Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat

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1 The interactions between nitric oxide (NO), prostacyclin and sensory neuropeptides in the maintenance of gastric mucosal integrity have been investigated in the anaesthetized rat.

2 Administration of either N^G-monomethyl-L-arginine (L-NMMA) to inhibit endothelium-derived NO formation, indomethacin to inhibit prostanoid biosynthesis or chronic capsaicin pretreatment to deplete sensory neuropeptides, did not induce acute mucosal injury.

3 In capsaicin-pretreated rats, however, L-NMMA ($12.5–100 \, mg \, kg^{-1}$ i.v.) dose-dependently induced acute mucosal damage, characterized as vasocongestion and haemorrhagic necrosis. The enatiomer D-NMMA ($100 \, mg \, kg^{-1}$ i.v.) did not induce any detectable mucosal damage.

4 This mucosal injury induced by L-NMMA was inhibited by concurrent administration of L-arginine ($300 \, mg \, kg^{-1}$ i.v.).

5 In indomethacin ($5 \, mg \, kg^{-1}$ i.v.)-pretreated rats, L-NMMA also induced mucosal damage. Furthermore, following indomethacin administration in capsaicin-pretreated rats, L-NMMA induced widespread, severe haemorrhagic necrotic damage.

6 These findings suggest a role for endogenous NO formed from L-arginine, acting in concert with prostacyclin and sensory neuropeptides, in the modulation of gastric mucosal integrity.

Introduction

The maintenance of gastric mucosal function and integrity depends critically on the status of the microcirculation. Limitations to microvascular perfusion, either by direct vasoconstriction or by removal of endogenous vasodilator tone, can lead to the development of mucosal damage, erosion or ulceration (Whittle et al., 1981; Whittle, 1986). Thus, local generation of thromboxane A_2 or intra-arterial administration of its stable mimetic U-46619, which induce vasoconstriction in the gastric microcirculation, lead to substantial mucosal damage (Whittle et al., 1981; 1985; Esplugues & Whittle, 1988). More recently, close-arterial infusion of the endothelium-derived vasoconstrictor peptide, endothelin-1 (Yanagisawa et al., 1988) has also been demonstrated to induce pronounced mucosal damage following local intra-arterial infusion (Whittle & Esplugues, 1988) and to potentiate ethanol-induced mucosal disruption (Wallace et al., 1989).

One local vasodilator mediator that can be formed by endothelial cells is prostacyclin and its synthesis can be inhibited by cyclo-oxygenase inhibitors such as indomethacin (Moncada et al., 1976). Inhibition of endogenous prostacyclin formation and a reduction in mucosal blood flow has been proposed to be a mechanism underlying the gastric damage induced by indomethacin (Whittle, 1977) and other non-steroid anti-inflammatory agents (see Whittle, 1986). Vascular endothelial cells also release a labile humoral vasodilator substance known as endothelium-derived relaxing factor or EDRF (Furchgott & Zawadzki, 1980; Furchgott, 1983). Nitric oxide (NO), formed by endothelial cells from the amino acid L-arginine, accounts for the biological properties of EDRF (Palmer et al., 1987; 1988a). The formation of NO by endothelial cells is selectively inhibited by the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; Palmer et al., 1988b; Rees et al., 1989a). Studies in the rabbit and rat have demonstrated that L-NMMA increases systemic arterial blood pressure, suggesting that endogenous NO biosynthesis can modulate resting vascular tone in vivo (Rees et al., 1989b; Whittle et al., 1989). L-NMMA has therefore been used to investigate the role of endogenous NO in the regulation of gastric mucosal integrity in the rat.

