Attenuation by nitrosothiol NO donors of acute intestinal microvascular dysfunction in the rat

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- 1 The effects of the nitric oxide (NO) donors, S-nitroso-glutathione (SNOG) and S-nitroso-N-acetyl-penicillamine (SNAP), on the acute intestinal microvascular dysfunction induced by N^G-nitro-L-arginine methyl ester (L-NAME) in combination with low doses of endotoxin were investigated in the anaesthetized rat.
- 2 Administration of L-NAME (5 mg kg⁻¹, s.c.) concurrently with *E. coli* lipopolysaccharide (LPS, 3 mg kg⁻¹, i.v.) provoked the leakage of radiolabelled albumin in the ileum and colon, as a measure of microvascular damage, determined 1 h after challenge.
- 3 Intravenous infusion of SNOG or SNAP (1–10 µg kg⁻¹ min⁻¹) dose-dependently attenuated the microvascular leakage induced by L-NAME and LPS.
- 4 Infusion of the lowest doses of SNOG or SNAP (1 µg kg⁻¹ min⁻¹, i.v.) that significantly reduced the albumin leakage, did not affect the increase in blood pressure in response to L-NAME in LPS-treated rats. Higher doses of SNOG or SNAP (5–10 µg kg⁻¹ min⁻¹, i.v.) dose-dependently reduced this increase in blood pressure.
- 5 In control studies, intravenous infusion of glutathione (10 µg kg⁻¹ min⁻¹) or N-acetyl-penicillamine (10 µg kg⁻¹ min⁻¹) had no effect on microvascular leakage in the ileum and colon induced by LPS and L-NAME.
- 6 Pretreatment with rabbit anti-rat neutrophil serum (0.4 ml kg⁻¹, i.p., 4 h before challenge), which reduced the neutrophil count in peripheral arterial blood, also inhibited the microvascular leakage in the ileum and colon.
- 7 The protective effects of the nitrosothiol NO donors in this model may reflect, in part, modulation of neutrophil interactions within the microcirculation or actions on endothelial cell integrity, in addition to any local vasodilator action.

Keywords: Nitric oxide; NO synthase inhibitors; N^G-nitro-L-arginine methyl ester; NO donors; S-nitroso-glutathione; S-nitroso-N-acetyl-penicillamine; neutrophils; rat intestinal vasculature; intestinal inflammation; endothelial injury; nitrosothiols

Introduction

The NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) provokes a substantial acute increase of vascular permeability in the rat small and large intestine following the concurrent administration of low doses of endotoxin, under conditions where neither L-NAME nor endotoxin alone caused such permeability changes (Laszlo *et al.*, 1994a). These changes in vascular permeability involve the acute release of platelet activating factor (PAF) and thromboxanes, mediators that can be released from neutrophils (Laszlo *et al.*, 1994b).

The nitrosothiol, S-nitroso-N-acetyl-penicillamine (SNAP) attenuates the acute gastrointestinal injury induced by PAF or high doses of endotoxin (Boughton-Smith *et al.*, 1990b; 1992). In the cat small intestine, NO donors reduce the microvascular injury caused by NO synthase inhibitors (Kubes & Granger, 1992) and attenuate the changes in epithelial cell permeability caused by such inhibitors administered alone or during ischaemia reperfusion (Kubes, 1992; Payne & Kubes, 1993). Infusion of acidified sodium nitrate also has beneficial effects in a shock syndrome induced by splanchnic artery occlusion in the rat (Aoki *et al.*, 1990). Low doses of SNAP have also been shown to exert protective actions against vascular injury induced by endothelin-1 in the rat gastric mucosa (Lopez-Belmonte *et al.*, 1993).

The aim of the present study was to evaluate the effects of the nitrosothiol NO donors, S-nitroso-glutathione (SNOG; Kowaluk & Fung, 1990; Radomski *et al.*, 1992) or SNAP (Ignarro *et al.*, 1981) on the acute microvascular leakage induced by L-NAME in combination with LPS in the intestine of the anaesthetized rat and to determine their concurrent actions on systemic arterial blood pressure. In addition, the effects of an anti-neutrophil antibody on such microvascular injury have been investigated.

Some of this work has been presented to the British Pharmacological Society (Laszlo *et al.*, 1994c).

