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Nitric oxide enhances prostaglandin production in ethanol-induced gastric mucosal injury in rats

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Abstract

The interaction between endogenous nitric oxide (NO), elicited by administration of *Escherichia coli* lipopolysaccharide, and cyclooxygenase system, in ethanol-induced injury in rat gastric mucosa, was investigated. Administration of graded doses of lipopolysaccharide reduced the gastric mucosal injury in response to ethanol. The ex vivo production of both nitrite and prostaglandin E_2 was increased in dose-related manner by lipopolysaccharide. Pretreatment with dexamethasone, L-N^6-(1-Iminoethyl)lysine(dihydrochloride) and L-N^G-nitro arginine methyl ester inhibited the protection associated with lipopolysaccharide treatment and the ex vivo production of both, nitrite and prostaglandin E_2. The pretreatment with L-arginine counteracted the decrease of nitrite and prostaglandin E_2 production in lipopolysaccharide-treated rats in which nitric oxide synthesis was blocked by L-N^6-(1-Iminoethyl)lysine(dihydrochloride). Administration of sodium nitroprusside and *S*-nitroso-*N*-acetyl-D,L-penicillamine caused a dose related enhancement in the accumulation of prostaglandin E_2. Indomethacin administration and *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide were ineffective in suppressing lipopolysaccharide-mediated protection against ethanol-induced damage, and in suppressing ex vivo increase of nitrite whereas the ex vivo increase of prostaglandin E_2 was prevented in a dose-related fashion. These results indicate that in ethanol-induced rat gastric injury, endogenous NO elicited by lipopolysaccharide or released by NO donors is able to activate the cyclooxygenase pathway, and the protective effect of lipopolysaccharide is dependent upon NO formation.

Keywords: Nitric oxide (NO); Prostaglandin E_2; Gastroprotection; Ethanol; Lipopolysaccharide

1. Introduction

Nitric oxide is formed from the terminal guanidine nitrogen atom of L-arginine by NO synthase in a number of tissues, including the gastric mucosa (Moncada et al., 1991; Brown et al., 1992). Three major isoforms have now been isolated, purified, cloned and expressed. The constitutive Ca^{2+}-dependent NO synthase isoform is present in endothelium and some neurones. Bacterial lipopolysaccharide or cytokines induce macrophages, vascular smooth muscle and other cells to express a different isoform of NO synthase (Moncada et al., 1991). NO formed by the constitutive enzyme plays an important role in the modulation of gastric mucosa integrity by interacting with sensory neuropeptide and endogenous prostaglandins (Whittle et al., 1990). The formation of a large amounts of NO by the inducible isoform of NO synthase accounts for the cytotoxicity of activated macrophages and is considered damaging to the gastrointestinal mucosa (Brown et al., 1994).

Prostaglandins, a family of mediators, have numerous effects. Cyclooxygenase, the first enzyme in the pathway of prostaglandin and other eicosanoids formation from arachidonic acid, also exists in both constitutive (cyclooxygenase-1) and cytokine/lipopolysaccharide-inducible (cyclooxygenase-2) isoforms (Fu et al., 1990; Xie et al., 1991). The enzymatic activity of both isoforms is inhibited by aspirin and other nonsteroidal antiinflammatory drugs (Meade et al., 1993). The gastric side effects associated with intake of these drugs in humans is thought to be related to inhibition of cyclooxygenase-1-derived prostanoids. It is now accepted that the constitutively expressed enzyme, cyclooxygenase-1, is involved in producing prostaglandins for cellular 'housekeeping' functions, including gastric cytoprotection; in contrast the inducible enzyme, cyclooxygenase-2, is thought to be involved in inflammation, cellular differentiation, and mito-

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genesis (DeWitt, 1991). Inducible NO synthase and cyclooxygenase-2 may co-exist in some cells after exposure to lipopolysaccharide or cytokines (Szabo et al., 1993; Corbett et al., 1993). It has been reported that NO activates the constitutive and inducible forms of cyclooxygenase leading to an augmented production of cyclooxygenase metabolites (Salvemini et al., 1993). Others have reported that nitric oxide reduce the release of prostanoids (Kanner et al., 1992; Stadler et al., 1993). However, the effects of NO on the cyclooxygenase pathway have not been fully clarified.

This study was designed to investigate in rat gastric mucosa after ethanol-induced damage, the potential interactions between NO synthase and cyclooxygenase. For this purpose, we have induced inducible NO synthase activity by administration of lipopolysaccharide and evaluated nitrite, a breakdown product of NO, and prostaglandin E_2 accumulation in ex vivo gastric mucosa. The animals were pretreated with inhibitors of NO synthase or inhibitors of prostaglandins synthesis before intragastric administration of ethanol and examined the effects on gastric mucosa. We also assessed the effects of NO donors on prostaglandin E_2 production in lipopolysaccharide-treated animals.

2. Materials and methods

2.1. Animal preparation

Interaction between the NO synthase and cyclooxygenase systems were investigated in male Sprague–Dawley rats (Charles River, Italy), weighing 220–240 g, which had been fasted for 24 h before the experiment with free access to water. Groups of rats were pretreated i.p. with graded doses of bacterial lipopolysaccharide from *Escherichia coli* (serotype 0111:B4, 1–3 mg kg^{-1} body weight); control rats received an equal volume of the saline. Gastric lesions were caused by intragastric instillation of 1.5 ml 95% ethanol 5 h after lipopolysaccharide administration. Other groups of rats were pretreated with lipopolysaccharide (3 mg kg^{-1} body weight) and the gastric lesions were caused at different time interval. Five min after the instillation of ethanol the animals were killed by inhalation of ether. The stomachs were quickly removed, opened along the greater curvature, and examined for lesions. A lesion index (l.i.) of gross mucosal damage was determined using a scoring system based on the number and length of haemorrhagic mucosal necrosis. The number of necrotic bands of more than 4 mm in length was multiplied by a factor of 3, lesions of 2–4 mm were multiplied by a factor of 2, and lesions of less than 2 mm by a factor of 1 (Franco, 1995).