The local release of vasodilator neuropeptides such as substance P or calcitonin gene-related peptide (CGRP) has also been implicated in the maintenance of mucosal integrity, since chronic pretreatment with capsaicin, which destroys primary sensory neurones and depletes their neuropeptide content (see Holzer, 1988), greatly augments mucosal damage induced by various challenging agents (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986; Esplugues et al., 1989). In order to investigate the interactions between these distinct local vasodilator mediators, NO, sensory neuropeptides and prostacyclin, in the gastric mucosa, we have evaluated the pro-ulcerogenic actions of L-NMMA in rats pretreated with capsaicin or treated with indomethacin.

Methods

Gastric mucosal damage

Male Wistar rats (230–260 g), deprived of food but not water for 18–20 h before the experiments, were anaesthetized with sodium pentobarbitone ($60 \, mg \, kg^{-1}$, i.p.) and the stomach exposed by a mid-line incision. After ligating the oesophagus and pylorus, 2 ml of acid saline ($100 \, mM \, HCl$) was instilled into the gastric lumen through the forestomach, followed by bolus intravenous administration of the agents under investigation. Forty-five min later, the stomachs were opened along the greater curvature, pinned to a wax block with the mucosal side up, immersed in neutral buffered formalin and then photographed on colour transparency film. The extent of macroscopically-visible damage was determined from these projected transparencies via computerized planimetry. The area of mucosal damage was calculated as the % of the total gastric mucosa showing macroscopically visible damage.

Two samples of the corpus ($0.5 \times 1.5 \, cm$) were excised from standardized regions of the dorsal and ventral aspects of the corpus mucosa, 0.5 cm below the limiting ridge, and were processed by routine techniques before being embedded in paraffin. Sections ($4 \, \mu m$) were stained with haematoxylin and eosin and examined under a light microscope. A 1 cm length of each histological section was assessed for epithelial cell damage (a

score of 1 being assigned); glandular disruption, vasoconges-
tion or oedema in the upper mucosa (a score of 2 being
assigned); haemorrhagic damage in the mid to lower mucosa
(a score of 3) and deep necrosis and ulceration (a score of 4).
Each section was evaluated on a cumulative basis to give the
histological index, the maximum score thus being 10. All
determinations were performed in a randomized manner with
both transparencies and histological sections coded to elimi-
nate observer bias.

Gastric damage in capsaicin-treated rats

Similar experiments were performed in animals pretreated
with capsaicin. Adult rats (190–210 g) were treated with capsa-
icin in increasing doses (20, 30 and 50 mg kg⁻¹, s.c.) on three
consecutive days, in a regimen that has been shown to deplete
neuropeptides in primary afferent neurones in adult rats
(Martling, 1987) and to enhance platelet-activating factor
(PAF)-induced gastric damage (Esplugues *et al.*, 1989). All
capsaicin injections were made under halothane anaesthesia
and, to counteract any respiratory impairment associated with
the administration of capsaicin, the rats were pretreated with
terbutaline (0.1 mg kg⁻¹, i.m.) and aminophylline (10 mg kg⁻¹,
i.m.) prior to capsaicin injection. The animals were used 2
weeks after completion of the capsaicin treatment. In control
studies, animals received a similar regimen of treatment with
the vehicle, although no significant difference could be
detected between any of the observations in vehicle-pretreated
or non-pretreated rats.

Systemic arterial blood pressure

Mean systemic arterial blood pressure (BP) was measured
from a cannula inserted into a carotid artery and connected to
a pressure transducer (Elcomatic) and a chart recorder
(Grass), with a resting value of 119 ± 2 mmHg ($n = 30$).

Materials

Indomethacin (Sigma Chemical Co) was freshly dissolved in
5% w/v NaHCO₃ solution and diluted to 1.25% with distilled
water. L-Arginine (Sigma Chemical Co) was prepared as the
acetate salt and dissolved freshly in isotonic saline. Nᴳ-
monomethyl-L-arginine acetate and its D-enantiomer, synthe-
sized as described previously (Patthy *et al.*, 1977) in the
Department of Medicinal Chemistry, Wellcome Research
Laboratories by Dr H. Hodson, were freshly dissolved in iso-
tonic saline before use. Capsaicin (Fluka Chemical AG) was
prepared as a 50 mg ml⁻¹ solution containing absolute
ethanol, Tween 80 and isotonic saline (10 : 10 : 80 : v/v/v).

