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Mechanisms of gastroprotection by lansoprazole pretreatment against experimentally induced injury in rats: role of mucosal oxidative damage and sulfhydryl compounds

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Abstract

This study investigated the mechanisms involved in the protective actions exerted by lansoprazole against experimental gastric injury. Following the intraluminal injection of ethanol–HCl, the histomorphometric analysis of rat gastric sections demonstrated a pattern of mucosal lesions associated with a significant increase in the mucosal contents of malondialdehyde and 8-*iso*-prostaglandin $F_{2\alpha}$ (indices of lipid peroxidation), as well as a decrease in the levels of mucosal sulfhydryl compounds, assayed as reduced glutathione (GSH). Pretreatment with lansoprazole 90 μmol/kg, given intraduodenally as single dose or once daily by intragastric route for 8 days, significantly prevented ethanol–HCl-induced gastric damage. The concomitant changes in the mucosal levels of malondialdehyde, 8-*iso*-prostaglandin $F_{2\alpha}$ and GSH elicited by ethanol–HCl were also counteracted by lansoprazole. In separate experiments, performed on animals undergoing 2-h pylorus ligation, lansoprazole did not enhance the concentration of prostaglandin E_2, bicyclo-prostaglandin E_2, or nitric oxide (NO) metabolites into gastric juice. Western blot analysis revealed the expression of both type 1 and 2 cyclooxygenase (COX) isoforms in the gastric mucosa of pylorus-ligated rats. These expression patterns were not significantly modified by single-dose or repeated treatment with lansoprazole. Lansoprazole also exhibited direct antioxidant properties by reducing 8-*iso*-prostaglandin $F_{2\alpha}$ generation in an in vitro system where human native low-density lipoproteins were subjected to oxidation upon exposure to $CuSO_4$. The present results suggest that the protective effects of lansoprazole can be ascribed to a reduction of gastric oxidative injury, resulting in an increased bioavailability of mucosal sulfhydryl compounds. It is also proposed that lansoprazole does not exert modulator effects on the gastric expression of COX isoforms as well as on the activity of NO pathways.

Keywords: Lansoprazole; Gastroprotection; Glutathione; Oxidative stress; Prostaglandins

Introduction

Benzimidazole derivatives, such as omeprazole, lansoprazole, and other related compounds, act as potent proton pump inhibitors, their action depending on the blockade of the α-subunit of the gastric H^+,K^+-ATPase, the enzyme responsible for the final step in the secretion of hydrochloric acid by parietal cells (Matheson and Jarvis, 2001; Sachs, 1997).

The more potent antisecretory effects of proton pump inhibitors, in comparison with other drugs, such as histamine H_2 receptor antagonists, are claimed to account for their efficacy in the treatment of peptic ulcer disease and other acid-related disorders of the upper gastrointestinal tract (Richardson et al., 1998). However, it has been recognized that the anatomical and functional integrity of gastric mucosa depend on the balance between aggressive and defensive mechanisms, and that the success of pharmacological treatments to prevent or heal ulcerative lesions may not depend only on the blockade of acid secretion, but

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also on the enhancement of mucosal protective factors (Dajani and Klamut, 2000; Peskar and Maricic, 1998). This ability of certain endogenous factors and drugs to counteract gastric mucosal damage through mechanisms unrelated to inhibition of acid secretion was previously designated as ''cytoprotection'', and is currently named ''gastroprotection'' (Szabo and Goldberg, 1990). In this respect, evidence was previously provided that lansoprazole and other proton pump inhibitors can afford acid-independent protection of gastric mucosa against a variety of necrotizing agents (for references, see Blandizzi et al., 1999a).

Although a large variety of endogenous factors, including prostaglandins, growth factors, gastrin, somatostatin, sensory peptides, nitric oxide (NO), and sulfhydryl compounds, have been implicated in gastroprotection (Højgaard et al., 1996; Wallace and Granger, 1996), the mechanisms involved in the protective actions of proton pump inhibitors remain largely to be clarified. In a previous study, we observed that, under appropriate experimental conditions, lansoprazole exerts gastroprotective actions, which seem significantly related to an enhancement of gastric mucus barrier. Indirect evidence was also obtained that an increased bioavailability of sulfhydryl compounds and possibly prostaglandins might account for the protective actions of this proton pump blocker at the level of gastric mucosa (Blandizzi et al., 1999a).