Fragments of rat gastric mucosa treated with lipopolysaccharide were carefully stripped, washed with ice-cold phosphate buffer, 0.1 M, pH 7.4 (PBS) and centrifuged at $3000 \times g$ at 4°C for 3 min. The PBS was replaced with 1 ml of fresh solution of Dulbecco's Modified Eagle's Medium (DMEM) and 1 mM EGTA, a calcium chelator, the samples were incubated for 5 h at 37°C under 5% CO_2 and 95% O_2. This fluid, which contained the released eicosanoids and NO, was removed for assay and the gastric mucosa was weighed.

Other groups of rats were treated by gavage (p.o.) with the selective inducible NO synthase inhibitor, L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (3–10–30 mg kg^{-1} body weight) 1 h prior to lipopolysaccharide treatment (3 mg kg^{-1} body weight) or L-N^G-nitro arginine methyl ester (10 mg kg^{-1} body weight s.c.). L-N^6-(1-Iminoethyl)lysine(dihydrochloride) is approximately 30 fold more selective for the inducible than for the constitutive form of NO synthase (Salvemini et al., 1995). To confirm its specificity a group of rats was also treated with L-arginine (300 mg kg^{-1} body weight s.c.) contemporaneously to L-N^6-(1-Iminoethyl)lysine(dihydrochloride). In a separate groups of experiment, rat were pretreated, 1 h before lipopolysaccharide treatment, with dexamethasone (2–5 mg kg^{-1} body weight s.c.), indomethacin (2–5–10 mg kg^{-1} body weight s.c.) and with selective cyclooxygenase-2 inhibitor, *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide (5–10 mg kg^{-1} body weight p.o.). Indomethacin (10 mg kg^{-1} body weight s.c.) was also administered 3 h before or contemporaneously to lipopolysaccharide-treatment. Gastric lesions were caused by intragastric instillation of 1.5 ml 95% ethanol 1 h and 5 h after lipopolysaccharide administration and after 5 min the animals were killed by inhalation of ether. In order to evaluated the role of exogenous nitric oxide on in vivo prostaglandin formation two nitric oxide donors namely sodium nitroprusside (5–10 mg kg^{-1} body weight) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (2–5 mg kg^{-1} body weight) were administered orally 1 h prior to lipopolysaccharide and 1 h prior to killing. We also studied the effects of exogenous NO, evoked by sodium nitroprusside (10 mg kg^{-1} body weight) in lipopolysaccharide-treated animals in which NO production was blocked by L-N^G-nitro arginine methyl ester (10 mg kg^{-1} body weight).

2.2. Mediators measured to assess the presence of the NO synthase and cyclooxygenase system

The production of nitrite, breakdown products of NO, or prostaglandin E_2, mediator released after activation of cyclooxygenase, were used, respectively as markers for the presence of the L-arginine to NO pathway and the cyclooxygenase pathway.

Nitrite accumulation in the culture supernatant was determined by first reducing the NO_3^- using a bacterial nitrate reductase prepared from E. Coli. Nitrite was assayed colorimetrically after reaction with the Griess reagent (Gross et al., 1991). The absorbance was evaluated with a microplate reader (Argus 400, Canberra Packard) at 550 nm (OD_{550}). All determinations were performed in dupli-

cate. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium. Results are expressed as μM nitrite.

2.3. Prostaglandin E_2 determinations

Cyclooxygenase activity was determined by measuring prostaglandin E_2 in the unextracted medium by enzyme-immunoassay (Cayman Chemicals, Ann Arbor, MI, USA) expressed as ng prostaglandin E_2/g of tissue per 5 h. Each experiment was performed in duplicate.

2.4. Western blot analysis

In a separate study we also examined the protein expression of cyclooxygenase 2 and inducible nitric oxide synthase. Gastric lesions were caused by intragastric instillation of ethanol 5 h after lipopolysaccharide (3 mg kg^{-1} body weight i.p.) administration. Other groups of rats were pretreated, 1 h before lipopolysaccharide, with dexamethasone (5 mg kg^{-1} body weight s.c.) and indomethacin (10 mg kg^{-1} body weight s.c.). Five min after the instillation of ethanol fragments of gastric mucosa were stripped and after incubation for 5 h the gastric biopsy were homogenized in a cold lysis buffer containing Tris 50 mM, pH 8; ethylenediamine tetraacetic acid 10 mM; Triton-X 100 1%; Leupeptin 20 μg/ml. Lysates were centrifuged at 4000 $\times$ *g* for 20 min at 4°C, then supernatants with equal amounts of protein were subjected to electrophoresis (125 V for 1.5 h with a Mini-Protean II Electrophoresis System by Biorad) on 10% (for cyclooxygenase 2) or on 7.5% (for inducible nitric oxide synthase) sodium dodecyl-sulphate-polyacrylamide gel. The separated proteins were transferred to nitrocellulose (100 V for 1.5–2 h). The membrane was blocked overnight with non-fat dry milk 5%, NaCl 0.9%, Tween-20 0.1% and then incubated with the primary antibody, prostaglandin H synthase 2 polyclonal antiserum or with inducible nitric oxide synthase polyclonal antiserum (1:1000 dilution) for 1 h. After three 15-min washes the blot was incubated (45 min) with biotinylated goat anti-rabbit immunoglobulins, (1:3000 dilution). After three 15-min washes the blot was incubated for 45 min with streptavidin–alkaline phosphatase conjugate (dilution 1:3000). The blot was developed with a premixed solution (3.75 mg 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in 250 μl *N,N*-dimethylformamide and 7.5 mg nitro blue tetrazolium in 250 μl *N,N*-dimethylformamide 70%, both in 25 ml of Tris 100 mM. $MgCl_2$ 50 mM, NaCl 150 mM, pH 9.5).

2.5. Drugs and chemicals

L-N^6-(1-Iminoethyl)-lysine (dihydrochloride), *S*-nitroso-*N*-acetyl-D,L-penicillamine, prostaglandin H synthase 2 polyclonal antiserum, inducible nitric oxide synthase polyclonal antiserum and *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide were obtained from Cayman Chemical. Biotinylated goat anti-rabbit immunoglobulins was obtained from Dako (Denmark). Streptavidin-alkaline phosphatase conjugate was obtained from Amersham International (England). Nitrocellulose BA85 was obtained from Schleicher and Schuell (Germany). All other compounds were from Sigma (St. Louis, MO, USA).