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons
between groups of parametric data were made by Student's *t*
test for unpaired data. Comparisons between groups of non-
parametric data (histological index) were made by Mann-
Whitney U-test. *P* values of less than 0.05 were taken as
significant.

Results

Effects of L-NMMA or capsaicin pretreatment

Intragastric instillation of acid saline (2 ml; 100 mM HCl) did
not lead to any macroscopically detectable mucosal damage
over the 45 min study period (Figure 1). On histological
examination, a minor degree of epithelial cell damage involv-
ing 13 ± 4% ($n = 4$) of the total section length was determined
(Table 1). Following intragastric instillation of acid saline,
intravenous administration of L-NMMA (12.5–100 mg kg⁻¹)
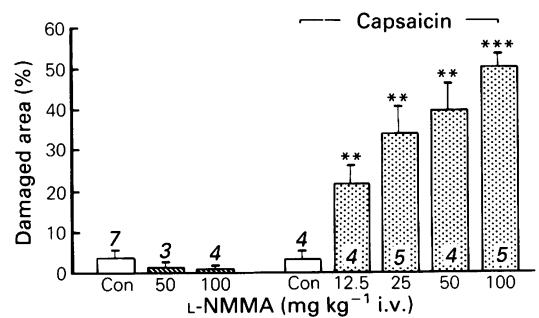did not induce any significant macroscopically detectable

Figure 1 Effects of Nᴳ-monomethyl-L-arginine (L-NMMA, 12.5–100 mg kg⁻¹ i.v.) on the gastric mucosa of vehicle- or capsaicin-pretreated rats. Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage 45 min after administration, are expressed as mean of *n* (number in columns) experiments where vertical lines show s.e.mean. Significant difference from the respective acid-control group (Con; intragastric 100 mM HCl) is shown as **$P < 0.01$, ***$P < 0.001$.

mucosal damage ($P > 0.05$; $n = 3$–5 per group), as shown for
the higher doses of L-NMMA in Figure 1. Likewise, no signifi-
cant increase in the minor histological damage was observed
following administration of these doses of L-NMMA (Table 1).

Capsaicin pretreatment alone did not induce any macro-
scopically apparent damage, following intragastric instillation
of acid-saline (Figure 1). Furthermore, no histologically
apparent damage could be located in the tissue sections fol-
lowing capsaicin pretreatment (Table 1).

Effect of L-NMMA following capsaicin pretreatment

In capsaicin-pretreated rats, L-NMMA (12.5–100 mg kg⁻¹)
induced dose-related macroscopic damage to the mucosa
(Figure 1). In a separate time-course study, distinct macro-
scopic damage could be detected in the corpus region 20 min
after L-NMMA (100 mg kg⁻¹) administration ($n = 3$), while
the degree of damage assessed 90 min after L-NMMA admin-
istration (65 ± 10% of total area, $n = 3$) was similar to that
observed after 45 min. With increasing doses of L-NMMA,
both the area of damage (Figure 1) and the severity of the
mucosal injury were significantly increased, this damage being
characterized macroscopically as surface cell sloughing, vaso-

Table 1 Effects of intravenous administration of Nᴳ-monomethyl-L-arginine (L-NMMA), D-NMMA and indo-methacin on the rat gastric mucosa, as assessed by histological evaluation

