Role of Nitric Oxide and Prostaglandin E₂ in Gastroprotection Induced by Lipopolysaccharide in Ethanol-Mediated Damage in Rats

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Key Words

Nitric oxide · Prostaglandin E₂ · Gastroprotection · Ethanol · Lipopolysaccharide

Abstract

Objective: This study was designed to investigate the role of endogenous nitric oxide (NO) and prostaglandin E₂ (PGE₂) in the gastroprotection induced by lipopolysaccharide (LPS). **Methods:** Ethanol was used to induce gastric lesions in control rats and in rats pretreated with graded doses of LPS administered at different time intervals and with inhibitors of NO synthase or prostaglandin synthesis. The ethanol-induced damage on gastric mucosa was assessed by measuring the extent of the lesion. We evaluated nitrite, a breakdown of NO, and PGE₂ accumulation in ex vivo gastric mucosa. **Results:** The ex vivo production of both NO and PGE₂ was increased in a dose-dependent manner by LPS injected 5 h before ethanol. Pretreatment with L-N⁶-(1-iminoethyl)lysine(dihydrochloride) inhibited the protection associated with LPS and the ex vivo increase of both NO and PGE₂. Indomethacin was ineffective in suppressing LPS-mediated protection against ethanol-induced damage and in suppressing ex vivo increase of nitrite whereas the ex vivo increase of PGE₂ was prevented in a dose-dependent manner. When ethanol was administered 30 min after LPS, there was a lack of protec-

tion and a lack of increase of NO and PGE₂. **Conclusion:** These results indicate that the reduction in ethanol-mediated damage in LPS-treated rats depends on endogenous PGE₂ formation and on endogenous NO produced by stimulation of inducible NO synthase.

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Introduction

The integrity of the gastrointestinal mucosa depends on the interaction of various protective and damaging factors to which mucosa is exposed. The major defence lines include the continuous mucus-alkaline secretion, the maintenance of the mucosal blood flow, and the restitution or proliferation of mucosal cells [1]. Although many chemical mediators participate in producing coordinated and effective mucosal responses to injury, there is overwhelming evidence that prostaglandins and nitric oxide (NO) are critical to this process [2, 3].

At least two isoforms of cyclooxygenase have been characterized [4, 5]. The predominant form in the stomach is cyclooxygenase-1, which is constitutively expressed and is thought to be a housekeeping gene product that produces the prostaglandins for maintaining the physiological functions such as gastric cytoprotection. In contrast, the inducible enzyme, cyclooxygenase-2, is expressed fol-

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1011-7571/01/0103-0140\$17.50/0

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lowing exposure of certain cytokines, mitogens and endotoxin, and is responsible for prostaglandin production at sites of inflammation [6].

NO synthase also exists in constitutive and inducible (iNOS) forms [7]. In the stomach the constitutive form results in the production of physiological levels of NO important in gastric mucosal functions, such as regulation of vascular tone and neurotransmission, and can be cytoprotective in the gastrointestinal mucosa by interacting with sensory neuropeptide and endogenous prostaglandins [2]. When appropriately stimulated, such as after exposure to endotoxins, iNOS can also be detected [8]. The iNOS can produce consistent, high concentrations of NO that have been shown to contribute to gastric damage, particularly when cells are exposed to large quantities of NO over an extended period of time [9, 10]. In contrast to these studies, it has been suggested that NO derived from iNOS plays an important role in maintaining mucosal integrity in some circumstances [11–13].

The aim of this study was to investigate the role of NO and prostaglandin synthesis in maintaining mucosal integrity. For this purpose, we have induced in rats iNOS activity by administration of lipopolysaccharide (LPS) and at different intervals we induced gastric mucosa damage by ethanol and evaluated nitrite, a breakdown product of NO, and prostaglandin E₂ (PGE₂) accumulation in ex vivo gastric mucosa. The animals were pretreated with inhibitors of NO synthase or inhibitors of prostaglandin synthesis before intragastric administration of ethanol and examined the effects on gastric mucosa.

Materials and Methods

Male Sprague-Dawley rats (Charles River, Italy) weighing 220–240 g were used for these studies. The animals had been fasted for 24 h before the experiment with free access to water. Groups of rats were pretreated intraperitoneally with graded doses of bacterial LPS from *Escherichia coli* (serotype 0111:B4, 1–3 mg/kg; Sigma, St. Louis, Mo., USA); control rats received an equal volume of saline. Gastric lesions were caused by intragastric instillation of 1.5 ml 95% ethanol 30 min or 5 h after LPS administration. Five minutes 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 of gross mucosal damage was determined using a scoring system based on the number and length of hemorrhagic mucosal necrosis. The number of necrotic bands more than 4 mm in length was multiplied by factor 3, lesions of 2–4 mm were multiplied by factor 2, and lesions of less than 2 mm by factor 1 [14].