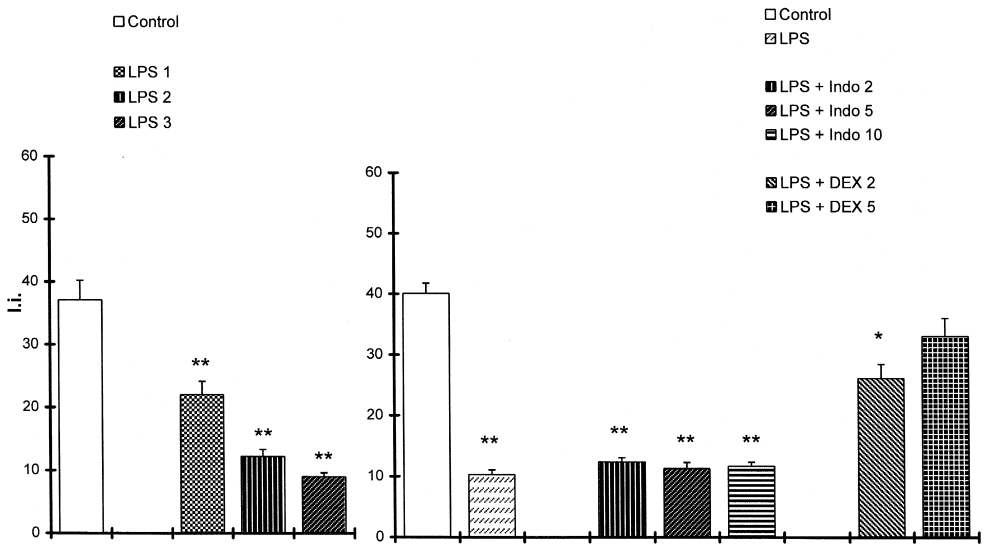

Fig. 1. Effect of lipopolysaccharide on ethanol-induced gastric mucosal damage in rats. lipopolysaccharide (1–2–3 mg kg^{-1}) was injected intraperitoneally 5 h before intragastric administration of 95% ethanol (1.5 ml) (left panel). Indomethacin (2–5–10 mg kg^{-1} s.c.) or dexamethasone (2–5 mg kg^{-1} s.c.) were administered 60 min before endotoxin (3 mg kg^{-1} i.p.) (right panel). Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and examined for lesions. Each column represents the mean $\pm$ S.E.M. no. 9–10 per group. Data were analyzed using Mann–Whitney *U*-test. $*$ $P < 0.01$; $**$ $P < 0.001$ significantly different from control rats receiving only ethanol.

Table 1
Time-course of lipopolysaccharide protection, NO_2^- and PGE_2 production in the rat gastric stomach

Treatment	lesion index	NO_2^- (μM)	PGE_2 (ng/g of tissue)
Control	41.3 ± 4.2	5.5 ± 1.7	358.2 ± 19.7
LPS 1 h	40.3 ± 3.9	7.1 ± 1.4	381.1 ± 17.4
LPS 3 h	32.6 ± 2.6	43.2 ± 2.7[a]	427.3 ± 22.5
LPS 5 h	7.4 ± 1.4[a]	120.7 ± 6.1[a]	715.8 ± 39.3[a]
LPS 24 h	8.4 ± 1.7[a]	110.8 ± 7.1[a]	680.2 ± 34.8[a]
LPS 48 h	23.1 ± 2.4[a]	90.2 ± 5.2[a]	480.6 ± 27.8[b]
LPS 72 h	31.9 ± 3.1	38.2 ± 2.7[a]	390.5 ± 22.4

Lipopolysaccharide (LPS) 3 mg kg^{-1} was injected intraperitoneally at different time before intragastric administration of 95% ethanol. Five minutes after ethanol, the stomach was removed and opened along the great curvature and examined for lesions and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate accumulation of NO_2^- by Griess method and PGE_2 accumulation by EIA immunoassay. Each point is the mean ± S.E.M. for no. 6 animals. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. [b]$P < 0.01$; [a]$P < 0.001$ significantly different from control group.

Indomethacin and *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide were dissolved in phosphate-buffered saline, 1% Tween 80, pH 7.4; all other compounds were dissolved in saline.

2.6. Statistical analysis

Statistical significance of difference between groups of parametric data was determined by one-way analysis of variance followed by Bonferroni *t*-test. Comparisons between groups of non-parametric data (lesion index) were made by the Mann–Whittney *U*-test. A probability (P) value of 0.05 or less was taken to indicate statistical significance. Results are expressed as mean ± S.E.M.

3. Results

Intragastric administration of 95% ethanol caused macroscopic hemorrhagic damage. Treatment of rats with graded doses of lipopolysaccharide resulted in a reduction of the damage associated with ethanol administration (Fig. 1, left panel). In the absence of ethanol, lipopolysaccharide administration was not associated with hemorrhagic damage when the gastric mucosa was examined 5 h after endotoxin injection (data not shown). The gastroprotective effect of lipopolysaccharide (3 mg kg^{-1}) on ethanol-mediated damage was not evident when determined 1 and 3 h after lipopolysaccharide injection. However, 5 and 24 h after, when there is a near maximal induction of NO, a significant increase in the protection was observed. This effect determined over 48 h was not significant different from control and gradually returns to control value (Table 1).

Dexamethasone administration, resulted in an increase in the degree of mucosal haemorrhage damage in 5 h post-lipopolysaccharide treated rats, while the gastroprotective effect of lipopolysaccharide was not significantly affected by pretreatment of rats with graded doses of indomethacin (2–5–10 mg kg^{-1}) 60 min before administration of lipopolysaccharide (Fig. 1, right panel) or at the dose of 10 mg kg^{-1} administered 3 h before or contemporaneously to the injection of lipopolysaccharide (Table 2). When ethanol was administered only 60 min after the contemporaneously administration of lipopolysaccharide and indomethacin, we observed a lack of protection suggesting that NO is involves in the protective actions of endotoxin.

