Endogenous nitric oxide and sensory neuropeptides interact in the modulation of the rat gastric microcirculation

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1 The effects of depletion of sensory neuropeptides from primary afferent neurones by capsaicin pretreatment, on the changes in resting gastric mucosal blood flow following administration of inhibitors of nitric oxide biosynthesis have been investigated in the pentobarbitone-anaesthetized rat.

2 Bolus administration of the NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 0.8–12.5 mg kg⁻¹ i.v.), induced a dose-dependent increase in systemic arterial blood pressure (BP) and a reduction in resting mucosal blood flow, as determined by laser Doppler flowmetry.

3 Concurrent administration of L-arginine (300 mg kg⁻¹ i.v.) attenuated the effects of L-NAME (6.25 mg kg⁻¹) on resting mucosal blood flow and BP. The enantiomer, D-NAME (50 mg kg⁻¹ i.v.), which does not inhibit NO biosynthesis, had no effect on either parameter.

4 The fall in mucosal blood flow induced by submaximal doses of L-NAME (0.8–3.2 mg kg⁻¹) was substantially augmented in rats pretreated 2 weeks earlier with capsaicin.

5 The fall in resting mucosal blood flow induced by the less potent NO-synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA; 1.6–25 mg kg⁻¹ i.v.) was likewise significantly augmented in capsaicin-pretreated rats.

6 Pretreatment (15 min) with indomethacin (5 mg kg⁻¹ i.v.) did not augment further the microvascular actions of L-NAME or L-NMMA in capsaicin-pretreated rats, suggesting the lack of interaction of endogenous prostanoids with these other mediators in regulating local blood flow. The effects of L-NAME on BP were not altered by capsaicin and indomethacin administration.

7 These findings indicate that endogenous sensory neuropeptides and NO can interact in the regulation of the gastric microcirculation.

Keywords: Nitric oxide; sensory neuropeptides; gastric mucosal blood flow; primary afferent neurones; capsaicin; N^G-nitro-L-arginine methyl ester

Introduction

The endothelium-derived vasodilator, nitric oxide (Palmer *et al.*, 1987; Khan & Furchgott, 1987; Ignarro *et al.*, 1987), formed from L-arginine (Palmer *et al.*, 1988a,b), plays an important role in the regulation of the resting gastric mucosal microcirculation (Pique *et al.*, 1989). Furthermore, endogenous nitric oxide (NO) interacts with sensory neuropeptides released from primary afferent neurones, in the modulation of the gastric mucosal integrity (Whittle *et al.*, 1990). Thus, in rats pretreated with capsaicin two weeks before the study to deplete sensory neuropeptides, acute administration of the inhibitor of NO biosynthesis N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989) induced extensive necrotic damage to the gastric mucosa (Whittle *et al.*, 1990).

Since the gastric microcirculation has a crucial role in the maintenance of mucosal integrity, the actions and interactions of local vasoactive mediators in the microvasculature are of great significance. Hence, in the present study the interactions between endogenous NO and sensory neuropeptides in the regulation of gastric mucosal blood, measured by laser Doppler flowmetry, have now been explored. Thus, the effects of N^G-nitro-L-arginine as the methyl ester (L-NAME), a potent inhibitor of NO biosynthesis (Palacios *et al.*, 1989; Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990; Rees *et al.*, 1990), on gastric mucosal blood flow have now been evaluated in both control and capsaicin-pretreated rats and compared to those of L-NMMA. In addition, by using indomethacin to inhibit mucosal cyclo-oxygenase (Whittle & Vane, 1987), the interaction of endogenous prostanoids with

these local mediators in the gastric microcirculation has also been investigated.

Methods

Measurement of gastric mucosal blood flow

Male Wistar rat (230–260 g body weight) were deprived of food but not water for 18–20 h before the experiment. Animals were anaesthetized with sodium pentobarbitone (60 mg kg⁻¹, i.p.) and the stomach exposed by a midline incision. A small bore (8.5 mm o.d.) plastic cannula was then inserted via a small incision in the forestomach and tied in place, to allow free access to the gastric lumen. Gastric blood flow was recorded continuously with a laser Doppler blood flow monitor (Model MBF3D, Moor Instruments Ltd, Devon) as described before (Tepperman & Whittle, 1991).

