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ROLE OF NITRIC OXIDE IN INDOMETHACIN-INDUCED GASTRIC MUCOSAL DYSFUNCTION IN THE RAT

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SUMMARY

The present study was undertaken to explore the role of nitric oxide (NO) in the pathogenesis of experimental non-steroidal anti-inflammatory drug (NSAID)-induced gastropathy. We assessed the role of NO inhibition and donation in indomethacin-induced gastric mucosal dysfunction. The stomach was perfused with vehicle (control) for 20 min, followed by indomethacin (10 mg ml^{-1} in 1·25 % sodium bicarbonate, pH 8·4) for 120 min. N^G-nitro-L-arginine methyl ester (L-NAME, 5 and 10 mg kg^{-1}, I.V. bolus), L-arginine, D-arginine (100 mg kg^{-1} I.V. bolus, 10 mg kg^{-1} h^{-1}, 2 h infusion) and the NO donor glyceryl trinitrate (GTN) were given at the same time (20, 40 and 80 μg kg^{-1} min^{-1}, 15 min infusion) as perfusion with indomethacin was started. Epithelial permeability was quantified by measuring blood-to-lumen clearance of ^{51}Cr-labelled EDTA. Indomethacin caused a 20-fold increase in ^{51}Cr-EDTA leakage compared with that of the control group. Treatment with L-NAME or L-arginine did not affect the indomethacin-induced alterations in mucosal permeability. Administration of GTN (20 μg kg^{-1} min^{-1}) significantly reduced the indomethacin-induced mucosal dysfunction. By contrast, higher doses of GTN (80 μg kg^{-1} min^{-1}) exacerbated epithelial dysfunction induced by indomethacin. Elevated levels of carbonyls and myeloperoxidase (MPO) observed after indomethacin administration were significantly reduced, to the control values, when GTN (20 μg kg^{-1} min^{-1}) was administered along with indomethacin. These data suggest that NO from exogenous sources can exert a dual action on the integrity of the gastric mucosa challenged by indomethacin. Low doses of GTN can prevent mucosal dysfunction induced by indomethacin, while higher doses of GTN may exacerbate the increases in epithelial permeability.

INTRODUCTION

The gastric mucosal barrier plays an important role in separating the luminal contents from the underlying mucosal interstitial fluid (Crissinger *et al.* 1990). Several endogenous mediators, including vascular leukocytes, mast cells, reactive oxygen metabolites and nitric oxide, have been implicated as the modulators of epithelial barrier integrity under physiological and pathophysiological states (Horton & Walker, 1993; Kubes, 1993; Kanwar *et al.* 1994*b*). Nitric oxide (NO) is a recently described endothelium-derived relaxing factor synthesized from the amino acid L-arginine via constitutive and inducible forms of NO synthase (Moncada *et al.* 1991). NO is produced by many cell types found within the gastric mucosa, and has been

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shown to contribute to the regulation of epithelial permeability in both stomach and small intestine (Kubes, 1993; Kanwar *et al*. 1994*a*; Coskun *et al*. 1996). These contentions are based on the observations that the inhibitor of NO synthesis, N^{G}-nitro-L-arginine methyl ester (L-NAME), rapidly increased permeability of the epithelial barrier to ^{51}Cr-labelled EDTA (^{51}Cr-EDTA), a small molecule used to quantitatively assess the normally tight mucosal barrier. NO has also been shown to have protective effects in some experimental ulcer models. These studies involved topical or parenteral administration of nitric oxide itself, nitric oxide donors or stimulators of endogenous nitric oxide synthesis. For example, Andrews *et al*. (1994) demonstrated that intravenous administration of sodium nitroprusside or acetylcholine before reperfusion results in a significant reduction in the extent of damage induced by gastric ischaemia–reperfusion. It has been also demonstrated that topical application to the gastric mucosa of a nitric oxide solution, sodium nitroprusside or glyceryl trinitrate inhibited the mucosal damage induced by subsequent exposure of the mucosa to ethanol (MacNaughton *et al*. 1989; Kitagawa *et al*. 1990). However, the role of NO in the modulation of the mucosal integrity may be more complex inasmuch as Lopez-Belmonte *et al*. (1993) demonstrated that unregulated release of high levels of NO within the microvasculature induced mucosal injury that appeared to be unrelated with systemic hypotensive effects of these compounds.

It is well documented that non-steroidal anti-inflammatory drugs (NSAIDs) cause clinically important gastrointestinal injury. Although the pathogenesis of the epithelial lesions responsible for this action are still unclear, the ability of these agents to inhibit prostaglandin synthesis (Vane, 1971), increased leukocyte infiltration into gastrointestinal mucosa (Wallace *et al*. 1990), release of several biogenic amines and reactive oxygen species (Pihan *et al*. 1987), disturbance of mucosal microcirculation and altered epithelial barrier function (Vaananen *et al*. 1991) have been used to explain the acute gastric damage associated with NSAID administration. Studies using inhibitors of nitric oxide synthesis have demonstrated that these inhibitors exacerbate gastric mucosal injury induced by indomethacin and, like NSAIDs, reduce mucosal blood flow and cause neutrophil adherence to the vascular endothelium (Kubes *et al*. 1991; Wallace *et al*. 1991). Based on the observations that NO donors prevent various aspects of gastric mucosal inflammation, investigators have recently developed derivatives of NSAIDs containing a moiety that generates NO. It has been reported that these derivatives reduced gastric mucosal injury without altering their effectiveness as anti-inflammatory agents or prostaglandin synthesis inhibitors. Moreover, a very profound attenuation in mortality was observed in rats treated with NO-NSAID derivatives (Reuter *et al*. 1994; Conforti *et al*. 1993). Although the role of NO in the pathogenesis of experimental NSAID gastropathy has been extensively studied, relatively little is known about the effects of NO on NSAID-induced gastric epithelial barrier dysfunction. The present study was undertaken to further explore the role of NO in the pathogenesis of experimental NSAID gastropathy. A perfused rat stomach preparation was used, and ^{51}Cr-EDTA leakage from blood to the gastric lumen was employed as an index of mucosal permeability. This procedure has previously been used as an index of mucosal permeability in the rat intestine and stomach (Crissinger *et al*. 1990; Coskun *et al*. 1996). The effects of NO inhibition and donation were assessed as an indication of the contribution of NO to the gastric mucosal dysfunction. An additional effort has been made to determine whether changes observed on mucosal permeability after NO inhibition and donation were related to the altered levels of reactive oxygen metabolites and tissue polymorphonuclear leukocytes (PMNs).