Under our conditions, pretreatment with graded doses of L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (3–10–30 mg kg^{-1}) 60 min before administration of lipopolysaccharide, resulted in a dose-dependent increase in the degree of mucosal damage, restoring the mucosal damage of 95% ethanol on the gastric mucosa. This effect of L-N^6-(1-Iminoethyl)lysine(dihydrochloride) was prevented by contemporaneously s.c. administration of L-arginine (300 mg kg^{-1}). Similarly pretreatment with L-N^G-nitro arginine methyl ester (10 mg kg^{-1}) re-established the effect of 95% ethanol on gastric mucosa of animals treated with endotoxin (3 mg kg^{-1}) (Fig. 2).

The ex vivo production of nitrite in the rats gastric mucosa incubated for 5 h was increased in dose-dependent

Table 2
Effects of indomethacin, on ethanol-induced gastric damage and on level of NO_2^- and PGE_2

Treatment	h before ethanol	lesion index	NO_2^- (μM)	PGE_2 (ng/g of tissue)
LPS	5	9.5 ± 1.3	108.5 ± 6.2	671.3 ± 38.8
Indomethacin 3 h before LPS	5	11.1 ± 2.0	101.7 ± 5.8	320.2 ± 33.2[a]
Indomethacin contemporaneously LPS	5	9.3 ± 1.8	97.3 ± 4.4	275.7 ± 27.5[a]
Indomethacin contemporaneously LPS	1	45.2 ± 4.1[a]	5.7 ± 0.9[a]	58.3 ± 6.2[a]

Lipopolysaccharide (LPS) 3 mg kg^{-1} was injected intraperitoneally either 5 h or 1 h before intragastric administration of 95% ethanol. Indomethacin (10 mg kg^{-1} s.c.) was administered 3 h before or contemporaneously to the injection of endotoxin. Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate NO_2^- accumulation by Greiss method and PGE_2 accumulation by EIA immunoassay. Each point is the mean ± S.E.M. for no. 6–7 animals. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. [a]$P < 0.001$ significantly different from lipopolysaccharide treated group.

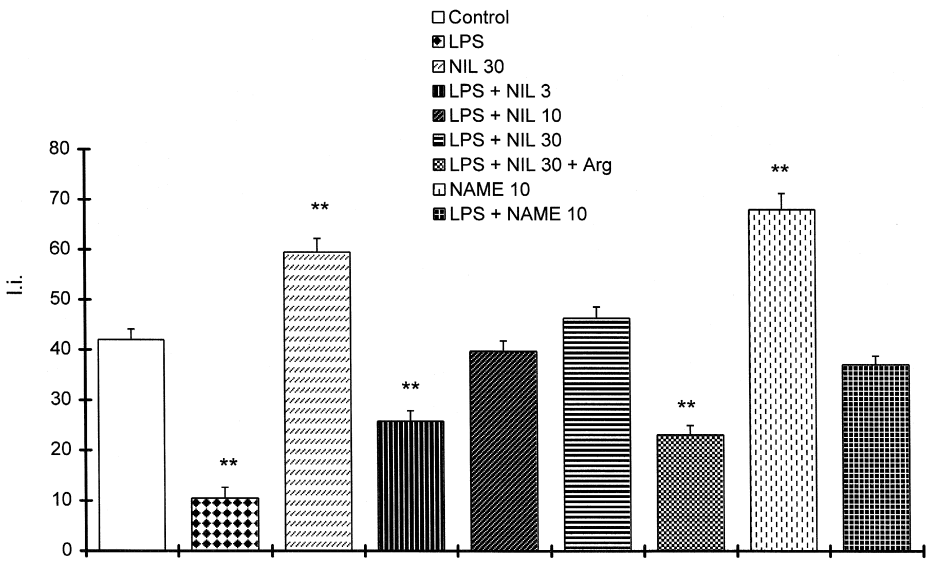

Fig. 2. Effect of lipopolysaccharide (3 mg kg^{-1} i.p.) on ethanol induced gastric damage in rats. L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (NIL; 3–10–30 mg kg^{-1} p.o.) or L-N^G-nitro arginine methyl ester (NAME; 10 mg kg^{-1} s.c.) were administered 60 min before endotoxin. L-arginine (300 mg kg^{-1} s.c.) was administered contemporaneously to L-N^6-(1-Iminoethyl)lysine(dihydrochloride). 5 h after rats were challenge with intragastric 95% ethanol (1.5 ml). The stomachs were excised 5 min later and evaluated for lesions. Each column represents the mean ± S.E.M. no. 8–10 per group. Data were analyzed using Mann–Whitney *U*-test. ∗∗ $P < 0.001$ significantly different from control rats receiving only ethanol.

manner in lipopolysaccharide treated rats. Indomethacin did not affect the increase of nitrite in the gastric mucosa after i.p. administration of 3 mg kg^{-1} lipopolysaccharide. When animals were treated with dexamethasone, the drug induced a significant dose-related inhibition of lipopolysaccharide-stimulated NO release (Fig. 3).

As shown the Fig. 4 the magnitude of the nitrite increase was reduced in animals pretreated with L-N^6-(1-