At present, the molecular mechanisms linking the gastric protective properties of lansoprazole to sulfhydryl compounds and prostaglandins are unclear. Moreover, endogenous sulfhydryl radicals and other antioxidant mechanisms appear to play significant roles in counteracting gastric injury associated with *Helicobacter pylori* infection (Jung et al., 2001; Kimura et al., 2001). On this basis, the present study was designed to examine the effects of lansoprazole on the following factors and mechanisms related to gastric protection: (1) mucosal oxidative damage and levels of sulfhydryl compounds; (2) gastric prostaglandin production and expression of cyclooxygenase (COX) isoforms; (3) release of stable NO metabolites (NO_x) into the gastric juice. In addition, the direct antioxidant properties of lansoprazole were assayed in an appropriate in vitro system.

Materials and methods

Animals and drug treatments. Albino male Wistar rats, 200–250 g body weight, were used throughout the study. They were fed standard laboratory chow and tap water ad libitum and were not used for at least 1 week after their delivery to the laboratory. The animals were housed, six in a cage, in temperature-controlled rooms on a 12-h light cycle at 22–24 °C and 50–60% relative humidity. Experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology (Society of Toxicology, 2002). Twenty-four hours before the experiments, animals were maintained in single cages and were deprived of food. Free access to water ad libitum was allowed until 1 h before the beginning of experiments.

Lansoprazole (90 μmol/kg) or its vehicle were administered by intraduodenal or intragastric route in accordance with two protocol designs. In the first set of experiments, lansoprazole was administered as a single dose by intraduodenal route. For this purpose, at the time of experiment, the animals were subjected to a midline laparotomy during a short anesthesia with diethyl ether (to minimize as much as possible the surgical stress), and the duodenum was exteriorized. Lansoprazole or its vehicle were directly injected into the distal portion of the duodenum by a 25-gauge needle, the abdominal incision was closed with clips, and the animals were allowed 10 min to recover from anesthesia. Animals were then subjected to subsequent experimental procedures as specifically indicated in the following sections. In the second series of experiments, animals received lansoprazole once daily by intragastric gavage for eight consecutive days, and then underwent further experimental procedures 12 h after administration of the last dose (Fig. 1). The intraduodenal route was selected for the single-dose protocol to prevent any putative interaction between lansoprazole and the mucosal necrotizing agent scheduled to be injected into the gastric lumen 30 min later. Such a route allowed maintaining an enteral access to lansoprazole, thus minimizing its pharmacokinetic changes in comparison with the intragastric administration used for the 8-day treatment protocol. In addition, the intraduodenal route ensured gastroprotective actions of lansoprazole and proton pump inhibitors comparable to those obtained with their intragastric administration (Blandizzi et al., 1999a; Okabe et al., 1988). The dose of lansoprazole tested in the present study was shown to be maximally effective in counteracting acute mucosal damage induced by necrotizing agents in previous reports (Blandizzi et al., 1999a; Satoh et al., 1989).

Animals subjected to gastric mucosal injury by ethanol–HCl

Induction of gastric mucosal damage. Gastric mucosal injury was induced by a mixture of ethanol and HCl according to the procedure previously reported (Blandizzi et al., 1999a). For this purpose, 30 min or 12 h after treatment with lansoprazole as single dose or repeated administration, respectively, 1 ml/200 g body weight of 60% ethanol (v/v) in 150 mM HCl (ethanol–HCl) was injected into the gastric lumen by intragastric gavage using a polyethylene orogastric catheter. In control experiments, animals were subjected to intragastric instillation of saline solution (154 mM NaCl). Ninety minutes after intragastric injection of ethanol–HCl or saline, the animals were euthanized by cervical dislocation, and their stomachs were rapidly removed and processed for the quantitative estimation of mucosal necrotic damage, or for the assay of malondialdehyde (MDA), 8-epiprostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$), and non-proteic sulfhydryl compounds in the mucosal layer (Fig. 1).

