion of nitroprusside can be attenuated by intravenous administration of the systemically active conjugate, SOD-PEG, that scavenges superoxide. The specificity of these actions of SOD-PEG was confirmed by its failure to affect the gastric damage induced by intra-arterial infusion of ET-1. These observations extend recent findings that mucosal damage provoked by NO donors can be abolished by concurrent local intra-arterial infusion of native SOD (Lamarque & Whittle, 1995), in doses shown previously to reduce the mucosal injury provoked by local infusion of the superoxide-generating system, xanthine oxidase-hypoxanthine (Esplugues & Whittle, 1989). Such findings therefore support the involvement of the superoxide anion in the tissue damage induced by nitroprusside or SNAP, which thus implicates the involvement of the cytotoxic moiety, peroxynitrite, formed from NO and superoxide (Beckman *et al.*, 1990).

Neutrophils are a source of superoxide generation and, moreover, play a key role in the gastrointestinal microvascular dysfunction provoked by ischaemia-reperfusion (Nilsson *et al.*, 1984; Grisham *et al.*, 1986), by infusion of PAF (Wallace & Whittle, 1986; Kubes *et al.*, 1990; Wood *et al.*, 1992) or by topical application of ethanol (Kvietys *et al.*, 1990). However, the involvement of neutrophils in the gastric injury induced by NO donors would appear unlikely since NO can prevent neutrophil aggregation (McCall *et al.*, 1988) and their adhesion to the microvasculature (Kubes *et al.*, 1991). Furthermore, in the present study, depletion of circulating neutrophils by over 90% by the use of anti-neutrophil antiserum failed to affect the mucosal damage provoked by nitroprusside. By contrast, the same treatment with this anti-neutrophil serum abolished the haemorrhagic mucosal injury sustained by a brief intragastric exposure to acid-ethanol. Such findings, while confirming the important role of the neutrophil in the acute mucosal damage provoked by this topical irritant, demonstrate that as with gastric antral lesions induced by indomethacin (Trevethick *et al.*, 1994), a neutrophil involvement is not an obligatory requirement for the provocation of all forms of gastric ulceration.

Commensurate with the lack of effect of neutrophil depletion, the local release of the neutrophil-derived gastric ulcerogen, PAF (Rosam *et al.*, 1986), does not appear to be involved since the PAF-receptor antagonists, WEB 2086 or BN 50521 failed to prevent nitroprusside-induced mucosal injury. Likewise, the generation of the pro-ulcerogenic mediator,

thromboxane A_2 (Whittle, 1993) by neutrophils, platelets or local tissue in the mucosal injury caused by NO donors can be excluded because of the lack of effect of the thromboxane synthase inhibitor, OKY 1581. Since both PAF and neutrophils play an important role in injury following ischameia-reperfusion injury in the stomach and intestine (Grisham *et al.*, 1986; Morgan-Smith *et al.*, 1987; Wallace *et al.*, 1990; Droy-Lefaix *et al.*, 1991), these present findings also suggest that NO-donor provoked gastric damage is not a result of similar ischaemic events in the mucosal microcirculation.

Xanthine oxidase, an enzyme found in endothelial cells (Ratych *et al.*, 1987; Zweier *et al.*, 1988; 1994) but not in the neutrophil (Jones *et al.*, 1985), is a prime source of superoxide generation. Allopurinol, administered by acute pretreatment by the intraperitoneal route, or following oral administration over 2 days, reduced the mucosal injury induced by local infusion nitroprusside or SNAP. As with the protective actions of allopurinol on gastric mucosal injury following hypovolaemic shock in the gastric mucosa and ischaemia reperfusion in the stomach and intestine (Granger *et al.*, 1981; Itoh & Guth, 1985; Perry *et al.*, 1986; Andrews *et al.*, 1992), such actions should reflect the inhibition of xanthine oxidase. The specificity of these actions of allopurinol in the present study was demonstrated by its failure to attenuate the mucosal injury induced by close-arterial infusion of endothelin-1. However, as in ischaemia-reperfusion injury in the cat small intestine (Nilsson *et al.*, 1994) a neutrophil- and xanthine oxidase-independent source of superoxide may also operate under these conditions, since mucosal injury was substantially reduced, but not abolished, by these doses of allopurinol.