METHODS

Wistar rats of both sexes (200–250 g) were maintained on a purified laboratory diet and deprived of food, but not water for 18–24 h before experiments. The study was approved by the Animal Care and Ethics Committee of Marmara University School of Medicine. The animals were anaesthetized with urethane (1·2 g kg^{-1}) and a tracheotomy was performed to facilitate breathing. A carotid artery was cannulated for arterial pressure recording (Nikon Kohden polygraph, model AP-621G) and blood sampling. The right jugular vein was also cannulated for the injection of radioisotope marker and various compounds. An abdominal incision was made, and plastic tubing was inserted into the stomach through an incision in the duodenum (5–10 mm distal to the pylorus), and secured with ligatures so that only the stomach would be exposed to the perfusion fluid. A second tube was passed into stomach through the mouth and oesophagus. The stomach was flushed with warmed (37 °C) saline to remove any particulate matter. Both renal pedicles were ligated to prevent rapid excretion of the radioisotope marker into urine. A thermometer was inserted into the rectum and the body temperature was maintained at 37 °C by a heating pad.

Mucosal permeability

After the surgery was completed, mucosal permeability was determined using the blood-to-lumen clearance of ^{51}Cr-ethylenediaminetetraacetic acid (EDTA) obtained from New England Nuclear (Boston). Briefly, ^{51}Cr-EDTA (100 μCi) in saline was administered intravenously as a bolus through jugular vein followed by a 20 min equilibration period, during which the stomach was perfused at a rate of 0·2 ml min^{-1} but no clearance measurements were taken. All perfusion fluids were warmed so that their temperature at the point of entering the stomach was 37 °C. The fluid was perfused via the oesophageal cannula and was collected from the duodenal cannula. The stomach was initially perfused for 20 min with isotonic saline, after which the perfusion system was flushed with air to remove any saline remaining in the stomach. The stomach was then perfused with indomethacin (10 mg ml^{-1} in 1·25 % sodium bicarbonate, pH 8·4) or the vehicle alone and the luminal perfusate was collected over 10 min periods for 2 h. The concentration of indomethacin used was based on experimental studies employing mucosal permeability measurements (Vaananen *et al*. 1991; Chmaisse *et al*. 1994). Blood samples (0·3 ml) were taken at 60 min intervals from the carotid artery and centrifuged (4500 *g*) for 4 min in an Eppendorf microfuge. The amount of radioactivity in the plasma and perfusate was then determined by gamma spectroscopy. The plasma-to-lumen clearance of ^{51}Cr-EDTA was calculated as:

$$\text{Clearance} = \text{c.p.m}_{\text{p}} \times \text{PR} \times 100/\text{c.p.m}_{\text{pl}} \times \text{wt},$$

where clearance of ^{51}Cr-EDTA is given in millilitres per minute per 100 grams tissue, c.p.m_{p} is counts per minute per millilitre of perfusate, PR is the perfusion rate, c.p.m_{pl} is counts per minute per millilitre of plasma, and wt is weight of the stomach in grams.

At the end of experiment, the rats were killed with an overdose of anaesthetic and the stomachs were removed, rinsed and weighed. The extent of macroscopically visible damage was measured and the length in millimeters was recorded. The damage score for the stomach was calculated as the sum of the lengths of all lesions. The tissue samples were then frozen at −70 °C for myeloperoxidase measurements.

In addition, the volume of luminal perfusate was measured at the times described above to determine alterations in net transmucosal fluid flux. This calculation was made by subtracting the amount of fluid entering the stomach from the amount collected from the distal end over the 10 min perfusion period. Net transmucosal fluid flux was calculated from the mean of all measurements and expressed as millilitres per minute per 100 g tissue weight.

In a group of animals, the NO synthesis inhibitor, N^{G}-nitro-L-arginine-methyl-ester (L-NAME, Bachem, Inc., Torrance, CA, USA) was given intravenously as a bolus (5 and 10 mg kg^{-1}), 5 min before the initiation of indomethacin administration to investigate the effects of NO inhibition on indomethacin-induced alterations in gastric mucosal permeability. In the latter experiments, L-arginine or D-arginine (Sigma) was given as a bolus (100 mg kg^{-1}) at the time of indomethacin administration and then infused at a rate of 10 mg kg^{-1} h^{-1} through the jugular vein throughout the experiment.

In order to evaluate the effect of exogenous NO on indomethacin-induced alterations in gastric mucosal permeability, glyceryl trinitrate (GTN; 20 μg kg^{-1} min^{-1}, GTN 20; 40 μg kg^{-1} min^{-1}, GTN 40 and 80 μg kg^{-1} min^{-1}, GTN 80; Schwarz Pharma) and sodium nitroprusside (SNP; 10 μg kg^{-1} min^{-1}, SNP 10; 30 μg kg^{-1} min^{-1}, SNP 30 and 60 μg kg^{-1} min^{-1}, SNP 60; Schwarz Pharma) were infused intra-

venously for 15 min, at the time when the gastric perfusion with indomethacin was started. The nitrovasodilators were dissolved freshly in isotonic saline and kept on ice protected from light. The doses of various drugs were selected based on previous studies in which they are employed as NO donors or inhibitors (Lopez-Belmonte *et al*. 1993).