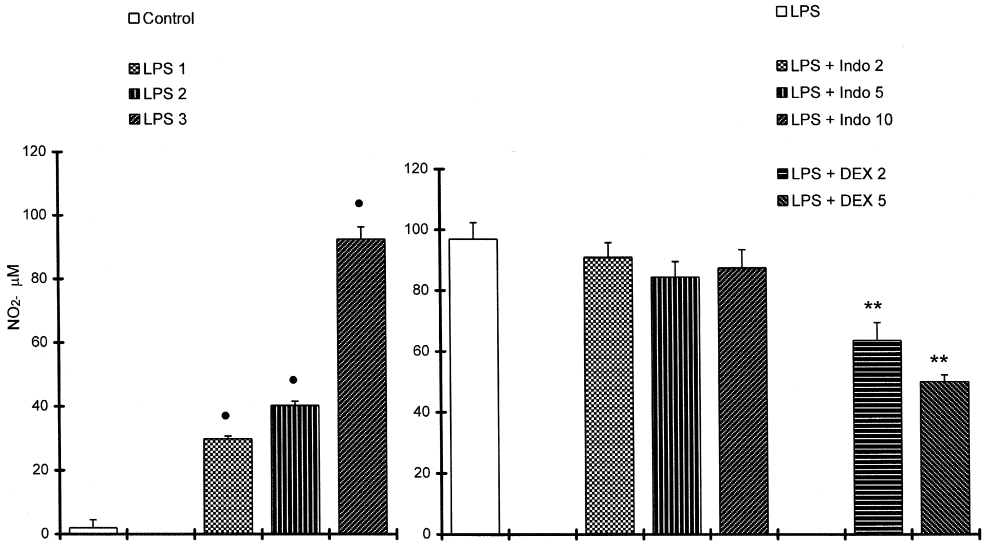

Fig. 3. Level of nitrite in the ex vivo gastric mucosa incubated in DMEM and 1 mM EGTA. Lipopolysaccharide (1–2–3 mg kg^{-1}) was injected intraperitoneally 5 h before intragastric administration of 95% ethanol (1.5 ml) (left panel). Indomethacin (2–5–10 mg kg^{-1} s.c.) or dexamethasone (2–5 mg kg^{-1} s.c.) were administered 60 min before endotoxin (3 mg kg^{-1} i.p.) (right panel). Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate nitrite accumulation by Greiss method. Each column represents the mean ± S.E.M. no. 9–10 per group. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. ∗∗ $P < 0.001$ significantly different from control lipopolysaccharide 3 mg kg^{-1} treated animals. ● $P < 0.001$ significantly different from control rats receiving only ethanol.

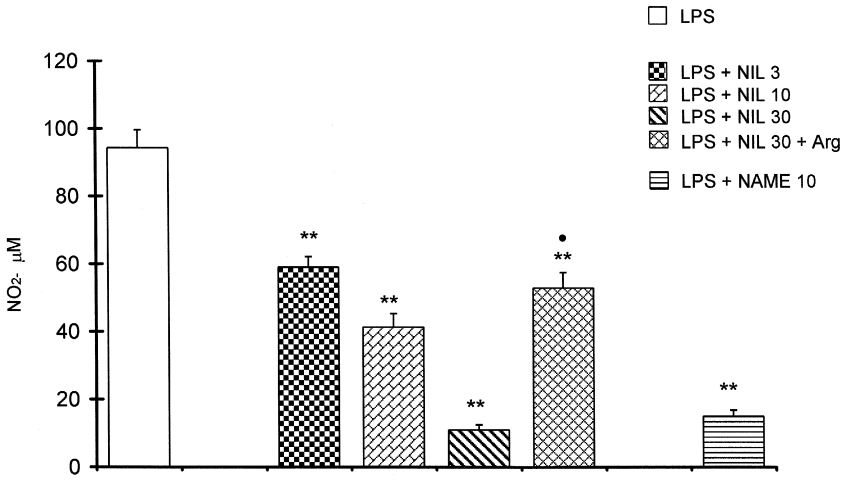

Fig. 4. Level of nitrite in the ex vivo gastric mucosa incubated in DMEM and 1 mM EGTA. Lipopolysaccharide (3 mg kg^{-1} i.p) was injected 5 h before intragastric administration of 95% ethanol. L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (NIL; 3–10–30 mg kg^{-1} p.o.) or L-N^G-nitro arginine methyl ester (NAME; 10 mg kg^{-1} s.c.) were administered 60 min before endotoxin. L-arginine (300 mg kg^{-1} s.c.) was administered contemporaneously to L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (30 mg kg^{-1}). Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate nitrite accumulation by Greiss method. Each column represents the mean ± S.E.M. no. 8–10 per group. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. $**$ $P < 0.001$ significantly different from control lipopolysaccharide 3 mg kg^{-1} treated animals. ● $P < 0.001$ significantly different from lipopolysaccharide plus NIL 30 treated group.

Iminoethyl)lysine(dihydrochloride) (3–10–30 mg kg^{-1}) in a dose related manner and in L-N^G-nitro arginine methyl ester group. Concurrent administration of L-arginine to animals receiving L-N^6-(1-Iminoethyl)lysine(dihydrochloride) prevented the decrease of nitrite. No significant changes in accumulation of nitrite in non lipopolysaccharide-treated animals (6.3 ± 1.2 μM) were observed after L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (30 mg kg^{-1}: 5.7 ± 0.9 μM) and after dexamethasone (5 mg kg^{-1}: 6.1 ± 1.4 μM).

An increase in prostaglandin E_2 accumulation in incubates of gastric mucosal fragments from lipopolysaccharide-treated animals was observed 5 h post-lipopolysaccharide injection (Fig. 5, left panel). Pretreatment with non-