The principle of laser Doppler flowmetry for assessment of gastric blood flow has been described previously in detail (Kiel *et al.*, 1985; Holm-Rutili & Berglin, 1988). A stainless steel laser optic probe (1.65 mm o.d.; Moor Instruments) was inserted into the gastric lumen via the plastic cannula and was allowed to rest gently on the gastric corpus mucosa. Changes in laser Doppler flow (LDF) were assessed in response to intravenous bolus injection (1 ml kg⁻¹) of isotonic saline or the compound under investigation. Average LDF values were determined for the 3 min control period just before the administration of L-NAME. Similarly the average LDF was calculated for a 3 min period, 10–15 min after L-NAME (0.8–12.5 mg kg⁻¹ i.v.) or in some further experiments, L-NMMA (1.6–25 mg kg⁻¹ i.v.), when values of LDF had stabilized. In some experiments, indomethacin (5 mg kg⁻¹ i.v.) in a dose inhibiting mucosal prostanoid biosynthesis by >90% (Whittle *et al.*, 1990) was injected 15 min before the administration of L-NAME, or L-NMMA.

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The mean systemic arterial blood pressure (BP) was measured from a cannula inserted into a carotid artery and connected to a pressure transducer (Bell & Howell, Ashford, U.K.) and a chart recorder (Grass model 7D polygraph). Changes in BP from resting levels are shown as Δ BP (mmHg).

Capsaicin-pretreatment

Adult rats (190–210 g) were treated with increasing doses of capsaicin for three consecutive days (20, 30 and 50 mg kg⁻¹, s.c.) to deplete neuropeptides in primary afferent neurones as described previously (Esplugues *et al.*, 1989). All capsaicin injections were made under halothane anaesthesia and, to counteract any respiratory impairment associated with administration of capsaicin, the rats were pretreated with terbutaline (0.1 mg kg⁻¹, i.m.) and aminophylline (10 mg kg⁻¹, i.m.) before capsaicin injection. The animals were used 2 weeks after completion of the capsaicin treatment. Control animals received a similar regimen of treatment with the vehicle alone.

Materials

N^G-nitro-L-arginine methyl ester (Sigma Chemical Co., Poole, U.K.) and its D-enantiomer (Bachem AG, Bubendorf, Switzerland) was dissolved in isotonic saline immediately before use. L-Arginine hydrochloride (Sigma Chemical Co) was dissolved freshly in isotonic saline. N^G-monomethyl-L-arginine acetate, synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories by Dr H. Hodson, was freshly dissolved in isotonic saline before use.

Capsaicin (Fluka Chemic AG, Buchs, Switzerland) was prepared in a 50 mg ml⁻¹ solution containing absolute ethanol, Tween 80 and isotonic saline (10:10:80 v/v/v). Terbutaline sulphate was obtained in an injectable form from Astra Pharmaceuticals, Kings Langley and aminophylline (Sigma Chemical Co.) was dissolved in isotonic saline. Indomethacin (Sigma Chemical Co.) was dissolved in sodium bicarbonate solution (5% v/v) and diluted 1:4 with distilled water to obtain an isotonic solution for intravenous administration.

Statistical analysis

All data are expressed as mean $\pm$ s.e.mean. Comparisons between groups were made by Student's *t* test for paired or unpaired data as appropriate, where *P* values of less than 0.05 were taken as significant.

Results

Effect of L-NAME on gastric mucosal blood flow

In the control series of studies, bolus intravenous injection of isotonic saline had no significant action on resting LDF (*n* = 4). Bolus intravenous administration of L-NAME (0.8–12.5 mg kg⁻¹) induced a dose-dependent and significant fall in resting LDF, reaching plateau levels within 15 min, with a maximal reduction of $49 \pm 2\%$ of basal (*n* = 7) being achieved with the highest dose (Figure 1). In contrast, the enantiomer D-NAME, in doses up to 50 mg kg⁻¹ i.v. had no significant effect on LDF, as shown in Figure 2.

The effects of L-NAME (6.25 mg kg⁻¹ i.v.) on LDF were inhibited (by $94 \pm 5\%$, *n* = 5; *P* < 0.001) by pretreatment (5 min) with L-arginine (300 mg kg⁻¹ i.v.). This dose of L-arginine itself had no significant effect on resting LDF (Figure 2).

Effects of capsaicin pretreatment

The effects of the threshold dose of L-NAME (0.8 mg kg⁻¹ i.v.) on resting LDF were significantly augmented in rats that had been pretreated with capsaicin two weeks earlier, as shown in Figure 1. Furthermore, the actions of L-NAME (1.6 and 3.2 mg kg⁻¹ i.v.) on LDF were likewise substantially enhanced

Figure 1 Effect of capsaicin-pretreatment, 2 weeks before the study, on the actions of N^G-nitro-L-arginine methyl ester (L-NAME, 0.8–12.5 mg kg⁻¹ i.v.) on resting laser Doppler flow (LDF) as an index of gastric mucosal blood flow in the anaesthetized rat. Results, shown as the fall in LDF (% of basal) following L-NAME in control (open columns) and capsaicin-pretreated (hatched columns) rats, are the mean of 5–7 experiments for each, vertical bars show s.e.mean. Significant difference from resting LDF is shown as **P* < 0.05 and from effects in control rats as [†]*P* < 0.05.