Fragments of rat gastric mucosa treated with LPS were carefully stripped, washed with ice-cold phosphate buffer, 0.1 M, pH 7.4 and centrifuged at 3,000 g at 4°C for 3 min. The phosphate-buffered

saline was replaced with 1 ml of fresh solution of Dulbecco's modified Eagle's medium and 1 mM EGTA, a calcium chelator, and the samples were incubated for 5 h at 37°C under 5% CO₂ and 95% O₂. This fluid, which contained the released eicosanoids and NO, was removed for assay and the gastric mucosa was weighed.

The production of NO was assayed by measurement of the accumulation of nitrite (NO₂⁻) in the culture medium by first reducing the NO₃⁻ using a bacterial nitrate reductase prepared from *E. coli*. Nitrite was assayed colorimetrically after reaction with the Griess reagent [15]. Briefly, 50 µl of the media (in duplicate) was mixed with 50 µl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2.5% orthophosphoric acid) and incubated for 10 min at room temperature and the absorbance was measured with a microplate reader (Argus 400, Canberra Packard) at 550 nm (OD₅₅₀). All determinations were performed in duplicate. Nitrite concentrations were calculated by comparison with OD₅₅₀ of standard solutions of sodium nitrite prepared in culture medium. Results are expressed as micromoles of nitrite.

PGE₂ in the medium was measured by enzyme immunoassay (Cayman Chemicals, Ann Arbor, Mich., USA) according to the manufacturer's protocol. The sensitivity of the assay was 30 pg/ml. The anti-PGE₂ antibody has <5% cross-reactivity with other eicosanoids as disclosed by the manufacturer. The data are expressed as nanograms of PGE₂ per gram of tissue in 5 h. Each experiment was performed in duplicate.

In a separate group of experiments, rats were treated by gavage (p.o.) with the selective iNOS inhibitor L-N⁶-(1-iminoethyl)lysine(dihydrochloride) (L-NIL) obtained from Cayman Chemicals (3–30 mg/kg) 1 h before LPS treatment (3 mg/kg). Ethanol was instilled into the stomach 5 h after LPS administration. To confirm its specificity a group of rats was also treated with L-arginine (300 mg/kg s.c.) contemporaneously with L-NIL.

Other groups of rats were pretreated with indomethacin (2–10 mg/kg s.c.) administered 1 h before LPS treatment (3 mg/kg). Gastric lesions were caused by intragastric instillation of 1.5 ml 95% ethanol 30 min or 5 h after LPS administration and 5 min after the instillation of ethanol the animals were killed by inhalation of ether.

Statistical Analysis

Statistical significance of differences between groups of parametric data was determined by one-way analysis of variance followed by Bonferroni t test. Comparisons between groups of nonparametric data (lesion index) were made by the Mann-Whitney U test. A probability (p) value of 0.05 or less was taken to indicate statistical significance. Results are expressed as mean ± SEM.

Results

Intragastric administration of 95% ethanol caused macroscopic hemorrhagic damage. Treatment of rats with graded doses of LPS, injected 5 h before ethanol, resulted in a reduction of the damage associated with 95% ethanol administration. The time interval between LPS administration and ethanol application was selected from previous studies in which we showed that 5 h was the optimal time required for the induction of prostaglandins and

Fig. 1. Effect of different doses of LPS injected 5 h (**A**) or 30 min (**B**) before intragastric administration of 95% ethanol, on gastric mucosal damage and on the level of NO₂⁻ and PGE₂ in rats. Each point is the mean ± SEM for 7 animals. Data were analyzed using Mann-Whitney U test for lesion index and by one-way analysis of variance followed by Bonferroni t test for the other data. ^a $p < 0.05$; ^b $p < 0.001$ versus control rats receiving only ethanol.

iNOS in the rat stomach [13]. The ex vivo production of nitrite and PGE₂ in the rats' gastric mucosa incubated for 5 h was increased in a dose-dependent manner in LPS-treated rats (fig. 1A). When ethanol was administered 30 min after the administration of LPS (fig. 1B) we observed a lack of gastroprotection associated with LPS (1–3 mg/kg) and a lack of the increase of nitrite and PGE₂. The accumulation of nitrite and PGE₂ did not change as compared to the group treated with ethanol.