Tissue myeloperoxidase (MPO) measurements

Tissue-associated MPO activity was determined in 0·2–0·5 g samples. The tissue samples were homogenized in 10 volumes of ice-cold potassium phosphate buffer (20 mM K_2HPO_4, pH 7·4). The homogenate was centrifuged at 4500 *g* for 20 min at 4 °C and the supernatant, which contained < 5 % of total MPO activity, was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mM acetic acid (pH 6·0) containing 0·5 % (w/v) hexadecyl trimethylammonium hydroxide (HETAB). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (Sigma). One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance of 1·0 min^{-1} at 655 nm and 37 °C (Coskun *et al*. 1996).

Determination of protein oxidation levels in plasma samples

Protein oxidation in venous plasma samples was quantified using the interaction between dinitrophenyl hydrazine (DNP, Sigma) and the carbonyls to yield a chromophore that absorbs strongly at 360 nm (Levine *et al*. 1990). Briefly, protein from the plasma samples obtained at the beginning and end of the experiments was precipitated by the addition of 20 % trichloroacetic acid (TCA). Precipitated protein was collected by centrifugation and resuspended in 0·5 ml of 10 mM DNP in 2 N HCl. The samples were incubated for 1 h at 25 °C with occasional mixing. Protein was then precipitated by the addition of 20 % TCA, collected by centrifugation, and the pellet was washed three times with 1 ml of an ethanol : ethyl acetate (1 : 1) solution to remove any unreacted DNP. The protein precipitate was solubilized by the addition of 1 ml of 1 N NaOH. The carbonyl content was calculated assuming a molar extinction coefficient of 22 000 (Levine *et al*. 1990).

Determination of total sulfhydryl (RSH) groups and lipid peroxide levels in plasma samples

Plasma samples were mixed with 1 ml of a solution containing 100 mM Tris·HCl (pH 8·2), 1 % sodium dodecyl sulfate, and 2 mM EDTA. The mixture was incubated for 5 min at 25 °C and centrifuged to remove any precipitate. 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 0·3 mM) was then added to each reaction volume and incubated for 15 min at 37 °C to allow for the formation of thionitrobenzoic acid (TNB) (Sedak & Lindsay, 1968). Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) as described previously (Kurtel *et al*. 1992).

Determination of tissue nitric oxide levels by chemiluminescence

Chemiluminescence measurements were done using a liquid scintillation counter (Tricarb 1500; Packard Instruments) in out-of-coincidence mode with a single active photomultiplier tube. Fresh gastric samples were gently transferred to precounted vials and NO detection was based upon the chemiluminescence reaction between NO and a purified luminol–H_2O_2 system (Kikuchi *et al*. 1993) at room temperature. Luminol was purified by recrystallization as a monosodium salt. The commercial luminol (5-amino-2,3-dihydro-1,4-phthalazine dione; Sigma) was dissolved in 1 N HCl and was treated with Norite. Adjustment of the pH to 3 with acetic acid yielded a crude pale yellow powder. The powder was dissolved in 1 N NaOH and again decolourized with Norite. Standing at 4 °C for 5 days produced a colourless powder and recrystallization twice from water resulted in a pure luminol sodium salt. The chemiluminescent probe contained 18 μM luminol, 150 μM desferrioxamine, 2·5 mM H_2O_2, and 2 mM K_2CO_3. Superoxide radical generation was quantified by adding lucigenin (bis-*N*, methylacridinium-nitrate; Sigma) at 0·2 mM final concentration to the vials (Van Dyke, 1987). Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 60 min corrected for tissue weight (counts min^{-1} mg^{-1}).

Statistics

All values are reported as means ± S.E.M. Student's *t* test and one-way analysis of variance with the Newman–Keuls (*post hoc*) test were used to determine whether data from different groups were statistically different. A P value of < 0·05 was considered statistically significant.

Table 1. *Effects of indomethacin and indomethacin plus* L-*NAME*, L-*arginine*, D-*arginine or glyceryl trinitrate* (*GTN*) *on lesion index, haematocrit, secretion/absorption and myeloperoxidase levels*

	n	Lesion index (mm)	Haematocrit (%)	Secretion/absorption (ml min^{-1} (100 g)$^{-1}$)	Myeloperoxidase (units (g wet wt)$^{-1}$)
Control	5	0·75 ± 0·7	53·7 ± 0·9	0·5 ± 0·07	198 ± 12
Indo	6	2·30 ± 1·7	53·0 ± 0·7	–0·005 ± 0·3 *	313 ± 10 **
NAME 5	5	1·50 ± 1·0	55·8 ± 3·3	–0·3 ± 0·3 **	348 ± 49 *
NAME 10	5	2·41 ± 2·0	54·6 ± 4·8	0·2 ± 0·09 *	310 ± 52 *
L-Arginine	5	3·50 ± 2·0	53·8 ± 1·2	–0·1 ± 0·05 *	238 ± 32 †
D-Arginine	6	0·60 ± 0·6	50·3 ± 4·3	n.t.	297 ± 36 *
GTN 20	6	0·50 ± 0·5	51·8 ± 2·7	0·96 ± 0·2 *†	199 ± 6 †
GTN 40	7	2·14 ± 1·3	53·8 ± 1·4	0·25 ± 0·21	322 ± 22 **
GTN 80	6	2·00 ± 1·5	54·0 ± 0·7	0·22 ± 0·17	299 ± 23 **

NAME 5, 5 mg kg^{-1} L-NAME; NAME 10, 10 mg kg^{-1} L-NAME; GTN 20, 20 μg kg^{-1} min^{-1} GTN; GTN 40, 40 μg kg^{-1} min^{-1} GTN; GTN 80, 80 μg kg^{-1} min^{-1} GTN. L- and D-Arginine application, 100 mg kg^{-1} bolus + 10 mg kg^{-1} h^{-1} infusion. A positive value for secretion/absorption data denotes net fluid absorption and a negative value indicates net secretion. n.t., not tested. * $P < 0·05$, ** $P < 0·01$; compared with control group. † $P < 0·05$; compared with indomethacin (Indo) group (Student's t test). Results are expressed as means ± S.E.M.