	Dose (mg kg⁻¹)	*Histological Index*	(*n*)
Control	—	1.5 ± 0.5	(4)
L-NMMA	100	0.7 ± 0.3	(3)
Indo	5	1.6 ± 0.6	(3)
Capsaicin-pretreated			
Control	—	1.5 ± 0.5	(4)
D-NMMA	100	1.6 ± 0.6	(3)
L-NMMA	25	4.4 ± 1.0*	(5)
	100	5.8 ± 1.4*	(5)
L-NMMA	100		
+	+		
L-Arginine	300	0.7 ± 0.3†	(3)
L-NMMA	100		
+	+		
Indo	5	9.2 ± 0.8†	(5)

Results, shown as the histological index of damage (0–10 scale), are mean ± s.e.mean of *n* animals, where statistical difference from the corresponding group control (intragastric 100 ml HCl), is shown as *$P < 0.05$ and from the L-NMMA (100 mg kg⁻¹) group in capsaicin-pretreated rats as †$P < 0.05$.

congestion and distinct areas of haemorrhagic necrosis. Such mucosal damage also involved the antral region.

In contrast, administration of the enantiomer, D-NMMA (100 mg kg^{-1}, i.v.) in capsaicin-pretreated rats did not induce any detectable mucosal injury (Figure 2). In further studies, administration of L-arginine (300 mg kg^{-1}, i.v.), immediately before L-NMMA (100 mg kg^{-1}, i.v.) caused a significant reduction in the damage (by 86 ± 2%, $n = 3$; $P < 0.01$) as shown in Figure 2.

Administration of L-NMMA (25 and 100 mg kg^{-1} i.v.) dose-dependently increased the histological index of damage, which was inhibited by concurrent administration of L-arginine (300 mg kg^{-1} i.v.), as shown in Table 1. The damage induced by L-NMMA was characterized histologically as epithelial cell and glandular disruption, with vasocongestion and haemorrhagic damage being also noted. D-NMMA (100 mg kg^{-1} i.v.) did not induce any histological injury in capsaicin-pretreated rats (Table 1).

Bolus intravenous administration of L-NMMA (12.5, 25, 50 and 100 mg kg^{-1}) in vehicle-pretreated rats induced a dose-dependent increase in mean systemic arterial BP of 23 ± 2, 33 ± 3, 41 ± 3 and 44 ± 4 mmHg above the basal value, respectively ($n = 4$–6 for each), which reached its peak value within 10 min and which was maintained over the 45 min observation period. Administration of D-NMMA (100 mg kg^{-1}) did not significantly increase systemic BP ($P > 0.05$; $n = 3$). The increase in BP induced by L-NMMA (100 mg kg^{-1}) was significantly attenuated (from 44 ± 4 to 18 ± 3 mmHg above the basal value; $n = 4$; $P < 0.05$) by prior administration of L-arginine (300 mg kg^{-1}, i.v.). There was no significant difference between the effects of L-NMMA (12.5 or 100 mg kg^{-1} i.v.) on BP in vehicle or capsaicin-pretreated rats ($P > 0.05$; $n = 4$ for each group).

Effects of indomethacin

Administration of indomethacin (5 mg kg^{-1} i.v.) immediately before intragastric acid instillation, did not significantly damage the mucosa over the 45 min study period (Figure 3). However, following indomethacin administration L-NMMA (100 mg kg^{-1}, i.v.) induced significant ($P < 0.05$) damage to the gastric mucosa (Figure 3).

In capsaicin-pretreated rats, indomethacin (5 mg kg^{-1}, i.v.) induced distinct mucosal injury, this damage being characterized as localized areas of linear haemorrhage and necrosis (Figure 3). Furthermore, following indomethacin administration, the mucosal damage induced by L-NMMA (100 mg kg^{-1}) in capsaicin-pretreated rats was substantially enhanced as shown in Figure 3, and involved virtually all of the mucosal surface. This damage was characterized macro-
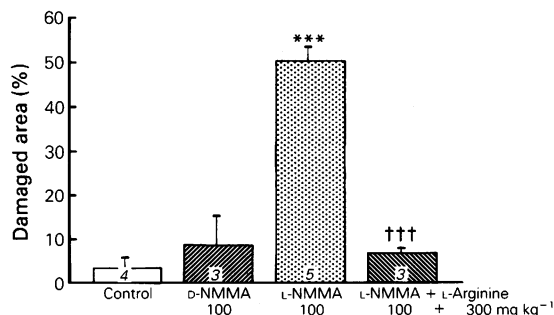