(Figure 1). The effects of near-maximal doses of L-NAME (6.25–12.5 mg kg⁻¹) were not, however, significantly potentiated in capsaicin-pretreated rats (Figure 1).

In further studies, the effects seen with L-NAME in capsaicin-pretreated rats, were compared to those of L-NMMA. The reduction in resting LDF by a threshold dose of L-NMMA (1.6 mg kg⁻¹ i.v.) in control rats was significantly greater in capsaicin-pretreated rats ($-0.4 \pm 0.2\%$ and $-34 \pm 7\%$ of basal respectively, *n* = 5 for each; *P* < 0.01). Likewise, the reduction in resting LDF by L-NMMA (3.2 mg kg⁻¹ i.v.) was significantly augmented by capsaicin pretreatment (Figure 3).

Effects on systemic arterial blood pressure

Intravenous administration of L-NAME (0.8–12.5 mg kg⁻¹) induced dose-dependent increases in resting BP (resting value,

Figure 2 Lack of effect of N^G-nitro-D-arginine methyl ester (D-NAME, 50 mg kg⁻¹ i.v.) on laser Doppler flow (LDF) as an index of gastric mucosal blood flow and the reversal of the effects of L-NAME (6.25 mg kg⁻¹ i.v.) by prior (5 min) administration of L-arginine (L-Arg, 300 mg kg⁻¹ i.v.) in a dose itself not affecting resting LDF. Results, shown as change in LDF (% of basal), are the mean of 5–7 experiments in each group, vertical bars show s.e.mean. Significant difference from resting values of LDF are shown as ***P* < 0.01 and inhibition of the effects of L-NAME as [†]*P* < 0.01.

Figure 3 Effect of pretreatment (15 min) with indomethacin (Indo, 5 mg kg⁻¹ i.v.), on the fall in laser Doppler flow (LDF), as an index of mucosal blood flow, induced by N^G-nitro-L-arginine methyl ester (L-NAME, 3.2 mg kg⁻¹ i.v.) and N^G-monomethyl-L-arginine (L-NMMA, 3.2 mg kg⁻¹ i.v.) in capsaicin-pretreated rats. Results, shown as change in LDF (% of basal) are the mean of 5–7 experiments in each group, vertical bars show s.e.mean. Significant difference from resting values is shown by †*P* < 0.05 and significant increase by capsaicin administration is shown by **P* < 0.05; there was no further significant change induced by indomethacin.

110 ± 5 mmHg) as shown in Table 1, reaching plateau values within 10 min, and which were well maintained for at least 60 min. The hypertensive effects of L-NAME (6.25 mg kg⁻¹ i.v.) were substantially attenuated (from Δ33 ± 4 to Δ12 ± 2 mmHg, *n* = 5; *P* < 0.05) by prior administration (5 min) of L-arginine (300 mg kg⁻¹ i.v.). This dose of L-arginine itself had no significant effect on BP (Δ3 ± 1 mmHg, *n* = 4). Intravenous administration of D-NAME (50 mg kg⁻¹) had no significant effect on BP (Δ1 ± 1 mmHg, *n* = 3).

Capsaicin-pretreatment had no significant action on the value of resting BP (115 ± 5 mmHg, *n* = 8). Furthermore, capsaicin-pretreatment had no significant effect on the hypertensive actions of L-NAME (0.8–12.5 mg kg⁻¹ i.v.), as shown in Table 1.

Effects of indomethacin

In a further series of studies, pretreatment (15 min) with indomethacin (5 mg kg⁻¹ i.v.) had no significant effect on the fall in LDF induced by L-NAME (3.2 mg kg⁻¹) or L-NMMA (3.2 mg kg⁻¹) in capsaicin-pretreated rats (Figure 3). Furthermore, the fall in LDF induced by a near-maximal dose L-NAME following capsaicin pretreatment (6.25 mg kg⁻¹) was not significantly altered by administration of indomethacin (−40 ± 4 and −38 ± 9% of basal respectively, *n* = 5 for each). Likewise, the augmented fall in LDF observed with a higher dose of L-NMMA (25 mg kg⁻¹ i.v.) induced by cap-

saicin pretreatment (from −25 ± 8% to −43 ± 6% of basal, *n* = 5, *P* < 0.01) was not significantly increased by indomethacin administration (−36 ± 10% of basal, *n* = 4). Indomethacin alone had no significant effect on resting LDF (−4 ± 2% of basal; *n* = 5).