RESULTS

The mean blood pressure of all rats at the begining of the experiment was 97 ± 5·26 mmHg ($n = 51$). There was no significant difference in the blood pressure of control and indomethacin-treated rats at the beginning and end of the experiments. L-NAME treatment at 5 and 10 mg kg^{-1} caused a significant ($P < 0·01$) increase in mean blood pressure to 136 ± 12 and 142 ± 8 mmHg at the beginning, which 30 min later slowly declined to 123 ± 14 and 128 ± 5 mmHg, respectively. Administration of the NO donor GTN at all doses significantly ($P < 0·01$) decreased mean arterial pressure throughout the infusion period compared with pretreatment values. For example, when GTN infusion was started intravenously at doses of 20 and 80 μg kg^{-1} min^{-1}, mean blood pressure immediately fell from 94 ± 5 and 92 ± 9 mmHg to 58 ± 2 and to 47 ± 3 mmHg, respectively ($P < 0·01$) and remained at this level throughout the infusion period. Similarly, when SNP infusion was started at doses of 10, 30 and 60 μg kg^{-1} min^{-1}, mean blood pressure fell to 45·2 ± 4·8, 45·6 ± 2·3 and 46 ± 4·7 mmHg, respectively ($P < 0·01$). There was no statistical significance between the groups during the infusion period. Upon termination of infusions, blood pressures returned to preinfusion values and gradually declined during the rest of the experiment. At the end of the experiment, mean blood pressure in GTN- and SNP-treated groups was significantly less than the values obtained at the beginning. For example, in the GTN 20 and GTN 80 groups, mean blood pressure values at the end of experiment were 70 ± 15 and 60 ± 5 mmHg, respectively. In the SNP 10, SNP 30, and SNP 60 groups, mean blood pressure values at the end of experiment were 76 ± 4, 61·6 ± 6 and 63·7 ± 8 mmHg, respectively ($P < 0·05$, compared with their corresponding pretreated values).

Table 1 summarizes the changes in lesion index, haematocrit, net transmucosal fluid flux and MPO values in various groups. Although there was an increase in lesion index after indomethacin treatment (2·3 ± 1·7 mm), this was not significantly different from the control group (0·75 ± 0·7 mm). Lesion indices in the control and indomethacin-treated groups were 20 and

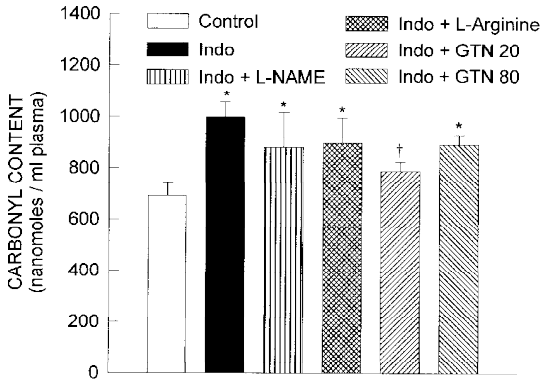

Fig. 1. Content of carbonyl in control groups ($n = 5$) and groups treated with indomethacin (Indo, 10 mg ml^{-1} in 1·25 % $NaHCO_3$ perfusion, $n = 6$) and indomethacin plus L-NAME (5 mg kg^{-1}, $n = 5$), L-arginine (100 mg kg^{-1} bolus, 10 mg kg^{-1} h^{-1} infusion, $n = 5$) and glyceryl trinitrate (GTN 20, 20 μg kg^{-1} min^{-1} $n = 6$; GTN 80, 80 μg kg^{-1} min^{-1}, $n = 4$). $*P < 0{\cdot}05$; compared with control group. $\dagger P < 0{\cdot}05$ compared with indomethacin-treated group. Results are expressed as means ± S.E.M.

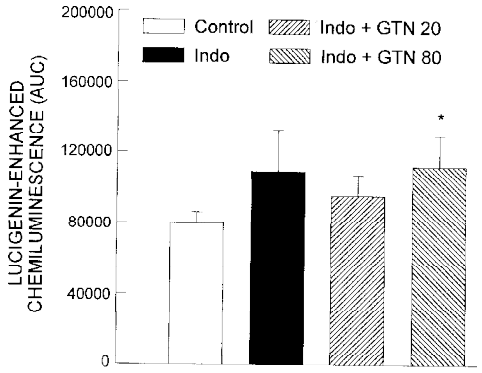

Fig. 2. Lucigenin-enhanced chemiluminescence intensity in control groups ($n = 7$) and groups treated with indomethacin (Indo, $n = 8$) and indomethacin plus glyceryl trinitrate (GTN 20, 20 μg kg^{-1} min^{-1} $n = 7$; GTN 80, 80 μg kg^{-1} min^{-1}, $n = 6$). $*P < 0{\cdot}05$; compared with control group. Results are expressed as means ± S.E.M.