Figure 3 Effect of indomethacin (Indo, 5 mg kg^{-1} i.v.), N^G-monomethyl-L-arginine (L-NMMA, 100 mg kg^{-1} i.v.) or their combination, on the gastric mucosa of vehicle- or capsaicin-pretreated rats. Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage, are expressed as mean of n (figures in columns) experiments, where vertical lines show s.e.mean. Significant difference from the appropriate acid-control group (intragastric 100 mM HCl) and from the corresponding non-capsaicin group is shown as *$P < 0.05$ and ††$P < 0.01$, †††$P < 0.001$, respectively.

scopically as both an increase in the number and area of the dark necrotic lesions, as well as the appearance of more widespread epithelial disruption, vasocongestion and haemorrhage, which was confirmed histologically (Table 1).

Discussion

We have demonstrated that although the administration of L-NMMA alone or capsaicin-pretreatment did not lead to acute gastric damage, administration of L-NMMA in capsaicin-pretreated rats did induce substantial mucosal injury, determined by both macroscopic and histological techniques. Furthermore, indomethacin enhanced the damage induced by L-NMMA under both control conditions and in capsaicin-pretreated rats. These findings thus suggest an interaction between NO, prostanoids and sensory neuropeptides in the regulation of gastric integrity.

The release of NO from endothelial cells and vascular tissue, which can be detected by both bioassay and chemical techniques, accounts for the biological activity of EDRF (Palmer *et al.*, 1987; Khan & Furchgott, 1987; Ignarro *et al.*, 1987; Kelm *et al.*, 1988). Thus, in those vascular beds in which endogenous endothelium-dependent vasodilators regulate vascular tone, inhibition of NO formation would be expected to reduce blood flow. Indeed, in the rabbit coronary circulation, *in vitro*, L-NMMA increased vascular tone (Amezcua *et al.*, 1989) while *in vivo*, L-NMMA increased systemic BP in the rabbit and rat (Rees *et al.*, 1989b; Whittle *et al.*, 1989), as confirmed in the present study. The effects of L-NMMA in reducing blood flow in specific vascular beds *in vivo* have recently been reported (Gardiner *et al.*, 1989). It is also of interest that enzymic disruption of endothelial cells in the rat gastric microcirculation attenuated the vasodilator response to acetylcholine, suggesting the local involvement of an endothelium-derived factor (Kitagawa *et al.*, 1987). Furthermore, a reduction in gastric mucosal blood flow following intravenous administration of L-NMMA in the rat has recently been demonstrated, indicating a microvascular role of endogenous NO in the mucosa (Pique *et al.*, 1989b).

Intravenous administration of L-NMMA alone did not induce mucosal damage over the acute observation period employed in the current study in the rat. Whether more chronic treatment with L-NMMA or other more prolonged inhibitors of NO formation would induce gastric injury will require further evaluation. However, the macroscopic mucosal damage induced by L-NMMA in capsaicin-pretreated rats was readily apparent within 20 min of administration, and was near-maximally developed within 45 min. The specific nature