Indomethacin administration had no significant effect on the hypertensive actions of L-NAME (0.8–12.5 mg kg⁻¹) in capsaicin-pretreated rats (Table 1).

Discussion

The present finding that the potent NO-synthase inhibitor, L-NAME substantially reduces resting laser Doppler flow (LDF) in the rat gastric mucosa, confirms a role of endogenous NO in the regulation of gastric mucosal blood flow. Furthermore, evidence has now been obtained for an interaction of NO with endogenous sensory neuropeptides from capsaicin-sensitive afferent neurones, in the modulation of the gastric microcirculation.

In previous studies, a reduction in resting gastric blood flow has been demonstrated following bolus intravenous administration of L-NMMA, assessed by both hydrogen gas clearance and radiolabelled microsphere techniques in the rat (Pique *et al.*, 1989; 1991; Pizcueta *et al.*, 1991), as also confirmed in the present study by use of laser Doppler flowmetry. Other workers, however, have failed to demonstrate a fall in resting LDF in the rat gastric mucosa following administration of L-NMMA or L-NAME, although this may reflect the overall dose given by infusion (Walder *et al.*, 1990). A reduction in pentagastrin-stimulated hyperaemia by L-NMMA and L-NAME was observed in that study (Walder *et al.*, 1990), as also seen with hydrogen gas clearance techniques following bolus administration of L-NMMA in doses having no direct action on acid secretion (Pique *et al.*, 1991). It was also demonstrated that mucosal blood flow under resting conditions was more resistant to reduction by L-NMMA than the elevated mucosal blood flow during secretory stimulation, which may reflect the relative contribution of NO to the local vascular tone under these different conditions (Walder *et al.*, 1990; Pique *et al.*, 1991).

Capsaicin pretreatment two weeks before the study, to deplete sensory neuropeptides from primary afferent neurones (Sternini *et al.*, 1987; Holzer, 1988; 1991; Green & Dockray, 1988), substantially augmented the effects of L-NAME on mucosal blood flow. This was particularly apparent with the threshold and lower doses of L-NAME, where the fall in mucosal blood flow was increased to an extent comparable to those obtained with maximal doses in non-capsaicin pretreated rats. The reduction in LDF induced by L-NMMA, which was less potent than L-NAME, was likewise augmented in capsaicin-pretreated rats. The response to the maximal dose of L-NAME was not enhanced further by capsaicin pretreatment, which probably reflects the maximal degree of indirect vasoconstriction that could be achieved in this microvascular bed under resting conditions. It could also possibly indicate that the NO synthase inhibitors and capsaicin pretreatment are

Table 1 Effect of capsaicin pretreatment and indomethacin on the hypertensive actions of N^G-nitro-L-arginine methyl ester (L-NAME)

L-NAME (mg kg ⁻¹)	ΔBP (mmHg)		
	L-NAME	L-NAME + Caps	L-NAME + Caps + Indo
0.8	9 ± 2*	7 ± 2*	5 ± 2*
1.6	13 ± 3*	11 ± 3*	11 ± 4*
3.2	18 ± 5*	18 ± 5*	18 ± 5*
6.25	33 ± 4**	31 ± 5**	33 ± 9*
12.5	43 ± 5**	41 ± 7**	42 ± 9**

Capsaicin (Caps) was administered 2 weeks before the study, and indomethacin (Indo, 5 mg kg⁻¹ i.v.) was administered 15 min before L-NAME (0.8–12.5 mg kg⁻¹ i.v.) in the anaesthetized rat. Results, shown as the increase in systemic arterial blood pressure (ΔBP; mmHg) are the mean ± s.e.mean of 5–7 experiments for each dose level. Statistically significant increase in BP from resting values is shown as **P* < 0.05, ***P* < 0.01; there was no significant effect of capsaicin pretreatment alone or with indomethacin administration on the responses to L-NAME.

acting through a final common mechanism that reduces NO release. Thus, sensory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are considered as endothelium-dependent vasodilators (Furchgott, 1983; Brain *et al.*, 1985), whose systemic vasodepressor actions may be attenuated by NO synthase inhibition (Whittle *et al.*, 1989; Whittle, 1990). Inhibition of NO formation would therefore be expected to reduce their local microvascular actions in the mucosa. However, capsaicin administration would act more profoundly to eliminate the local actions of these neuropeptides, while the release of NO is likely to be under a complex regulation by several local mediators.