Methods

Experimental protocol

Male Wistar rats (225–275 g) were deprived of food overnight, but received water *ad libitum*. The rats were anaesthetized with pentobarbitone sodium (60 mg kg⁻¹, i.p.) and *E. coli* lipopolysaccharide (LPS, 3 mg kg⁻¹, i.v.), ¹²⁵I-labelled human serum albumin ([¹²⁵I]-HSA, 2 µCi kg⁻¹, i.v.) and L-NAME (5 mg kg⁻¹, s.c.) were administered through a tail vein (Laszlo *et al.*, 1994a). Plasma leakage in the ileum and colon was determined 1 h after endotoxin injection. The dose of L-NAME was selected as producing near-maximal effects from previous studies in this model (Laszlo *et al.*, 1994b).

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Plasma leakage

Plasma leakage of [125 I]-HSA was determined in the ileum and colon, as an index of vascular endothelial damage. Blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0.318%) and centrifuged (10,000 g, 10 min, 4°C). The [125 I]-HSA content of the plasma and segments of ileal and colonic tissue was determined in a gamma-spectrometer (Nuclear Enterprises NE 1600) and the albumin content in the intestine was calculated, taking into account any changes in intestinal blood volume as described previously (Boughton-Smith *et al.*, 1993). Values from control tissue were subtracted from the values of treated tissue and the data were expressed as plasma leakage, μl plasma g^{-1} tissue.

Effect of SNOG or SNAP infusion on blood pressure and intestinal plasma leakage response induced by LPS and L-NAME

The effect of L-NAME (5 mg kg^{-1} , s.c.) administered concurrently with LPS (3 mg kg^{-1} , i.v.) on rat intestinal plasma leakage was determined after 1 h. Intravenous infusion of SNOG (1–10 μg kg^{-1} min^{-1}) or SNAP (1–10 μg kg^{-1} min^{-1}) was initiated concurrently with LPS and L-NAME administration and plasma leakage in the ileum and colon were determined 1 h later.

Systemic arterial blood pressure (BP) was measured from the right carotid artery of a separate group of rats with a blood pressure transducer (Elcomatic) connected to a Grass Polygraph. LPS and L-NAME were administered concurrently with the initiation of intravenous infusion of SNOG (1–10 μg kg^{-1} min^{-1}) or SNAP (1–10 μg kg^{-1} min^{-1}) and the blood pressure was monitored over 1 h. The effects on BP of LPS or L-NAME administration alone or in combination, and of the NO donors alone, was also measured during the 1 h investigation period.

Effect of glutathione or N-acetyl-penicillamine infusion on intestinal plasma leakage induced by LPS and L-NAME

In control studies, infusion of glutathione (10 μg kg^{-1} min^{-1} , i.v.) or N-acetyl-penicillamine (10 μg kg^{-1} min^{-1} , i.v.) was initiated concurrently with the administration of LPS and L-NAME and plasma leakage in the ileum and colon was determined after 1 h.

Effect of anti-neutrophil antiserum on intestinal plasma leakage induced by LPS and L-NAME

Rabbit anti-rat neutrophil antiserum (0.4 ml kg^{-1} , i.p.) was administered 4 h before the concurrent injection of LPS (3 mg kg^{-1} , i.v.) and L-NAME (5 mg kg^{-1} , s.c.). Plasma leakage in the ileum and colon was determined 1 h after this challenge. Neutrophil count in peripheral arterial blood (collected from the abdominal aorta) was determined by the staining of blood smears (Diff-Quick, Camlab Ltd., Cambridge, U.K.).

Materials

E. coli lipopolysaccharide (0111:B4), L-NAME and glutathione were obtained from Sigma Chemical Co. (Poole, Dorset). SNOG and SNAP were synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories. 125 I-labelled human serum albumin was from Amersham International (U.K.). Rabbit anti-rat neutrophil antiserum was obtained from Accurate Chemical and Scientific Corporation (Westbury, New York, U.S.A.). N-acetyl-penicillamine was generated by the incubation of SNAP solution in ambient light for 48 h at 37°C to deplete its NO content. The

compounds were dissolved freshly in isotonic saline immediately prior to use.

Statistical evaluation

Data are shown as the mean $\pm$ s.e.mean of (*n*) studies. For statistical comparisons, analysis of variance with the Bonferroni test was utilised, where $P < 0.05$ was taken as significant.

Results

Effect of SNOG or SNAP infusion on intestinal plasma leakage

Neither LPS (3 mg kg^{-1} , i.v.) nor L-NAME (5 mg kg^{-1} , s.c.) alone affected plasma leakage in the ileal ($\Delta 0 \pm 5$ and $\Delta 6 \pm 11 \mu\text{l}$ g^{-1} tissue, respectively; $n = 4-5$) or colonic tissue ($\Delta 0 \pm 4$ and $\Delta 8 \pm 14 \mu\text{l}$ g^{-1} tissue, respectively; $n = 4-5$) over 1 h. In contrast, the administration of L-NAME concurrently with LPS significantly provoked plasma leakage in the ileum and colon after 1 h (Figure 1).