50 %, respectively. Treatment with L-NAME, L- and D-arginine and the NO donors GTN and SNP did not significantly change the lesion index. Lesion indices were $1{\cdot}86 \pm 1$, $0{\cdot}88 \pm 0{\cdot}4$ and $0{\cdot}66 \pm 0{\cdot}6$ mm in the SNP 10, SNP 30 and SNP 60 groups, respectively. Similarly, haematocrit values did not differ among the groups, indicating that no significant haemoconcentration or bleeding developed following this type of experimental procedure for inducing gastric injury. However, the ability of the stomach to absorb fluid was significantly reduced and there was net fluid loss into the lumen after perfusion with indomethacin or indomethacin plus L-NAME, L-arginine and SNP. Net fluid flux values were $-1{\cdot}02 \pm 0{\cdot}55$, $-0{\cdot}83 \pm 0{\cdot}35$ and $0{\cdot}05 \pm 0{\cdot}5$ ml min^{-1} (100 g)$^{-1}$ in SNP 10, SNP 30 and SNP 60 groups, respectively (not significantly different from indomethacin alone). Moreover, impaired water absorption was significantly restored after infusion with 20 mg kg^{-1} min^{-1} GTN ($P < 0{\cdot}05$).

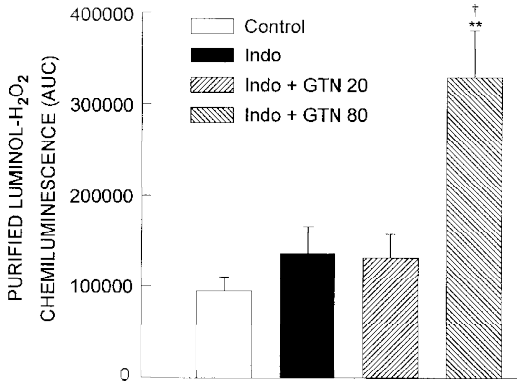

Fig. 3. Changes in tissue luminol–H_2O_2 chemiluminescence intensity induced by indomethacin in animals treated with glyceryl trinitrate (GTN20, 20 μg kg^{-1} min^{-1} $n = 7$; GTN80, 80 μg kg^{-1} min^{-1}, $n = 6$). $^{**}P < 0{\cdot}01$; compared with control group. †$P < 0{\cdot}05$; compared with indomethacin-treated group. Results are expressed as means ± S.E.M.

When the relationship between the net transmucosal water flux and blood-to-lumen clearances of ^{51}Cr-EDTA was investigated, no correlation was found between these parameters ($r = -0{\cdot}55$, slope = $-0{\cdot}14$, n.s. from 0). In terms of granulocyte accumulation into the gastric wall, tissue MPO levels were found to be significantly increased in the indomethacin-treated group compared with controls ($P < 0{\cdot}01$). The elevated MPO levels that were observed in the indomethacin-treated group were significantly reduced in animals which were treated with L-arginine or GTN20 ($P < 0{\cdot}05$). Tissue-associated MPO levels were 370 ± 41, 334 ± 42 and 372 ± 72 units (g wet wt)$^{-1}$ in animals treated with SNP10, SNP30 and SNP60 groups, respectively (not significantly different from indomethacin alone).

Figure 1 illustrates the content of plasma carbonyl groups in control animals and rats treated with indomethacin alone or indomethacin plus L-NAME, L-arginine, D-arginine, GTN20- or GTN80. Carbonyl content significantly increased in the indomethacin group compared with controls ($P < 0{\cdot}05$). GTN20 treatment significantly reduced the elevated carbonyl content observed after indomethacin administration ($P < 0{\cdot}05$). Carbonyl levels were also not different in GTN40- and SNP-treated rats compared with the indomethacin-treated group (data not shown). Plasma levels of TBARS and total sulfhydryls did not significantly differ between any of the groups tested (data not shown).

Figure 2 demonstrates the changes in tissue chemiluminescence intensity in response to administration of indomethacin alone or indomethacin plus various doses of GTN. Lucigenin-enhanced tissue chemiluminescence intensities were significantly increased in animals treated with indomethacin plus GTN80, compared with control values, indicating an enhanced production of reactive oxygen metabolites in the intestinal samples.

Figure 3 illustrates the changes in tissue luminol–H_2O_2 chemiluminescence intensity induced by indomethacin in animals treated with either GTN20 or GTN80. Administration of indomethacin alone or indomethacin plus GTN20 did not change the chemiluminescence values compared with control values. However GTN at a dose of 80 μg kg^{-1} min^{-1} significantly increased luminol–H_2O_2 chemiluminescence intensity compared with other groups. Intensities of lucigenin-enhanced and luminol–H_2O_2 chemiluminescences were not tested in GTN40 and SNP groups.