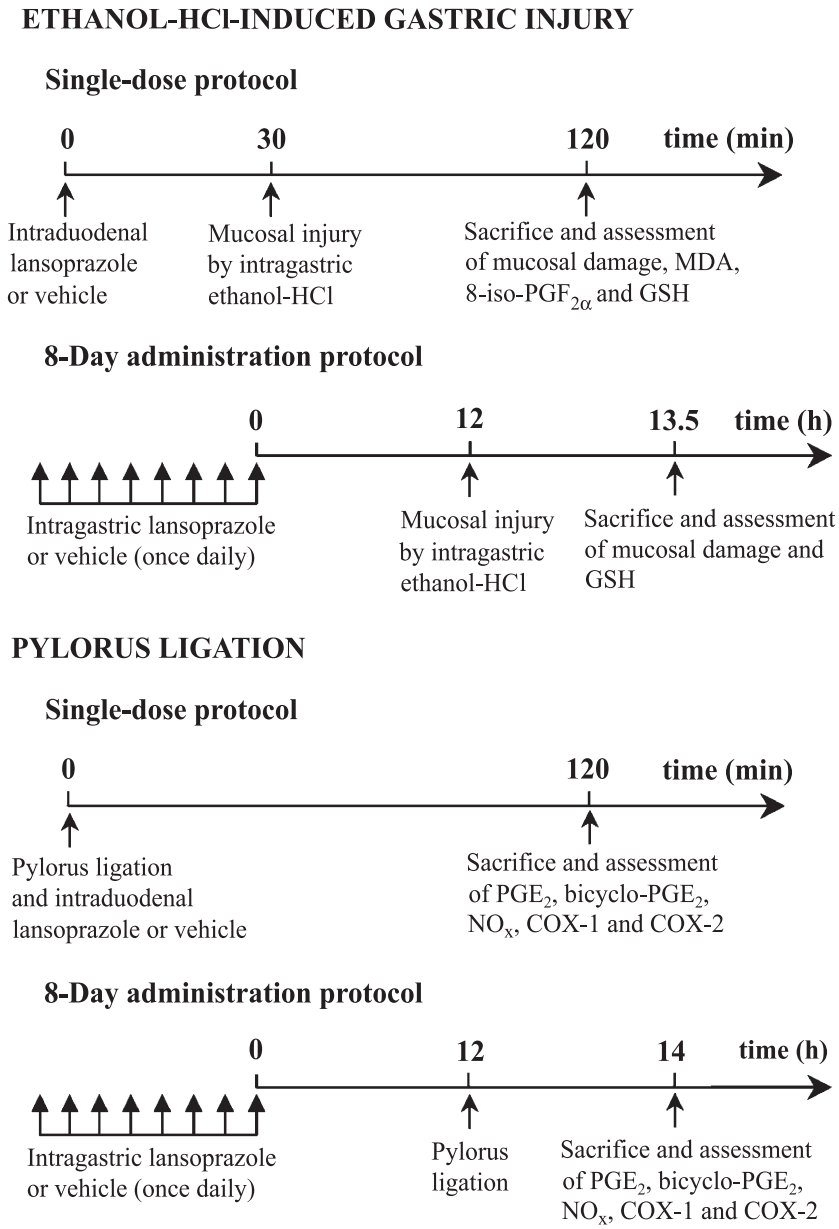

Fig. 1. Schematic diagram indicating the time-courses of experiments on ethanol–HCl-induced gastric injury and pylorus ligation. Because in single-dose experiments on the ethanol–HCl model, 2 h elapsed between intraduodenal administration of lansoprazole and euthanized animal, in the pylorus ligation model, the same time interval was allowed between pylorus ligation (with concomitant intraduodenal administration of lansoprazole) and euthanized animal.

Histomorphometric evaluation of gastric mucosal damage. The morphometric evaluation of gastric mucosal damage was carried out according to the method by Natale et al. (2001). Briefly, the stomach was opened along the greater curvature, pinned upon a cork plate, and fixed in 10% formalin buffered with phosphate for 24 h at 4 °C. Each stomach was dissected in parallel strips perpendicular to the lesser curvature, at distance of 2 mm. The strips obtained from each stomach were sequentially placed on a glass slide and oriented with the side distal to the pylorus upward. A solution of melted 3% agar was poured on the strips and cooled at 4 °C. Then, the agar block was dehydrated and embedded in paraffin wax. Three micrometer-thick paraffin sections were cut using an HM 330 Microm microtome (Heidelberg, Germany) and stained with hematoxylin and eosin. Sections were observed by light microscopy and the length of both total and damaged mucosa was evaluated by a micrometric scale. The lesion index was estimated as the length fraction of damaged mucosa over the total length of mucosa, and expressed in percentage values. Moreover, taking into account the depth of the mucosal damage, three types of lesions were also discriminated. Type I lesion consists of lysis of mucosal cells on the luminal-free surface, gastric pit cells being undamaged. Type II lesion