The cellular distribution of the xanthine oxidase that may be involved in the pathogenesis of the gastric injury has not been identified, but histochemical studies show a localization in endothelial cells in the intestinal lamina propria (Jarash *et al.*, 1986). The primary events leading to the proposed xanthine oxidase activation and the release of superoxide in the present studies are not known. However, it is possible that NO donors may stimulate this enzyme, either through initial NO release or by early cellular perturbation, since the conversion of cytoplasmic xanthine dehydrogenase to the superoxide-producing oxidase form occurs rapidly after cell disruption (Jarasch *et al.*, 1986).

The present studies thus implicate the involvement of superoxide, generated by xanthine oxidase activity, in the mechanisms leading to tissue injury induced by local infusion of NO donors. The cytotoxic actions of NO donors have also been demonstrated in studies *in vitro* on colonic epithelial cells (Tepperman *et al.*, 1994). Superoxide production by xanthine oxidase may also be involved in such cellular damage, as well as in the gastro-intestinal microvascular injury associated with the excessive production of NO following induction of NO synthase (Laszlo *et al.*, 1994a,b). Knowledge of the regulation of xanthine oxidase activity as a source of superoxide under such conditions, should therefore provide a greater understanding of the cytotoxic and pathological role of NO.

References

ANDREWS, F.J., MALCONTENTI, C. & O'BRIEN, P.E. (1992). Sequence of gastric mucosal injury following ischaemia and reperfusion: Role of reactive oxygen metabolites. *Dig. Dis. Sci.*, **32**, 1356–1361.

BECKMAN, J.S., BECKMAN, T,W, CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620–1624.

BOUGHTON-SMITH, N.K., EVANS, S.M., LASZLO, F., WHITTLE, B.J.R. & MONCADA, S. (1993a). The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. *Br. J. Pharmacol.*, **110**, 1189–1195.

BOUGHTON-SMITH, N.K., DEAKIN, A.M., FOLLENFANT, R.L., WHITTLE, B.J.R. & GARLAND, L.G. (1993b). Role of oxygen radicals and arachidonic acid metabolites in the reverse passive Arthus reaction and carrageenin paw oedema in the rat. *Br. J. Pharmacol.*, **110**, 896–902.

BOUGHTON-SMITH, N.K., HUTCHESON, I.R., DEAKIN, A.M., WHITTLE, B.J.R. & MONCADA, S. (1990). Protective effect of S-nitroso-N-acetyl-penicillamine in endotoxin-induced acute intestinal damage in the rat. *Eur. J. Pharmacol.*, **191**, 485–488.

BOUGHTON-SMITH, N.K., HUTCHESON, I. & WHITTLE, B.J.R. (1989). Relationship between PAF-acether and thromboxane A_2 biosynthesis in endotoxin-induced intestinal damage in the rat. *Prostaglandins*, **38**, 319–333.

CASALS-STENZEL, J. (1987). Protective effect of WEB 2086, a novel antagoinst of platelet-activating factor, in endotoxin shock. *Eur. J. Pharmacol.*, **135**, 117–122.

DROY-LEFAIX, M.T., DROUET, Y., GERAUD, G., HOSFORD, D. & BRAQUET, P. (1991). Superoxide dismutase (SOD) and the PAF-antagonist (BN 52021) reduce small intestinal damage induced by ischaemia-reperfusion. *Free Radical Res. Commun.*, **12–13**, 725–735.

ESPLUGUES, J.V. & WHITTLE, B.J.R. (1989). Gastric damage following local intra-arterial administration of reactive oxygen metabolites in the rat. *Br. J. Pharmcol.*, **97**, 1085–1092.

GRANGER, D.N., RUTILI, G. & MCCORD, J.M. (1981). Superoxide radicals in feline intestinal ischaemia. *Gastroenterology*, **81**, 22–29.

GRISHAM, M., LUCRECIA, B., HERNANDEZ, A. & GRANGER, D.N. (1986). Xanthine oxidase and neutrophil infiltration in intestinal ischaemia. *Am. J. Physiol.*, **251**, G567–G574.

HUTCHESON, I.R., WHITTLE, B.J.R. & BOUGHTON-SMITH, N.K. (1990). Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br. J. Pharmacol.*, **101**, 815–820.