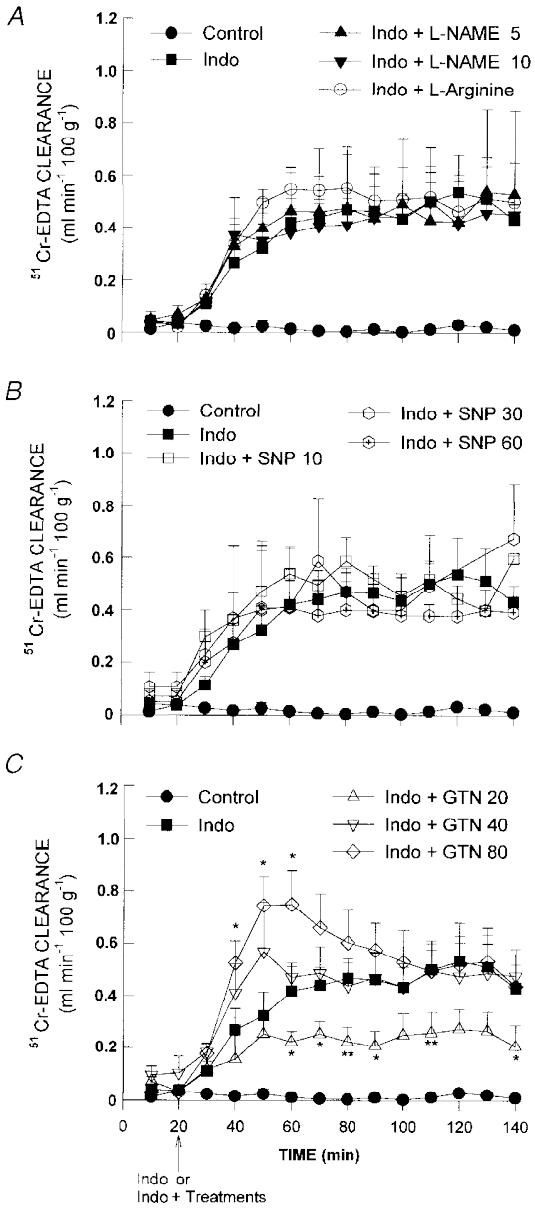

Fig. 4. Mucosal permeability values assessed in control rats (*A*, $n = 5$) and rats treated with indomethacin (Indo, $n = 6$) or indomethacin plus L-NAME (5 mg kg^{-1}, $n = 5$ and 10 mg kg^{-1}, $n = 5$), L-arginine (100 mg kg^{-1} bolus, 10 mg kg^{-1} h^{-1} infusion, $n = 5$) and sodium nitroprusside (SNP10, 10 μg kg^{-1} min^{-1}, $n = 4$; SNP30, 30 μg kg^{-1} min^{-1}, $n = 4$; SNP60, 60 μg kg^{-1} min^{-1}, $n = 4$) (*B*) and glyceryl trinitrate (GTN20, 20 μg kg^{-1} min^{-1}, $n = 7$; GTN40, 40 μg kg^{-1} min^{-1}, $n = 7$; GTN80, 80 μg kg^{-1} min^{-1}, $n = 6$) (*C*). Mucosal permeability increased significantly within 10 min of indomethacin administration ($P < 0{\cdot}05$), reached peak permeability values (~20-fold) at 40 min and remained at this level for the rest of the experiment. A similar result was obtained when ^{51}Cr-EDTA clearance was measured after L-NAME and L-arginine infusions. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$; compared with indomethacin-treated group. Results are expressed as means ± S.E.M.

Figure 4 shows the mucosal permeability values assessed in control groups and groups treated with indomethacin or indomethacin plus L-NAME, L-arginine, SNP and GTN. In control animals, basal ^{51}Cr-EDTA clearance did not change significantly throughout the 2 h measurement period. Indomethacin administration caused a significant mucosal dysfunction such that mucosal permeability increased significantly within 10 min of indomethacin administration ($P < 0{\cdot}05$), reached peak permeability values (~20-fold) at 40 min and remained at this level for the rest of the experiment. A similar result was obtained when ^{51}Cr-EDTA clearance was measured after L-NAME and L-arginine infusions. There was no statistical significance between the groups (Fig. 4*A*). SNP treatment at doses of 10, 30 and 60 μg kg^{-1} min^{-1} did not significantly change the mucosal permeability to ^{51}Cr-EDTA molecule compared with the indomethacin-treated group at all time points (Fig. 4*B*). However, when GTN at the dose of 20 μg kg^{-1} min^{-1} was infused, the elevated permeability values normally observed after indomethacin treatment were significantly reduced ($P < 0{\cdot}05$, $P < 0{\cdot}01$). Yet, higher doses of GTN (80 μg kg^{-1} min^{-1}) significantly exacerbated ^{51}Cr-EDTA clearance for the first 40 min of the experiment when compared with indomethacin group alone ($P < 0{\cdot}05$, Fig. 4*C*).

DISCUSSION

The ability of NSAIDs to increase gastrointestinal epithelial permeability has been well established in human studies as well as in studies employing experimental animal models (Chmaisse *et al*. 1994; Mion *et al*. 1994). Despite extensive research, the pathogenesis of this condition remains poorly understood. In the present study, we have used a rat model of indomethacin-induced gastric injury to test the hypothesis that NO plays a role in the pathogenesis of gastric barrier dysfunction. The results of our study show that perfusion of the stomach with indomethacin at a concentration of 10 mg ml^{-1} dramatically increases (~20-fold) mucosal permeability to radiolabelled EDTA. Furthermore, the NO donor GTN at a dose of 20 μg kg^{-1} min^{-1} significantly inhibits the indomethacin-induced increases in epithelial permeability, while by contrast higher doses of GTN (80 μg kg^{-1} min^{-1}) exacerbate the mucosal dysfunction induced by indomethacin. In terms of macroscopically visible mucosal damage, neither perfusion of the stomach with indomethacin nor treatment with L-NAME, L-arginine, D-arginine, SNP or GTN caused any significant lesions compared with control groups. Using animal models, investigators have previously shown that a reduction in luminal pH is necessary for the exacerbation of indomethacin-induced mucosal injury (Vaananen *et al*. 1991; Chmaisse *et al*. 1994). It has been demonstrated that exposure of the stomach to 100 mM HCl following indomethacin perfusion greatly exacerbates the mucosal injury and causes significant macroscopic lesions, indicating that increased luminal acidity makes indomethacin more toxic and the mucosa more susceptible to injury. However, in the present study, indomethacin dramatically increased gastric mucosal permeability when perfused with 1·25 % sodium bicarbonate at a pH of 8·4 throughout the experiment, indicating that even at high luminal pH levels indomethacin is still able to distrupt mucosal integrity. The lack of correlation between lesion indices and permeability measurements indicates that gastric morphology is not severely disrupted by indomethacin before epithelial permeability is increased.