Figure 2 Prevention of the gastric mucosal damage induced by N^G-monomethyl-L-arginine (L-NMMA, 100 mg kg^{-1} i.v.) by concurrent administration of L-arginine (300 mg kg^{-1} i.v.) in capsaicin-pretreated rats. D-NMMA (100 mg kg^{-1} i.v.) did not induce mucosal damage. Results, shown as % of the total mucosal area that exhibited macroscopically assessed damage, are expressed as mean of n (numbers in columns) experiments where vertical lines show s.e.mean. Significant difference from acid-control group (intragastric 100 mM HCl) is shown as ***$P < 0.001$ and from the L-NMMA group by †††$P < 0.001$.
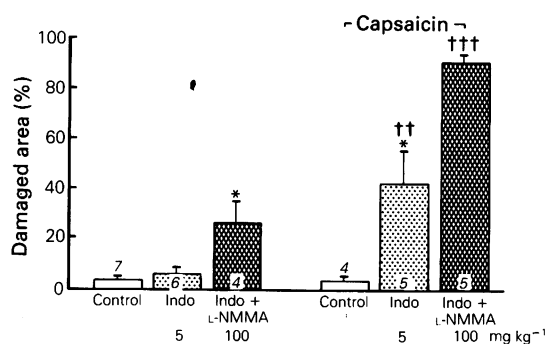
of this action of L-NMMA was demonstrated by the lack of injurious effect of D-NMMA, its enantiomer which does not inhibit NO synthesis by endothelial cells (Palmer et al., 1988b). These findings thus implicate a contribution of endogenous NO to the protective mechanisms within the mucosa. Recently the nitrovasodilator, nitroprusside, has been demonstrated to inhibit mucosal damage following intravenous administration (Wallace et al., 1989). The mechanisms of mucosal protection by endogenous NO or that released by nitrovasodilators remain to be explored, but are likely to involve actions on the microvasculature.

The severe mucosal injury induced by L-NMMA under these conditions of capsaicin-preatment, characterized macroscopically and confirmed histologically as epithelial cell sloughing, vasocongestion and haemorrhagic necrosis, was inhibited by concurrent intravenous administration of L-arginine. Previous studies have demonstrated that L-arginine can reverse the inhibitory actions of L-NMMA on NO synthesis by endothelial cells, in culture or on isolated vascular tissue, and by isolated neutrophils (Palmer et al., 1988b; Rees et al., 1989a; McCall et al., 1989), suggesting the currently observed protective effect of L-arginine was not simply due to non-specific actions of this systemically administered amino acid. Furthermore, L-arginine attenuates the hypertension induced by L-NMMA in the rabbit and rat (Rees et al., 1989b; Whittle et al., 1989) as confirmed in the present study using comparable doses. These findings thus suggest that L-NMMA acts by competing for the uptake or utilization of L-arginine, the substrate for NO biosynthesis. The doses of L-NMMA that were found to provoke mucosal injury in the rat are similar to those previously shown to inhibit endothelium-dependent vasodilatation in vivo induced by such agents as acetylcholine or bradykinin in the rat and rabbit (Whittle et al., 1989; Rees et al., 1989b). Furthermore, these doses of L-NMMA have been shown to inhibit the ex vivo generation of NO by vascular tissue, detected by bioassay and chemiluminescence techniques (Rees et al., 1989b). The present findings with L-NMMA thus suggest that the gastric mucosa can form endogenous NO derived from L-arginine, but its direct detection awaits further study.

Capsaicin-sensitive neurones containing vasoactive neuropeptides including substance P and CGRP have been located by immunohistochemical techniques in the rat stomach, particularly in association with the submucosal microvasculature (Sharkey et al., 1984; Ekblad et al., 1985; Green & Dockray, 1988). In previous studies in the rat, capsaicin pretreatment did not modify systemic arterial BP or resting gastric mucosal blood flow, and as also observed in the current study, did not induce damage to the gastric mucosa (Pique et al., 1989a,b; Esplugues et al., 1989). However, capsaicin pretreatment substantially potentiated the reduction in mucosal blood flow induced by the intravascular ulcerogen, PAF, which was accompanied by a significant increase in mucosal injury (Pique et al., 1989a). Such increased gastric damage in capsaicin-pretreated rats has previously been observed following challenge with other ulcerogens (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986). Thus, the release of neuropeptides from capsaicin-sensitive primary sensory neurones appears to be involved in the regulation of a local microvascular protective response to challenge.