consists of damage confined to the upper part of the lamina propria involving the cells lying on both surface mucosa and gastric pits, gastric gland cells being undamaged. Type III lesion consists of damage that involves the lower part of the lamina propria, as well with injury of gastric glands associated with detachment of whole layers of necrotic superficial mucosa (Natale et al., 2001).

Assay of MDA and 8-iso-PGF$_{2\alpha}$ in the gastric mucosa. The concentrations of MDA and 8-*iso*-PGF$_{2\alpha}$ in samples of gastric mucosa were determined to obtain quantitative estimations of membrane lipid oxidative damage. For this purpose, specimens of mucosa were scraped from the underlying tissue layers of gastric wall using two glass slides kept cold on ice. The mucosa was weighed, minced by forceps, homogenized in 2 ml of cold buffer (Tris–HCl 20 mM, pH 7.4) using a politron homogenizer (Cole Parmer Homogenizer, Vernon Hills, IL, USA), and centrifuged at 1500 × *g* for 10 min at 4 °C. Aliquots of the supernatants were then used for the subsequent assay procedures. The mucosal concentrations of MDA were estimated by a kit for colorimetric assay (Oxis International, Portland, OR, USA), and the results were expressed as nanomole of MDA per milligram of gastric mucosa. Gastric mucosal levels of 8-*iso*-PGF$_{2\alpha}$were determined by a kit for competitive enzyme-linked immunoassay (Cayman Chemicals, Ann Arbor, MI, USA), and the results were expressed as picogram of 8-*iso*-PGF$_{2\alpha}$ per milligram of gastric mucosa.

Assay of sulfhydryl compounds in the gastric mucosa. The concentrations of reduced glutathione (GSH) in specimens of gastric mucosa were determined to quantitatively estimate the tissue content of nonprotein sulfhydryl compounds (Tashima et al., 2000). For this purpose, samples of mucosa were scraped from the underlying tissue layers of gastric wall using two glass slides kept cold on ice. The scraped mucosa was weighed, minced by forceps, homogenized in 2 ml of cold buffer [0.4 M 2-(*N*-morpholino)ethanesulfonic acid, 0.1 M phosphate, and 2 mM ethylenediaminetetraacetic acid (EDTA), pH 6] using the politron homogenizer, and centrifuged at 10 000 × *g* for 15 min at 4 °C. Aliquots of supernatants were deproteinated by a solution containing 1.25 M metaphosphoric acid and 4 M triethanolamine to avoid interference due to particulate components or protein sulfhydryl groups, and then used for the subsequent assay procedures. Gastric mucosal levels of GSH were determined by a kit for enzymatic colorimetric assay (Cayman Chemicals), and the results were expressed as nanomole of GSH per milligram of gastric mucosa.

Animals subjected to pylorus ligation

Procedure for pylorus ligation. Pylorus ligation was carried out as previously described (Blandizzi et al., 1999a). Briefly, during a short anesthesia with diethyl ether, the abdomen was opened by a midline laparotomy, and the pylorus was ligated. The abdominal incision was then closed with clips, and the animals were allowed to recover from anesthesia for 10 min. In animals receiving single-dose, the injection of lansoprazole or its vehicle into the duodenal lumen was performed at the time of pylorus ligation (Fig. 1). In a subgroup of experiments, animals not receiving lansoprazole were subjected to intragastric instillation of bacterial lipopolysaccharide (5 mg/kg) 30 min before pylorus ligation. The lipopolysaccharide dose was selected in accordance with Beubler et al. (2001). Two hours after pylorus ligation, animals were euthanized by cervical dislocation, the esophageal–gastric junction was ligated, and the whole stomach was excised (Fig. 1). Following injection of 1-ml phosphate-buffered saline (pH 7.4) into the lumen, the gastric content was emptied and carefully collected in graduated centrifuge tubes. After addition of indomethacin, at a final concentration of 100 μM, to the collected gastric content, to avoid the spontaneous synthesis of prostaglandins in vitro, each sample was centrifuged at 1500 × *g* for 10 min at 4 °C. The volume of supernatant was then measured and aliquots were stored at −80 °C until the assay of prostaglandin E_2 (PGE_2), bicyclo-PGE_2, and NO_x. The net volume of fluid secreted into the gastric lumen during 2 h of pylorus ligation was estimated by subtraction of 1-ml buffer saline from the total volume of supernatant collected from stomach. The glandular portion of gastric mucosa was gently rinsed with saline and stored at −80 °C to perform Western blot assay for the protein expression of COX isoforms, COX-1 and COX-2.