In an attempt to determine whether reactive metabolites of oxygen contribute to the pathogenesis of indomethacin-induced mucosal dysfunction, in our experimental model, we have determined the plasma levels of carbonyls (as an indicator of protein oxidation), TBARS

(lipid peroxidation) and total sulfhydryl groups at the end of the perfusion period. Although we did not observe any significant changes in the levels of TBARS and sulfhydryls between the groups, indomethacin perfusion significantly increased carbonyl formation, indicating an oxidative modification of plasma proteins which is known to alter their function and turnover (Oliver *et al*. 1987). Our results are in agreement with a previously published report demonstrating that tissue levels of carbonyls, but not glutathione or TBARS, increase after indomethacin administration (Alican *et al*. 1995). The elevated plasma carbonyl content in the indomethacin-treated group also suggests that oxidation of these proteins may be an early component of the sequence of events leading to tissue damage. The finding that carbonyl formation and MPO levels increase in the indomethacin-treated group, compared with controls, is consistent with the hypothesis that PMNs may be an important source of reactive oxygen metabolites. In order to further demonstrate the enhanced oxygen radical production, we determined lucigenin-enhanced chemiluminescence in tissue samples after indomethacin administration. The lucigenin-dependent chemiluminescence assay has often been used to study the oxidative process, in particular the generation of superoxide (Van Dyke, 1987). Although chemiluminescence intensity was not significantly different from control values when indomethacin was applied alone, addition of GTN at a dose of $80\,\mu g\,kg^{-1}\,min^{-1}$ significantly increased chemiluminescence intensity compared with controls, suggesting increased superoxide production after GTN administration.

In the present study, we have shown that administration of L-NAME had no effect on indomethacin-induced mucosal dysfunction. It has been previously shown that L-NAME treatment alone causes significant mucosal dysfunction in both stomach and intestine (Kubes, 1993; Coskun *et al*. 1996). The observation that L-NAME has no additive effect on indomethacin-induced mucosal dysfunction supports the contention that perhaps same inflammatory mechanisms are involved. It has been suggested that L-NAME may have a direct effect on interepithelial junctions or may cause the activation of inflammatory cells (e.g. mast cells, PMNs) and the release of reactive oxygen metabolites (Kanwar *et al*. 1994*b*). Previous studies showing that superoxide dismutase (SOD) significantly reduced the increase in mucosal permeability and the extent of macroscopically visible mucosal damage induced by indomethacin imply that reactive oxygen metabolites are also involved in this process (Vaananen *et al*. 1991). Thus, it is conceivable that the progressive increase in superoxide generation after indomethacin administration could inactivate NO production, an event that could then disrupt mucosal integrity. A profound reduction in NO levels could explain why L-NAME failed to exhibit an effect in indomethacin-induced mucosal dysfunction. Alternatively, inactivation of NO synthase may explain the lack of effect of L-NAME on epithelial permeability. However it is not clear whether indomethacin inactivates calcium-dependent NO synthase or damages the NO synthase-containing cells. Another possible explanation for the reduced epithelial effects of L-NAME may be that tissue levels of L-arginine, the substrate for NO synthase may also be reduced after indomethacin administration. However, the fact that exogenous administration of L-arginine did not affect the mucosal dysfunction induced by indomethacin suggests that it is an unlikely explanation. Recent studies employing the measurement of tissue L-arginine levels in the intestine have demonstrated that production of NO does not appear to be substrate limited since L-arginine levels do not change in the intestine before and after ischaemia–reperfusion, which is known to alter NO levels (Kubes, 1993). Other investigators have implicated NO-independent effects of L-arginine by demonstrating that L-arginine but not D-arginine reduces gastric blood flow and this effect cannot be inhibited by the NO inhibitor, L-NAME (Jose-Geraldo *et al*. 1994). Although it seems in the present study that the

inhibitory effect of L-arginine on elevated MPO levels after indomethacin administration is a NO-dependent effect (since D-arginine did not exert a similar response), alternative mechanisms should also be considered.

Another objective of this study was to examine the effects of nitrovasodilator agents that release NO, either following metabolic transformation, as with GTN, or spontaneously, as with SNP, on indomethacin-induced gastric mucosal dysfunction (Ignarro *et al*. 1981). Our results show that GTN at a dose of 20 μg kg^{-1} min^{-1} significantly reduces the elevated epithelial permeability observed after indomethacin administration. These findings are consistent with the observations that derivatives of NSAIDs, containing a moiety that generates NO, reduce the gastric damage score (Reuter *et al*. 1994). The mucosal injury induced by oral administration of aspirin has also been shown to be reduced by the NO-releasing aspirin derivative nitroxybutylester (Wallace *et al*. 1995). Administration of NO or NO donors likewise could protect the mucosa against damage induced by ethanol (McNaughton *et al*. 1989), or acid (Kitagawa *et al*. 1990). In contrast to the effects of GTN 20, infusion of SNP at doses of 10, 30, 60 μg kg^{-1} min^{-1} did not alter the elevated epithelial permeability observed after indomethacin administration. This discrepancy between the effects of the two NO donors cannot be explained by hypotensive effects since both agents produced comparable falls in systemic blood pressure. Although it is generally accepted that organic (GTN) and inorganic (SNP) nitrates share a final common pathway with NO, important differences exist in the biotransformation of these compounds. For example, a concommitant local release of cyanide from SNP is needed before NO can be released, whereas with GTN enzymatic activation involving sulfhydryls is required for the release of NO (Anderson *et al*. 1994). It is possible that the different effects of NO donors used in this experimental model may be due to the type of nitrate preparation used, the local concentrations that are produced at the site of inflammation or even the sensitivity of the vascular bed (arteriolar or venous) to these compounds.