Substance P and CGRP have been demonstrated to induce endothelium-dependent vasodilatation in vitro (Furchgott, 1983; Brain et al., 1985). Furthermore, the fall in systemic BP following intravenous administration of substance P in the rat can be attenuated by L-NMMA, suggesting a role of endogenous NO in the vasodilator responses of such neuropeptides in vivo (Whittle et al., 1989). If such mechanisms operated in the mucosal microcirculation, L-NMMA would be expected to attenuate any vasodilator actions of these endogenous neuro-

peptides, as well as other endothelium-dependent vasodilators. Capsaicin pretreatment does not affect resting gastric mucosal blood flow (Pique et al., 1989a), nor does it alter the increase in systemic BP induced by L-NMMA. However, it will be of interest to determine whether capsaicin pretreatment augments the changes in gastric blood flow induced by L-NMMA (Pique et al., 1989b), since such microcirculatory changes could underlie the resulting mucosal damage.

Endogenous endothelium-independent vasodilators such as prostacyclin, whose systemic hypotensive actions are not modified by L-NMMA (Whittle et al., 1989), may synergistically interact with these other vasodilator mediators in the local regulation of the microcirculation. Indeed, following intravenous administration of indomethacin, in a dose sufficient to induce near-maximal inhibition of mucosal prostacyclin formation (Whittle, 1986), the gastric injury induced by L-NMMA was significantly augmented, although not to the same degree as by capsaicin-pretreatment. In capsaicin pretreated rats, this low dose of indomethacin rapidly induced mucosal damage, as demonstrated previously following its intraperitoneal injection (Holzer & Sametz, 1986). Furthermore, the present findings show that in capsaicin-pretreated rats, indomethacin substantially augmented the damage induced by L-NMMA which involved virtually all of the mucosal area, including the antral region. Parenteral indomethacin has been demonstrated to limit blood flow in the gastric microcirculation (see Whittle, 1986) and whether such actions are substantially accentuated in capsaicin-pretreated rats or following NO inhibition will require evaluation.

Although the possibility that other actions of indomethacin may contribute to this enhanced injurious response cannot be entirely discounted, these present findings support a contribution of endogenous prostaglandins in the regulation of mucosal integrity, particularly under conditions where the synthesis or release of NO or the neuropeptides are concurrently reduced. Indeed, if these latter conditions occurred clinically, non-steriod anti-inflammatory agents would be expected to be considerably more injurious to the gastric mucosa.

The crucial importance of the gastric microcirculation is thus re-inforced by the current findings, that suggest that at least three chemically and physiologically distinct classes of local vasodilator mediators of diverse biochemical origins act in concert in the maintenance of mucosal viability. Thus, endogenous NO, sensory neuropeptides and a prostanoid, probably prostacyclin, all appear to subserve a modulator function in the regulation of gastric mucosal integrity. These mediators may not only exert local vasodilator actions on the microcirculation, essential for adequate microvascular blood flow under physiological conditions, but may act to enhance or preserve endothelial cell function and continuity, especially under conditions of challenge. It is therefore of relevance that both NO and prostacyclin can be synthesized by endothelial cells (Palmer et al., 1988a), and it is possible that neuropeptides originating from the afferent sensory neurones in the vicinity of the microvessels are involved in the regulation of their release.

Since damage to the endothelial cell can be a prime event leading to mucosal injury induced by irritants (Guth et al., 1984; Szabo et al., 1985), this could reflect a derangement in the formation or release of these vasoactive mediators. Although selective inhibition of either mediator alone induced no acute mucosal injury, concurrent pharmacological interference with more than one of these mediators induced rapid and widespread tissue damage. It will therefore be necessary to consider the nature of the interactions of these vasoactive mediators when assessing the physiological regulation of gastric function and integrity, and the pathogenesis of mucosal damage and peptic ulceration.

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