The effects of capsaicin pretreatment itself on resting mucosal blood flow could not be evaluated in the present investigation because of the relative, rather than absolute values of blood flow changes determined by LDF measurement. However, previous studies using hydrogen gas clearance have suggested that depletion of sensory neuropeptides by capsaicin does not influence the absolute levels of resting mucosal blood flow (Pique *et al.*, 1990). Although CGRP, the predominant sensory neuropeptide located in rat gastric tissue, can induce vasodilatation in the mucosa (Holzer & Guth, 1991) and can attenuate the injurious actions of the vasoconstrictor endothelin-1 (Whittle & Lopez-Belmonte, 1991), such sensory neuropeptides alone do not appear to play a major role in the modulation of the resting microvascular blood flow. Thus, the augmented reduction of LDF would not appear to simply reflect the summation of the removal of the influence of these two types of mediators on resting vasodilator tone, and suggests that such a microvascular role for the sensory neuropeptides under basal conditions may only operate under conditions of reduced NO availability.

The reduction in resting LDF induced by submaximal doses of L-NAME was reversed by pretreatment with L-arginine, confirming that its vasoconstrictor actions in the microcirculation were as a consequence of the inhibition of NO biosynthesis from this precursor amino acid. The enantiomer D-NAME did not affect LDF, while as also observed by others, bolus administration of L-NAME but not D-NAME induced a dose-dependent increase in rat systemic BP, which was attenuated by the administration of L-arginine (Gardiner *et al.*, 1990; Rees *et al.*, 1990). In contrast to its effects in the gastric microcirculation, the hypertensive actions of L-NAME were not significantly altered in capsaicin-pretreated rats, indicating that the interactions between endogenous NO and sensory neuropeptides in the gastric microcirculation are not generalized effects in all vascular territories. It will be of interest, therefore, to explore in detail such interactions with NO in those vascular beds in which sensory neuropeptides are thought to play a significant role in modulating blood flow.

An interaction between NO and sensory neuropeptides in the regulation of gastric mucosal integrity has previously been observed, with L-NMMA inducing acute mucosal injury in capsaicin-pretreated rats (Whittle *et al.*, 1990). Since microvascular blood flow is a critical determinant of mucosal integrity, it is possible that the augmented changes in mucosal blood flow induced by administration of NO inhibitors following capsaicin-pretreatment, as observed in the present study, contributes to such damage. Indomethacin pretreatment substantially augmented this mucosal injury in capsaicin-pretreated rats (Whittle *et al.*, 1990), yet in the present study had no effect on the enhanced changes in LDF induced by L-NMMA or L-NAME. These findings could suggest that endogenous prostanoids do not interact with NO and sensory neuropeptides in the modulation of resting mucosal blood flow, yet do influence the tissue susceptibility to local ischaemia. Such actions may reflect protective actions of prostanoids, possibly prostacyclin, on the microvascular endothelium. Since there is evidence that NO can reduce gastro-intestinal vascular damage in endotoxic shock (Hutcheson *et al.*, 1990; Boughton-Smith *et al.*, 1990), it is feasible that NO and the sensory neuropeptides can also interact to promote microvascular integrity in the gastric mucosa under physiological conditions, by mechanisms additional to an enhancement of local blood flow.

The rat gastric mucosa possess high levels of the constitutive NO-synthase (Whittle *et al.*, 1991), but whether this calcium- and NADPH-dependent enzymic activity is solely located in the microvascular endothelium awaits studies on its mucosal cell distribution. The capsaicin-sensitive primary afferent neurones that store and release sensory neuropeptides have been demonstrated in close proximity to the submucosal microvasculature (Ekblad *et al.*, 1985), the vessels that regulate mucosal blood flow, and thus are appropriately located to influence the microcirculation. It is also pertinent that using immunohistochemical techniques, NO synthase has been localized in neuronal cell bodies and nerve fibres within the myenteric plexus of the rat duodenum (Bredt *et al.*, 1990). Furthermore, neuronally-derived NO has been proposed as a mediator of the non-cholinergic non-adrenergic relaxation on the rat stomach musculature following electrical stimulation (Li & Rand, 1990; Boeckxstaens *et al.*, 1991) and the reflex relaxation of guinea-pig stomach following distension (Desai *et al.*, 1991). It is therefore possible that the interactions between endogenous sensory neuropeptides and NO observed in the present study in the gastric mucosa may involve actions on local neuromodulator processes that regulate the mucosal microvasculature.

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