ITOH, M. & GUTH, P.H. (1985). Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat. *Gastroenterology*, **88**, 1162–1167.

JARASCH, E.D., BRUDER, G. & HEID, H.W. (1986). Significance of xanthine oxidase in capillary endothelial cells. *Acta Physiol. Scand. Suppl.*, **548**, 39–46.

JONES, H.P., GRISHAM, M.B., BOSE, S.K., SHANNON, V.A., SCHOTT, A. & MCCORD, J.M. (1985). Effect of allopurinol on neutrophil superoxide production, chemotaxis, or degranulation. *Biochem. Pharmacol.*, **34**, 3673–3676.

KITAGAWA, H., TAKEDA, F. & KOHEI, H. (1990). Effect of endothelium-derived relaxing factor on the gastric lesion induced by HCl in rats. *J. Pharmacol. Exp. Ther.*, **253**, 1133–1137.

KOLATI, M., HOSFORD, D., GUINOT, P., ESANU, A. & BRAQUET, P. (1991). Platelet activating factor (PAF). A review of its effects, antagonists and possible future clinical implications (Part I). *Drugs*, **42**, 9–29.

KUBES, P. & GRANGER, D.N. (1992). Nitric oxide modulates microvascular permeability. *Am. J. Physiol.*, **262**, H611–H615.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1990). Platelet-activating factor-induced microvascular dysfunction: role of adherent leukocytes. *Am. J. Physiol.*, **258**, G158–G163.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4651–4655.

KVIETYS, P.R., TWOHIG, B., DANZELL, J. & SPECIAN, R.D. (1990). Ethanol-induced injury to the gastric mucosa: Role of neutrophils and xanthine oxidase-derived radicals. *Gastroenterology*, **98**, 909–920.

LAMARQUE, D. & WHITTLE, B.J.R. (1995). Role of oxygen-derived metabolites in the rat gastric mucosal injury induced by nitric oxide donors. *Eur. J. Pharmacol.*, **277**, 187–194.

LASZLO, F., WHITTLE, B.J.R. & MONCADA, S. (1994a). Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. *Br. J. Pharmacol.*, **111**, 1309–1315.

LASZLO, F., WHITTLE, B.J.R. & MONCADA, S. (1994b). Dual beneficial or detrimental actions of nitric oxide synthase inhibitors on endotoxin-induced gastric microvascular injury. In *The Biology of Nitric Oxide*. ed. Moncada, S., Feelisch, M., Busse, R. & Higgs, E.A. pp. 276–279. London: Portland Press.

LASZLO, F., WHITTLE, B.J.R. & MONCADA, S. (1994c). Interactions of constitutive nitric oxide with PAF and thromboxane on rat intestinal vascular integrity in acute endotoxaemia. *Br. J. Pharmacol.*, **113**, 1131–1136.

LOPEZ-BELMONTE, J., WHITTLE, B.J.R. & MONCADA, S. (1993). The actions of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa. *Br. J. Pharmacol.*, **108**, 73–78.

MACNAUGHTON, K., CIRINO, G. & WALLACE, J.L. (1989). Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach. *Life Sci.*, **45**, 1869–1876.

MCCALL, T.B., WHITTLE, B.J.R., BOUGHTON-SMITH, N.K. & MONCADA, S. (1988). Inhibition of FMLP-induced aggregation of rabbit neutrophils by nitric oxide. *Br. J. Pharmacol.*, **95**, 571P.

MORGAN-SMITH, S., HOLM-RUTILI, L., PERRY, M.A., GRISHAM, M.B., ARFORS, K.E., GRANGER, D.N. & KVIETYS, P.R. (1987). Role of neutrophils in haemorrhagic shock-induced gastric mucosal injury in the rat. *Gastroenterology*, **93**, 466–471.

NILSSON, U.A., SHOENBERG, M.H., ANEMAN, A., POCH, B., MAGADUM, S., BEGER, H.G. & LUNDGREN, O. (1994). Free radicals and pathogenesis during ischaemia and reperfusion of the cat small intestine. *Gastroenterology*, **106**, 629–936.

PARKS, D.A. & GRANGER, D.N. (1983). Ischaemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. *Am. J. Physiol.*, **245**, G285–G289.