The gastroprotective effect of LPS, injected 5 h before ethanol, was not significantly affected by pretreatment of rats with graded doses of indomethacin (2–10 mg/kg) administered 1 h before the injection of LPS (fig. 2A). Indomethacin did not affect the increase of nitrite in the gastric mucosa after i.p. administration of 3 mg/kg LPS, but inhibited dose-dependent PGE₂ accumulation in response to LPS although they were consistently higher if compared to the indomethacin group.

When ethanol was administered 30 min after the administration of LPS, indomethacin (2–10 mg/kg) increased the mucosal damage and induced a significant dose-dependent inhibition of PGE₂, while the accumulation of nitrite was not affected (fig. 2B).

Pretreatment of L-NIL 30 mg/kg induced a slight increase of gastric lesion index ($p < 0.05$) as compared to the ethanol control group. Pretreatment with graded doses of L-NIL (3–30 mg/kg) 60 min before administration of LPS resulted in a dose-dependent increase in the degree of mucosal damage as compared to LPS-treated animals, restoring the mucosal damage of 95% ethanol on the gastric mucosa. The magnitude of the nitrite increase and the increment in the release of PGE₂ induced by LPS was reduced in a dose-dependent manner by administration of L-NIL. These effects were prevented by contemporaneous s.c. administration of *L*-arginine (300 mg/kg, fig. 3).

2

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Fig. 2. Effect of indomethacin on ethanol-induced gastric damage and on the level of NO₂⁻ and PGE₂ in rats pretreated with LPS (3 mg/kg) 5 h (A) or 30 min (B) before intragastric administration of 95% ethanol. Indomethacin 2–10 mg/kg was administered s.c. 1 h before LPS treatment. Each point is the mean ± SEM for 7 animals. Data were analyzed using Mann-Whitney U test for lesion index and by one-way analysis of variance followed by Bonferroni t test for the other data. ^a $p < 0.05$; ^b $p < 0.001$ versus control rats receiving only ethanol; ^c $p < 0.001$ versus ethanol plus LPS-treated control group; ^d $p < 0.001$ versus rats treated with indomethacin 10 mg/kg.

Fig. 3. Effect of L-NIL on ethanol-induced gastric damage and level of NO₂⁻ and PGE₂ in rats pretreated with LPS (3 mg/kg) 5 h before intragastric administration of 95% ethanol. L-NIL 3–30 mg/kg was administered p.o. 1 h before LPS treatment. L-Arginine 300 mg/kg was administered s.c. contemporaneously with L-NIL. Each point is the mean ± SEM for 7 animals. Data were analyzed using Mann-Whitney U test for lesion index and by one-way analysis of variance followed by Bonferroni t test for the other data. ^a $p < 0.05$; ^b $p < 0.001$ versus control rats receiving only ethanol; ^c $p < 0.001$ versus ethanol plus LPS-treated control rats; ^d $p < 0.001$; ^e $p < 0.05$ versus rats treated with LPS plus L-NIL 30 mg/kg.

Discussion

In agreement with other data [11–13], pretreatment of rats with different doses of bacterial LPS 5 h prior to ethanol treatment reduced the extent of mucosal hemorrhagic damage in response to intraluminal ethanol. It is well known that iNOS is responsible for the increase in the synthesis of NO that occurs some 3–6 h after exposure to endotoxin [16]. The induction of NO synthase by LPS caused a great increase in the release of NO and PGE₂. The prevention of NO production by the use of inhibitors of NO synthase reduced the protective activity of LPS and markedly attenuated PGE₂ release in a dose-dependent fashion, but the PGE₂ levels were never lower than control. Pretreatment with a nonselective cyclooxygenase-1/cyclooxygenase-2 inhibitor, indomethacin, at a dose effecting a 90% inhibition of gastric mucosa prostaglandin formation did not affect the gastric mucosa protection by LPS and the increase of the ex vivo formation of NO by gastric mucosa while it significantly reduced the increment in the release of PGE₂ formation. These data suggest that NO is involved in the protective actions of endotoxin and plays an important role in this protection. The gastro-

protective effect of LPS on ethanol-mediated damage was not evident when determined 30 min after LPS injection and the accumulation of nitrite and PGE₂ is not different from the control group, suggesting that the protection is linked to NO produced by iNOS.

It has been reported that NO activates cyclooxygenase, leading to an augmented production of cyclooxygenase metabolites [13, 17]. The observation that NO can influence the stimulation of the gastric mucosa to activate cyclooxygenase, leading to an augmented production of prostaglandins, is thought to be related to the beneficial role of NO [13].

In conclusion, the results indicate that the reduction in ethanol-mediated damage in LPS-treated rats depends on endogenous prostaglandin formation and endogenous NO produced by stimulation of iNOS.

Acknowledgments

This study was supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica 60% (grant 1998–1999).

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