Concurrent infusion of SNOG (1–10 μg kg^{-1} min^{-1} , i.v.) dose-dependently reduced plasma leakage in the ileum and colon induced by LPS and L-NAME. The lower dose of SNOG (1 μg kg^{-1} min^{-1} , i.v.) produced a $36 \pm 8\%$ and $45 \pm 9\%$ reduction in albumin leakage provoked by LPS and L-NAME in the ileum and colon, respectively ($P < 0.01$; $n = 5$), whereas the highest dose of SNOG (10 μg kg^{-1} min^{-1} , i.v.) abolished such leakage in both tissues (Figure 1).

Similarly, concurrent infusion of SNAP (1–10 μg kg^{-1} min^{-1} , i.v.) dose-dependently reduced such plasma leakage in the ileum and colon. The lower dose of SNAP (1 μg kg^{-1} min^{-1} , i.v.) produced a $73 \pm 3\%$ and $74 \pm 4\%$ reduction in albumin leakage provoked by LPS and L-NAME in the ileum and colon, respectively ($P < 0.001$; $n = 4$), while the highest dose of SNAP (10 μg kg^{-1} min^{-1} , i.v.) abolished such leakage in both tissues (Figure 2).

Effect of SNOG or SNAP infusion on blood pressure

Intravenous administration of LPS (3 mg kg^{-1}) did not significantly affect BP over the 1 h investigation period; the changes in BP (compared to the 0 min value) were 8 ± 2 , 2 ± 4 , -11 ± 7 and -11 ± 8 mmHg 15, 30, 45 and 60 min following LPS administration ($n = 5$). Concurrent injection of L-NAME (5 mg kg^{-1} , s.c.) in endotoxin-treated rats elevated arterial BP, with a maximum increase of 44 ± 8 mmHg at 30 min ($P < 0.001$; $n = 6$) as shown in Figure 3.

Infusion of SNOG (1–10 μg kg^{-1} min^{-1} , i.v.) caused a dose-dependent decrease of L-NAME-induced BP elevation over the 1 h period in endotoxin-treated rats. The lower dose of SNOG (1 μg kg^{-1} min^{-1} , i.v.) had no effect on the increase in BP by L-NAME, whereas the highest dose of SNOG (10 μg kg^{-1} min^{-1}) abolished this BP response ($P < 0.01$, $n = 4$) as shown in Figure 3.

Likewise, infusion of SNAP (1–10 μg kg^{-1} min^{-1} , i.v.) caused a dose-dependent decrease of L-NAME-induced BP elevation over the 1 h period in endotoxin-treated rats. The lower dose of SNAP (1 μg kg^{-1} min^{-1} , i.v.) had no effect on this increase in BP, while the highest dose of SNAP (10 μg kg^{-1} min^{-1} , i.v.) significantly attenuated the BP elevation ($P < 0.01$, $n = 4$) as shown in Figure 4.

Infusion of SNOG (5 and 10 μg kg^{-1} min^{-1} , i.v., for 1 h) in control rats not receiving LPS or endotoxin affected the resting BP by a maximal value -4 ± 7 (NS; $n = 4$) and by -17 ± 4 mmHg ($P < 0.05$; $n = 4$), respectively. Likewise, infusion of SNAP (5 and 10 μg kg^{-1} min^{-1} i.v. for 1 h) in control rats affected resting BP by a maximal value of -10 ± 5 and by -23 ± 7 mmHg ($P < 0.05$; $n = 4$) respectively.

Effect of glutathione or N-acetyl-penicillamine infusion on intestinal plasma leakage

In these control studies, the concurrent infusion of glutathione ($10\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v.) or N-acetyl-penicillamine ($10\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v.) did not affect ileal and colonic plasma leakage induced by LPS and L-NAME after 1 h ($n=4$; Figures 1 and 2).

Figure 1 Effects of a 1 h infusion of S-nitroso-glutathione (SNOG, $1\text{--}10\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v.) or glutathione (GSH, $10\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v.) on vascular leakage of radiolabelled albumin provoked by the concurrent administration of N^G -nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1} , s.c.) and lipopolysaccharide (LPS, 3 mg kg^{-1} , i.v.) in the rat (a) ileum and (b) colon. Albumin leakage (expressed as plasma leakage, $\Delta\mu\text{l g}^{-1}\text{ tissue}$) was determined 1 h after challenge. Data are given as the mean $\pm$ s.e.mean of 4–5 rats per group; statistical significance is shown as $*P < 0.05$ compared to the control LPS + L-NAME (Cont).