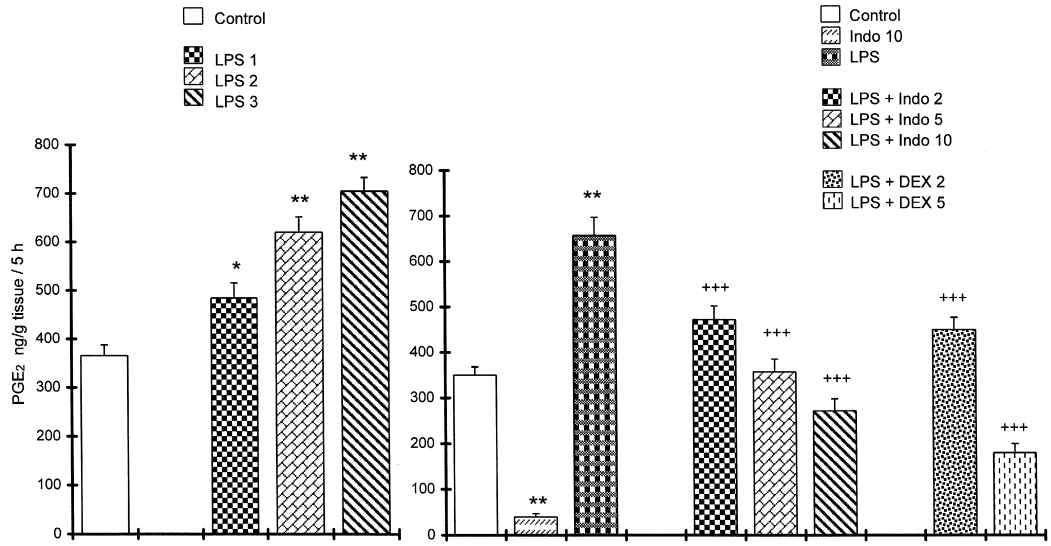

Fig. 5. Accumulation of prostaglandin E_2 in the ex vivo gastric mucosa incubated in DMEM and 1 mM EGTA. Lipopolysaccharide (1–2–3 mg kg^{-1}) was injected intraperitoneally 5 h before intragastric administration of 95% ethanol (1.5 ml) (left panel). Indomethacin (2–5–10 mg kg^{-1} s.c.) or dexamethasone (2–5 mg kg^{-1} s.c.) were administered 60 min before endotoxin (3 mg kg^{-1} i.p.) (right panel). Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate prostaglandin E_2 accumulation by EIA immunoassay. Each column represents the mean ± S.E.M. no. 9–10 per group. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. $*$ $P < 0.01$; $**$ $P < 0.001$ significantly different from control group. $^{+++}P < 0.001$ significantly different from lipopolysaccharide 3 mg kg^{-1} treated animals.

Table 3
Effects of *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398), on ethanol-induced gastric damage and on level of NO_2^- and PGE_2

Treatment	lesion index	NO_2^- (μM)	PGE_2 (ng/g of tissue)
Control	43.5 ± 3.3	6.0 ± 1.2	347.3 ± 18.8
LPS	12.1 ± 2.0[a]	82.7 ± 4.7	720.2 ± 43.7
LPS ± NS-398 (5 mg kg^{-1})	10.3 ± 1.8[a]	105.3 ± 5.2	520.5 ± 37.5[b]
LPS ± NS-398 (10 mg kg^{-1})	13.4 ± 1.7[a]	90.8 ± 4.5	410.3 ± 20.1[b]

Lipopolysaccharide (LPS) 3 mg kg^{-1} was injected intraperitoneally 5 h before intragastric administration of 95% ethanol. NS-398 (5–10 mg kg^{-1} p.o.) was administered 60 min before endotoxin. Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate NO_2^- accumulation by Greiss method and PGE_2 accumulation by EIA immunoassay. Each point is the mean ± S.E.M. for no. 8–10 animals. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. [a]$P < 0.001$ when compared with control group; [b]$P < 0.001$ when compared with LPS treated group.

selective cyclooxygenase-1/cyclooxygenase-2 inhibitor, indomethacin (10 mg kg^{-1}) abolished the release of prostaglandin E_2 in non-lipopolysaccharide treated rats. As expected, indomethacin (2–5–10 mg kg^{-1}) inhibited dose-relatedly prostaglandin E_2 accumulation in response to lipopolysaccharide. Also the administration of dexamethasone reduced in dose-dependent manner the lipopolysaccharide-induced release of prostaglandin E_2 (Fig. 5, right panel).

The cyclooxygenase-2 selective inhibitor, *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide, did not affect both the gastroprotective effect of lipopolysaccharide on ethanol-mediated damage and the increase of nitrite, while clearly reduced the increment in the release of prostaglandin E_2 (Table 3).

Fig. 6 shows that the increment in the release of prostaglandin E_2 induced by lipopolysaccharide was reduced in a dose-dependent manner (3–10–30 mg kg^{-1}) by administration of L-N^6-(1-Iminoethyl)lysine(dihydrochloride). However concurrent administration of L-arginine prevented the decrease of prostaglandin E_2. Also the administration of other NO synthase inhibitor such as L-N^G-nitro arginine methyl ester at the dose of 10 mg kg^{-1} was able to prevent the increase of prostaglandin E_2 elicited by lipopolysaccharide alone.

We next tested if the administration of NO donors could affect prostaglandin E_2 accumulation in incubates of gastric mucosal fragments from lipopolysaccharide-treated animals. As expected, the administration of lipopolysaccharide (3 mg kg^{-1}) increased prostaglandin E_2 amount. The administration of sodium nitroprusside (5–10 mg kg^{-1}) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (2–5 mg kg^{-1}) have a protective effect and enhanced the accumulation of prostaglandin E_2 in dose related fashion (Fig. 7). When

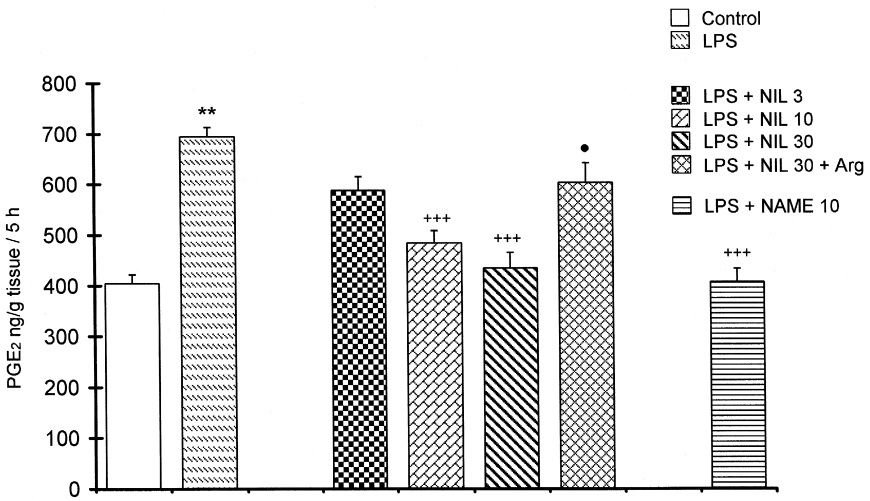

Fig. 6. Accumulation of prostaglandin E_2 in the ex vivo gastric mucosa incubated in DMEM and 1 mM EGTA. Lipopolysaccharide (3 mg kg^{-1} i.p.) was injected 5 h before intragastric administration of 95% ethanol. L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (NIL; 3–10–30 mg kg^{-1} p.o.) or L-N^G-nitro arginine methyl ester (NAME; 10 mg kg^{-1} s.c.) were administered 60 min before endotoxin. L-arginine (300 mg kg^{-1} s.c.) was administered contemporaneously to L-N^6-(1-Iminoethyl)lysine(dihydrochloride) (30 mg kg^{-1}). Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate prostaglandin E_2 accumulation by EIA immunoassay. Each column represents the mean ± S.E.M. no. 8–10 per group. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. ** $P < 0.001$ significantly different from control group. $^{+++}P < 0.001$ significantly different from lipopolysaccharide 3 mg kg^{-1} treated group. ● $P < 0.001$ significantly different from lipopolysaccharide plus L-N^6-(1-Iminoethyl)lysine(dihydrochloride) 30 treated group.