PARKS, D.A., BUCKLEY, G.A., GRANGER, D.N., HAMILTON, S.R. & MCCORD, J.M. (1982). Ischaemic injury in the cat small intestine: role of superoxide radicals. *Gastroenterology*, **82**, 9–15.

PERRY, M.A., WADHWA, A., PARKS, D.A., PICKARD, W. & GRANGER, D.N. (1986). Role of oxygen radicals in ischaemia-induced lesions in the cat stomach. *Gastroenterology*, **80**, 362–367.

RACHMILEWITZ, D., STAMLER, J.S., KARMELLI, F., MULLINS, M.E., SINGEL, D.J., LOSCALZO, J., XAVIER, R.J. & PODOLSKY, D.K. (1993). Peroxynitrite-induced rat colitis - A new model of colonic inflammation. *Gastroenterology*, **105**, 1681–1688.

RATYCH, R.E., CHUKNYISKA, R.S. & BULKLEY, G.B. (1987). The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. *Surgery*, **102**, 122–130.

ROSAM, A.-C., WALLACE, J.L. & WHITTLE, B.J.R. (1986). Potent ulcerogenic actions of platelet-activating factor on the stomach. *Nature*, **319**, 54–56.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distruction and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.

TEPPERMAN, B.L., BROWN, J.F., KOROLKIEWICZ, R. & WHITTLE, B.J.R. (1994). Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: Effect of endotoxin challenge. *J. Pharmacol., Exp. Ther.*, **271**, 1477–1482.

TEPPERMAN, B.L., VOZZOLO, B.L. & SOPER, B.D. (1993). Effect of neutropenia on gastric mucosal integrity and mucosal nitric oxide synthesis in the rat. *Dig. Dis. Sci.*, **38**, 2056–2061.

TREVETHICK, M.A., CLAYTON, N.M., BAHL, A.K., SANJAR, S. & STRONG, P. (1994). Neutrophil depletion does not prevent indomethacin-induced ulceration of the rat gastric antrum. *Agents Actions*, **41**, Special Conference Issue: C226-C227.

WALLACE, J.L., HOGABOAM, C.M. & MCKNIGHT, G.W. (1990). Platelet-activating factor mediates gastric damage induced by hemorrhagic shock. *Am. J. Physiol.*, **259**, G140–G146.

WALLACE, J.L. & WHITTLE, B.J.R. (1986). Picomole doses of platelet-activating factor predispose the gastric mucosa to damage by topical irritants. *Prostaglandins*, **31**, 989–998.

WHITTLE, B.J.R. (1993). Neuronal and endothelium-derived mediators in the modulation of the gastric microcirculation: integrity in the balance. *Br. J. Pharmacol.*, **110**, 3–17.

WHITTLE, B.J.R. (1994). Nitric oxide in gastrointestinal physiology and pathology. *Physiology of the Gastrointestinal Tract.*, ed. Johnson, L.R. pp. 267–294. New York: Raven Press.

WHITTLE, B.J.R. & ESPLUGUES, J.V. (1988). Induction of rat gastric damage by the endothelium-derived peptide, endothelin. *Br. J. Pharmacol.*, **95**, 1011–1013.

WHITTLE, B.J.R. LOPEZ-BELMONTE, J. & MONCADA, S. (1990). Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. *Br. J. Pharmacol.*, **99**, 607–611.

WOOD, J.G., YAN, Z.Y. & CHEUNG, L.Y. (1992). Gastric microvascular actions of platelet-activating factor: role of leukocytes. *Am. J. Physiol.*, **262**, G537–G544.

YASUE, N. & GUTH, P.H. (1988). Role of exogenous acid and retransfusion in hemorrhagic shock-induced gastric lesions in the rat. *Gastroenterology*, **94**, 1135–1143.

ZWEIER, J.L., BRODERICK, R., KUPPUSAMY, P., THOMPSON-GORMAN, S. & LUTTY, G.A. (1994). Determination of the mechanisms of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *J. Biol. Chem.*, **269**, 24156–24162.

ZWEIER, J.L., KUPPUSAMY, P. & LUTTY, G.A. (1988). Measurement of endothelial cell free radical generation: Evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4046–4050.

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