Figure 2 Effects of a 1 h infusion of S-nitroso-N-acetyl-penicillamine (SNAP, $1\text{--}10\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v.) or N-acetyl-penicillamine (AP, $10\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v.) on vascular leakage of radiolabelled albumin provoked by the concurrent administration of N^G -nitro-L-arginine methyl ester (L-NAME 5 mg kg^{-1} , s.c.) and lipopolysaccharide (LPS, 3 mg kg^{-1} , i.v.) in the rat (a) ileum and (b) colon. Albumin leakage (expressed as plasma leakage, $\Delta\mu\text{l g}^{-1}\text{ tissue}$) was determined 1 h after challenge. Data are given as the mean $\pm$ s.e.mean of 4–5 rats per group; statistical significance is shown as $*P < 0.05$ compared to the control LPS + L-NAME group (Cont).

Effect of anti-neutrophil antiserum on intestinal plasma leakage

The intraperitoneal administration of an anti-neutrophil serum (0.4 ml kg^{-1}), 4 h before challenge, significantly attenuated LPS and L-NAME-induced plasma leakage in the ileum and colon (by $49 \pm 6\%$ and by $43 \pm 9\%$, respectively; $P < 0.01$; $n = 5$) as shown in Figure 5. The anti-neutrophil serum reduced the neutrophil count in the peripheral arterial blood by $85 \pm 5\%$ after 4 h following its administration ($n = 5$). Higher doses of the antiserum were not investigated since in preliminary studies, its specificity was reduced, with significant reductions in other blood cell types. Pre-immune rabbit serum (0.4 ml kg^{-1}) had no significant effect on neutrophil count or the increase in plasma leakage ($n = 4$).

Figure 3 Reduction by S-nitroso-glutathione infusion ($1\text{ }[\blacksquare]$, $5\text{ }[\blacklozenge]$ and $10\text{ }[\blacktriangle]\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v., from 0 min) of blood pressure elevation induced by N^G -nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1} , s.c., at 0 min) (O, broken line) following the administration of lipopolysaccharide (3 mg kg^{-1} , i.v., at 0 min). Arterial blood pressure (expressed as ΔmmHg) was measured over a 1 h period. Data are shown as the mean $\pm$ s.e.mean of 4–6 rats per group; statistical significance is given as $*P < 0.05$ compared to the control L-NAME group.

Figure 4 Reduction by S-nitroso-N-acetyl-penicillamine infusion ($1\text{ }[\blacksquare]$, $5\text{ }[\blacklozenge]$ and $10\text{ }[\blacktriangle]\mu\text{g kg}^{-1}\text{ min}^{-1}$, i.v., from 0 min) of blood pressure elevation induced by N^G -nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1} , s.c., at 0 min) (O, broken line) following the administration of lipopolysaccharide (3 mg kg^{-1} , i.v., at 0 min). Arterial blood pressure (expressed as ΔmmHg) was measured over a 1 h period. Data are shown as the mean $\pm$ s.e.mean of 4–6 rats per group; statistical significance is given as $*P < 0.05$ compared to the control L-NAME group.

Figure 5 Inhibition of (a) ileal and (b) colonic leakage of radiolabelled albumin provoked by the concurrent administration of N^G -nitro-L-arginine methyl ester (L-NAME, 5 mg kg^{-1} , s.c.) and lipopolysaccharide (LPS, 3 mg kg^{-1} , i.v.) by the pretreatment of anti-neutrophil antiserum (ANS, 0.4 ml kg^{-1} , i.p., 4 h before challenge). Albumin leakage (expressed as plasma leakage, $\Delta \mu\text{l g}^{-1}$ tissue) was determined 1 h after challenge. Data are given as the mean $\pm$ s.e. mean of 4–5 rats per group; statistical significance is shown as * $P < 0.05$ compared to the LPS-alone group and # $P < 0.05$ compared to the LPS + L-NAME group.