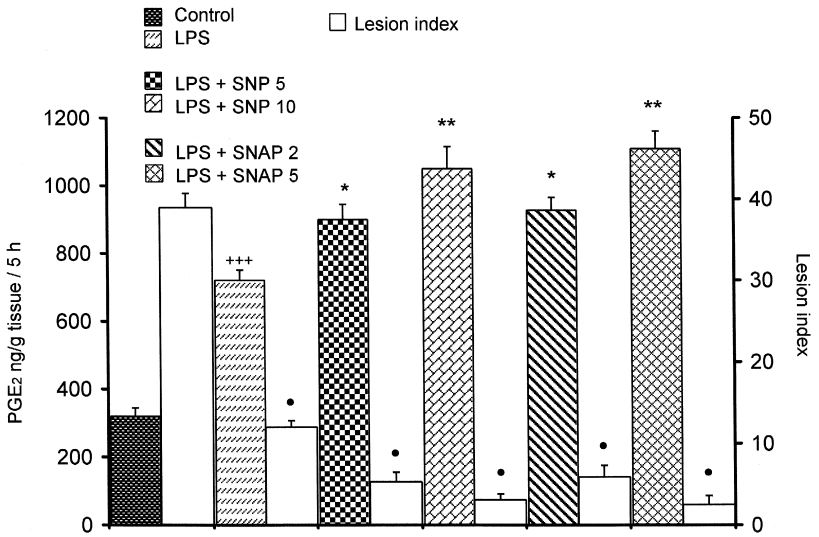

Fig. 7. Effect of administration of sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) on ethanol induced gastric damage and on accumulation of prostaglandin E_2 in ex vivo gastric mucosa incubated in DMEM and 1 mM EGTA. Lipopolysaccharide (3 mg kg^{-1} i.p) was injected 5 h before intragastric administration of 95% ethanol. Sodium nitroprusside (5–10 mg kg^{-1}) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (2–5 mg kg^{-1}) were administered 60 min before endotoxin and 1 h prior to the sacrifice. Five minutes after administration of ethanol, the stomach was removed and opened along the great curvature and fragment of gastric mucosa were carefully stripped and incubated. After 5 h incubation the medium was used to estimate prostaglandin E_2 accumulation by EIA immunoassay. Each column represents the mean ± S.E.M. 8–10 per group. Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. $^{+++}P < 0.001$ significantly different from control group. $*\ P < 0.01$; $**\ P < 0.001$ significantly different from lipopolysaccharide 3 mg kg^{-1} treated group. Lesion index data were analyzed using Mann–Whitney *U*-test. ● $P < 0.001$ significantly different from control rats receiving only ethanol.

sodium nitroprusside (10 mg kg^{-1}) was co-administered together with L-N^G-nitro arginine methyl ester (10 mg kg^{-1}) 60 min before lipopolysaccharide, exogenous NO (evoked by sodium nitroprusside) was able to counteract (580 ± 45 ng/g of tissue) the decrease of prostaglandin E_2 in lipopolysaccharide-treated gastric mucosa in which NO synthesis was blocked by L-N^G-nitro arginine methyl ester (330 ± 27 ng/g of tissue; $P < 0.001$).

When sodium nitroprusside 10 mg kg^{-1} and *S*-nitroso-*N*-acetyl-D,L-penicillamine 5 mg kg^{-1} were administered alone without lipopolysaccharide, they showed a gastroprotective activity against ethanol (l.i. 15 ± 2.7 and 18 ± 3.1 respectively and the release of PGE_2 are respectively 610 ± 45.1 and 570 ± 39.8 ng/g of tissue).

Western blot studies confirmed the presence of cyclooxygenase 2 and inducible nitric oxide synthase and were in agreement with the measurement of prostaglandins and nitric oxide (Figs. 8 and 9).

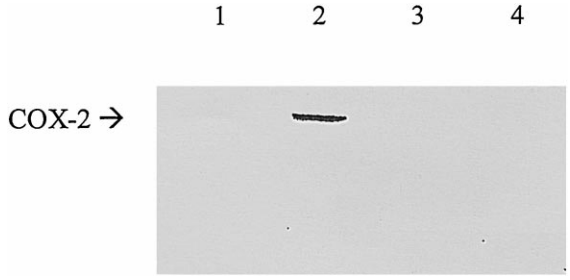

Fig. 8. Effect of dexamethasone and indomethacin on cyclooxygenase-2 (COX-2) protein expression in the rat gastric mucosa treated with lipopolysaccharide. Lane 1 contains extract from untreated cells. Lane 2 contains extract from cells treated with lipopolysaccharide (3 mg kg^{-1}) for 5 h before intragastric administration of 95% ethanol. Lane 3 contains extract from cells treated with dexamethasone (5 mg kg^{-1} s.c.) 1 h prior to treatment with lipopolysaccharide. Lane 4 contains extract from cells treated with indomethacin (10 mg kg^{-1} s.c.) 1 h prior to lipopolysaccharide. Equal amounts of protein were loaded to each lane.