The mechanisms by which NO donors decrease mucosal dysfunction remain unclear but several possibilities have been proposed including improved perfusion of the tissues due to vasodilatation, inactivation of superoxide and inhibition of platelet or PMN adherence (Kubes, 1992). Our observation that administration of GTN significantly reduces the indomethacin-induced elevations in PMN accumulation supports the contention that these agents interfere with the infiltration of PMN and production of reactive oxygen metabolites. However, it is not entirely clear to what extent PMN accumulation contributes to indomethacin-induced permeability changes. The observation that L-arginine treatment does not alter the changes in mucosal permeability induced by indomethacin, while significantly decreasing the elevated levels of MPO, suggests that epithelial permeability is not entirely associated with the increased number of PMNs in the gastric wall. The data from a recent report by Kubes (1992) reveal that PMNs play a critical role in microvascular but not mucosal dysfunction. This is based on the fact that pretreatment with an anti-CD18 antibody, which prevents leukocyte adhesion to postcapillary venules, significantly prevents the increase in microvascular permeability but does not affect the rise in mucosal permeability.

It is conceivable that fluid secretion into the lumen associated with increased interstitial fluid pressure as a result of vascular dysfunction may promote a leaky mucosal barrier. Indeed, the ability of the gastric mucosa to absorb water was significantly impaired and there was net fluid secretion into the gastric lumen after indomethacin administration. Moreover GTN (20 μg kg^{-1} min^{-1}) treatment prevented fluid loss into the lumen, suggesting that the beneficial effects of the NO donor on epithelial permeability may be related to decreased fluid loss into

the lumen. However, the lack of correlation between transmucosal fluid flux and permeability measurements argues against this possibility and suggests that alternative mechanisms must also be considered. It is also noteworthy that the beneficial effects of GTN on epithelial permeability occur 40 min after its administration and indomethacin-induced mucosal dysfunction is not entirely prevented, implying that a therapeutic increase in NO levels during indomethacin administration does not interfere with the events that are responsible for the early rise in epithelial permeability. These findings, coupled with the observation that indomethacin-induced permeability changes occur within minutes, suggest that at least some of the alterations in mucosal integrity caused by indomethacin can be attributed to its direct local toxicity. Indomethacin is known from *in vitro* studies to be one of the NSAIDs most toxic to cultured epithelial cells (Allen *et al*. 1991). It has been also shown that indomethacin can change the cellular synthesis of adenosine triphosphate through the anaerobic glycolytic pathway, a process which is known to increase epithelial tight junction permeability (Duffey *et al*. 1981; Kurtel *et al*. 1992). Further investigations are required to explain the involvement of these mechanisms in the early permeability changes induced by indomethacin.

In the present study, GTN at a dose of 80 μg kg^{-1} min^{-1} exacerbated epithelial dysfunction induced by indomethacin while producing a hypotensive effect on systemic arterial pressure that was comparable to the effect produced by GTN20, suggesting that the hypotensive effect on systemic arterial pressure does not appear to be the primary mechanism by which the alterations in epithelial permeability are initiated. These results are consistent with the data of Lopez-Belmonte *et al*. (1993). These authors have demonstrated that exogenous NO can protect the rat gastric mucosa from damage induced by the vasoconstrictor peptide endothelin-1, but the unregulated release of high levels of NO within the microvasculature induces mucosal injury. It has also been suggested that release of large amounts of NO from the donor drugs causes mucosal injury because of the cytotoxic properties of the NO radical or the peroxynitrite radical that has been shown to be formed through the interaction of NO with superoxide (Beckman *et al*. 1990). In order to test this hypothesis we aimed to detect tissue NO concentrations by chemiluminescence measurements based on a luminol–H_2O_2 system. Kikuchi *et al*. (1993) recently demonstrated that NO reacts with H_2O_2 in the aqueous phase giving peroxynitrate, a potent reagent for luminol chemiluminescence at both alkaline and neutral pH. In this reaction system, peroxynitrate produced in tissue samples cannot be distinguished from NO because the chemiluminescence signal derives both from the conversion of NO to peroxynitrate by H_2O_2 and from peroxynitrate already produced. Our results suggest that GTN administration at a dose of 80 μg kg^{-1} min^{-1} significantly increases chemiluminescence intensity compared with other groups, indicating enhanced formation of NO. These findings, together with our data on the increased superoxide production at this dose of GTN, suggest that at least some of the peroxynitrate measured in this system is produced by GTN. Thus, our data suggest that NO derived from exogenous sources can exert a dual action on the integrity of the gastric mucosa challenged by indomethacin. Low doses of the NO donor GTN can prevent mucosal dysfunction induced by indomethacin; by contrast, higher doses of GTN may provoke the indomethacin-induced increases in epithelial permeability.

In summary, the results of the present study support the contention that the integrity of the gastric epithelial layer, as assessed by ^{51}Cr-EDTA clearance, is extremely sensitive to indomethacin administration. Present results also demonstrate that luminal acidity and macroscopically visible lesions are not a prerequisite for the indomethacin-induced alterations in mucosal dysfunction. Moreover, our results have shown for the first time that permeability

changes induced by indomethacin administration can be inhibited by a NO donor, GTN, at low doses, but that higher doses of GTN can exacerbate the mucosal injury. These results support the contention that NO donors might be effective in the treatment of conditions characterized by epithelial dyfunction, but also underscore the care that must be taken in evaluating the dosage of the exogenous sources of NO.

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