Discussion

This study confirms the previous observation (Laszlo *et al.*, 1994a,b) that following the administration of NO synthase inhibitors, challenge with low doses of endotoxin causes acute increases in intestinal microvascular permeability to albumin. Such microvascular leakage is indicative of local injury and is associated with the macroscopic appearance of intestinal mucosal injury (Laszlo *et al.*, 1994b). Concurrent infusion of NO donors, SNOG or SNAP dose-dependently reduced ileal and colonic microvascular leakage induced by a combination of LPS and L-NAME over the 1 h investigation period. In control studies, the concurrent infusion of the corresponding carrier molecules devoid of NO, glutathione or N-acetyl-penicillamine did not affect the microvascular plasma leakage following endotoxin challenge. The doses of SNAP found to be effective in this model were 50 fold lower than those previously demonstrated to attenuate acute microvascular injury following challenge with high doses of endotoxin (Boughton-Smith *et al.*, 1990b).

NO may prevent microvascular leakage and injury in the ileum and colon by counteracting the vasoactive or tissue damaging actions of pro-inflammatory mediators, such as PAF and thromboxanes. The early release of these mediators into intestinal tissue has been shown previously in experimental models of intestinal inflammation (Whittle *et al.*, 1987; Boughton-Smith *et al.*, 1989; 1990a). Furthermore, the inhibition of thromboxane synthesis or the administration of PAF-receptor antagonists prevents the development of the acute intestinal microvascular plasma leakage and mucosal damage induced by the concurrent administration of LPS and L-NAME (Laszlo *et al.*, 1994b). In the present study, the lowest dose of SNOG or SNAP significantly inhibited micro-

vascular leakage without affecting the increase in BP induced by L-NAME in endotoxin-treated rats. Although changes in intestinal blood flow by these low doses of the NO donors cannot yet be excluded, these present findings could suggest that mechanisms are involved other than local vasodilatation in the protection of the microcirculation. One such mechanism could involve inhibition of neutrophil-dependent vascular endothelial damage, since in addition to being a vasodilator (Moncada *et al.*, 1991), NO can attenuate the aggregation of neutrophils and their adhesion to the microvasculature (McCall *et al.*, 1988; Kubes & Granger, 1992) as can SNAP (Xin-Liang *et al.*, 1993).

Exposure to PAF or endotoxin enhances the adherence of neutrophils to the endothelium (Matsuda *et al.*, 1991; Koltai *et al.*, 1991; Worthen *et al.*, 1992) and such leukocytes play a key role in the elevation of vascular permeability during inflammation (Wedmore & Williams, 1981). Moreover, neutrophils are considered to be involved in the evolution of microvascular injury of the gut under acute pathological conditions (Hernandez *et al.*, 1987; Kvietys *et al.*, 1990; Chmisse *et al.*, 1994). During the development of such acute microvascular damage, neutrophils adhere to the vascular endothelium, releasing cytotoxic agents such as oxygen radicals and pro-inflammatory mediators including PAF (Sacks *et al.*, 1978; Pabst & Johnston, 1980; Tauber & Babor, 1985; Hernandez *et al.*, 1987; Kubes *et al.*, 1990; 1991; Lefer & Lefer, 1993). In addition to inhibiting neutrophil adhesion, NO donors can also inhibit the release of superoxide anions and PAF from neutrophils (Moilanen *et al.*, 1993). Endogenous NO appears to play a similar modulatory role since the increased neutrophil adherence and migration in mesenteric venules of the anaesthetized rat that follows NO synthase inhibition is significantly reduced by PAF and leukotriene receptor antagonists (Arndt *et al.*, 1993).

Administration of an anti-neutrophil serum protects the intestinal mucosa against ischaemia-reperfusion and indomethacin-induced damage (Hernandez *et al.*, 1987; Chmisse *et al.*, 1994) and reduces the susceptibility of the gastric mucosa to injury by ethanol (Kvietys *et al.*, 1990; Tepperman *et al.*, 1993), indomethacin (Wallace *et al.*, 1990) or PAF (Etienne *et al.*, 1988). The involvement of neutrophils in at least part of the intestinal vascular injury in the current acute model is suggested by the findings that the administration of an anti-neutrophil serum significantly inhibited the microvascular leakage.

Our findings thus demonstrate that the NO donors, SNOG or SNAP can attenuate microvascular permeability changes in the ileum and colon. This could reflect protective effects of NO on the microvascular bed, in addition to any local vasodilator action, which may involve modulation of neutrophil activity, as well as a possible more-direct action on endothelial barrier integrity through elevation of cyclic GMP levels (Buchan & Martin, 1992). While studies on intestinal microvascular blood flow and cellular adhesion will help to clarify the mechanisms involved, the present observations suggest that parenteral administration of these nitrosothiol NO donors can prevent local microvascular injury independently of any change in systemic haemodynamics.

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