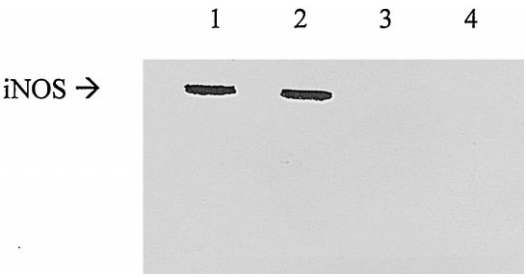

Fig. 9. Effect of dexamethasone and indomethacin on inducible nitric oxide synthase (iNOS) protein expression in the rat gastric mucosa treated with lipopolysaccharide. Lane 1 contains extract from cells treated with lipopolysaccharide (3 mg kg^{-1} i.p.) for 5 h before intragastric administration of 95% ethanol. Lane 2 contains extract from cells treated with indomethacin (10 mg kg^{-1} s.c.) 1 h prior to treatment with lipopolysaccharide. Lane 3 contains extract from cells treated with dexamethasone (5 mg kg^{-1} s.c.) 1 h prior to treatment with lipopolysaccharide. Lane 4 contains extract from untreated cells. Equal amounts of protein were loaded to each lane.

4. Discussion

Our results strongly suggest that NO released endogenously from lipopolysaccharide or released by NO donors, plays a critical role in the release of prostaglandin E_2 induced by lipopolysaccharide, possibly through an activation of cyclooxygenase. In agreement with other data (Tepperman and Soper, 1994; Barrachina et al., 1995), pretreatment of rats with different doses of bacterial lipopolysaccharide, which by itself did not damage the gastric mucosa examined 5 h after administration, reduced the extent of mucosal haemorrhagic damage in response to intraluminal ethanol. Our data indicates that gastric mucosa is capable of producing ex vivo high levels of NO after pretreatment of different doses of lipopolysaccharide. It is well known that the inducible NO-synthase is responsible for the increase in synthesis of NO that occurs some 3–6 h after exposure to endotoxin, and its expression is inhibited by glucocorticoids (Whittle, 1994). This finding thus confirms and extends previous studies that have identified the presence of Ca^{2+}-independent NO synthase activity in epithelial cells isolated from rat gastric mucosa (Brown et al., 1994). The induction of NO synthase by lipopolysaccharide caused a great increase in the release of nitrites and prostaglandin E_2, both products of NO synthase and cyclooxygenase, respectively. Besides, the prevention of NO production by the use of inhibitors of NO synthase, namely L-N^6-(1-Iminoethyl)lysine(dihydrochloride) or L-N^G-nitro arginine methyl ester markedly attenuated prostaglandin E_2 release in a dose dependent fashion. The observations that administration of NO donors such as sodium nitroprusside and *S*-nitroso-*N*-acetyl-D,L-penicillamine, elicited an enhancement on prostaglandin E_2 production by administration of lipopolysaccharide together with the fact that co-administration of L-N^G-nitro arginine methyl ester was able to prevent the above effects, indicate a interaction between NO and cyclooxygenase in rat gastric mucosa. In a previous research it has been reported that NO regulates the activity of cyclooxygenase enzymes, enhancing (Salvemini et al., 1993) or inhibiting (Kanner et al., 1992; Stadler et al., 1993) their synthesis. Here we show that in ethanol-induced injury in rat gastric mucosa, endogenous NO produced by inducible NO synthase or released by NO donors can influence the stimulation of the gastric mucosa to activate cyclooxygenase, leading to an augmented production of prostaglandin E_2. Increasing evidence is emerging that the link between the cyclooxygenase and NO synthase pathways may be of widespread importance in a number of systems, including the gastric mucosa.

In our study dexamethasone, a well-known inhibitor of lipopolysaccharide-induction of NO synthase in endothelial cell (Palmer et al., 1992) and macrophages (Di Rosa et al., 1990), is able to reduce both nitrite accumulation, as well as prostaglandin E_2. Although, it is known that dexamethasone might reduce prostaglandin E_2 synthesis via the inhibition of phospholipase A_2 activity (Flower, 1988), the existence of an inducible cyclooxygenase that is expressed only in the context of inflammation and that is under glucocorticoid regulation has been postulated (Masferrer et al., 1992). The ability of glucocorticoids to suppress PGs production may occur through various mechanisms and a multiple levels, including both phospholipase A_2 and cyclooxygenase enzyme activities. Our results with dexamethasone may support the hypothesis that inhibition of endotoxin-dependent inducible NO synthase leads to a reduction of the protective action of lipopolysaccharide treatment and a decrease in prostaglandins synthesis in rat gastric mucosa, probably by the lack of NO. Similarly the NO synthase inhibitor L-N^6-(1-Iminoethyl)lysine(dihydrochloride) and L-N^G-nitro arginine methyl ester also reduced the protective influence of lipopolysaccharide and this was reversed by co-administration of L-arginine.

Pretreatment with a non selective cyclooxygenase-1/cyclooxygenase-2, indomethacin, in a dose resulting in 88% inhibition of gastric mucosa prostaglandin formation was ineffective in reducing the gastroprotective influence of lipopolysaccharide administration and did not affect the increase of the ex vivo formation of NO by gastric mucosa while the prostaglandin E_2 production was reduced. Also the selective cyclooxygenase-2 inhibitor, *N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide, was ineffective in reducing the gastroprotective influence of lipopolysaccharide and did not affect the increase of the NO, while the prostaglandin E_2 production was reduced.

These data suggest that the reduction in ethanol-mediated damage in lipopolysaccharide-treated rats is due to maintenance of NO produced by the inducible enzyme, although previous reports have indicated that inducible NO synthase by itself has damaging actions on the gastrointestinal tract (Tepperman et al., 1993; Boughton-Smith et al., 1993). A number of studies have demonstrated that endogenous NO or NO donors reduce the severity of ethanol-induced gastric damage (MacNaughton et al., 1989; Franco and Velo, 1994, 1996). The mechanism of this protective action by inducible NO synthase is uncertain but may include effects on the microvasculature, including maintenance of blood flow (Tepperman and Whittle, 1992; Masuda et al., 1995), reduction in leukocyte adhesion and superoxide formation (Kubes et al., 1993). Our results demonstrated that endogenous NO released after administration of lipopolysaccharide and NO donors stimulate cyclooxygenase activity in rat gastric mucosa after ethanol-induced damage.

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