Assay of PGE_2 and bicyclo-PGE_2 in the gastric juice. Concentrations of PGE_2 and bicyclo-PGE_2 in samples of gastric juice were determined by kits for competitive enzyme-linked immunoassay (both from Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). Before assay, samples were subjected to purification by Amprep-C_{18} columns (Amersham Pharmacia Biotech). For this purpose, 0.5 ml of 1:4 water/ethanol and 10 μl of glacial acetic acid were added to 0.5 ml of each sample. After 5-min incubation at room temperature, samples were centrifuged at 2500 × *g* for 2 min. The supernatant was removed and applied to an Amprep-C_{18} column, which had been previously primed twice with 1 ml of 10% ethanol. The column was then washed with 1 ml of distilled water and 1 ml of hexane. PGE_2 or bicyclo-PGE_2 was eluted twice with 0.75 ml of ethyl acetate. Eluate fractions were collected and evaporated to dryness under a nitrogen stream. Dried samples were stored at −80 °C until performance of immunoenzyme assay. After assessment of PGE_2 and bicyclo-PGE_2 concentration (ng/ml) in gastric juice, their outputs over 2 h were calculated by multiplication for the net volume of fluid secretion, and the resulting values were then normalized to 1 h. Accordingly, data were expressed as nanogram per hour.

Assay of NO_x in the gastric juice. The stable breakdown products of NO, released into the gastric juice, were

estimated as previously described (Blandizzi et al., 1999b), with minor modifications. Before assay, all samples were ultrafiltered by centrifuge (1000 × *g* for 60 min at room temperature) using a 30-kDa molecular weight cut-off filter (Amicon Inc., Beverly, MA, USA). The ultrafiltrate, recovered from each sample, was stored at −80 °C until the assay. NO_x concentration in aliquots of the ultrafiltrate was measured by the Griess method (Green et al., 1982), after reduction of nitrates to nitrites with nitrate reductase in the presence of 5 mM NADPH for 3 h at 25°C. Nitrites were incubated with Griess reagent [0.1% *N*-(1-naphthyl)ethylenediamine dyhydrochloride and 1% sulfanilamide in 2.5% H_3PO_4)] for 10 min at 25 °C, and the absorbance was measured at 540 nm by an Uvikon 930 Spectrophotometer (Kontron Instruments, Milan, Italy). Standards were prepared with sodium nitrate and taken through the assay. The results were expressed as output of NO_x in nanomole per hour, in accordance with the procedure reported above for PGE_2 and bicyclo-PGE_2.

Western blot assay of COX-1 and COX-2 in the gastric mucosa. Specimens of mucosa were scraped from the underlying tissue layers of gastric wall using two glass slides kept cold on ice. Mucosal samples were weighed and homogenized in lysis buffer containing HEPES 10 mM, NaCl 30 mM, EDTA 0.2 mM, phenylmethylsulphonyl fluoride 2 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml, sodium fluoride 1 mM, sodium orthovanadate 1 mM, glycerol 2%, $MgCl_2$ 0.3 mM, and Triton-X 100 1%, using the politron homogenizer. Mucosal homogenates were centrifuged at 20000 × *g* for 15 min at 4 °C, and the resulting supernatants were then separated from pellets and stored at −80 °C. Protein concentration was determined in each sample by the Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA, USA). In order to perform Western blot analysis of COX-1 and COX-2, equivalent amounts of protein lysates (50 μg) were separated by electrophoresis on sodium dodecylsulfate polyacrylamide gel (8%) and transferred onto a nitrocellulose membrane. The blots were then blocked overnight with 5% nonfat dried milk in phosphate-buffered saline, and incubated overnight at room temperature with goat polyclonal antiserum raised against rat COX-1 or COX-2 (dilution 1:1000). After repeated washings with 0.1% Tween-20 in Tris-buffered saline, a peroxidase-conjugated rabbit anti-goat antibody (dilution 1:10000) was added for 1 h at room temperature. After repeated washings with 0.1% Tween-20 in Tris-buffered saline, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences Europe GmbH, Cologno Monzese, Italy). The relative expression of COX-1 or COX-2 was quantified by densitometric analysis with NIH Image computer program (Scion Corporation, USA).

In vitro assay of antioxidant activity of lansoprazole. To evaluate whether lansoprazole is endowed with direct antioxidant properties, the drug was incubated in a reaction mixture where human native low-density lipoproteins (LDLs) were subjected to oxidation upon exposure to $CuSO_4$ at 37 °C for 120 min, in accordance with a standardized method (Lubrano et al., 2003). Lansoprazole was assayed at the concentrations of 10 and 100 μM. The reaction mixture consisted of 2 ml of a phosphate buffer (10 mM KH_2PO_4/K_2HPO_4, pH 5.3) containing LDLs 150 μg/ml and $CuSO_4$ 1 μM. At the end of incubation, the oxidative reaction was stopped by addition of buthyldihydroxytoluene 0.5 M in acetonitrile, and the extent of LDL oxidation was estimated by measurement of 8-*iso*-$PGF_{2\alpha}$ concentrations. For this purpose, 100-μl aliquots of the reaction mixture were withdrawn and used to assay 8-*iso*-$PGF_{2\alpha}$ by a kit for competitive enzyme-linked immunoassay, as reported above for gastric mucosal tissues. The results were expressed as picogram of 8-*iso*-$PGF_{2\alpha}$ per milliliter.

Statistics. The results are given as mean ± standard error of the mean (SEM). The statistical significance of data was

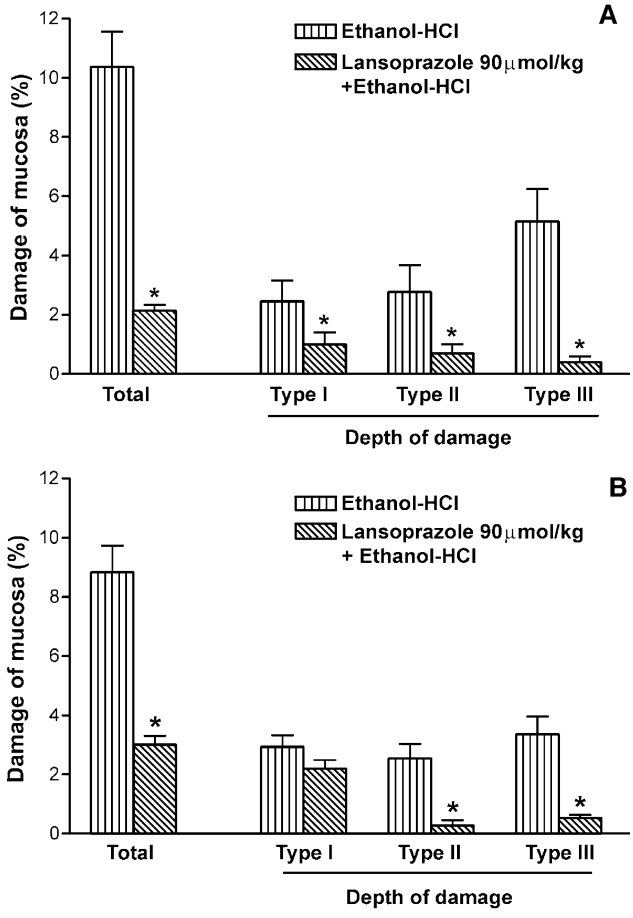

Fig. 2. Morphometric evaluation of gastric mucosal damage in rats subjected to intragastric administration of ethanol–HCl. (A) Effects of a single intraduodenal dose of lansoprazole 90 μmol/kg. (B) Effects of intragastric lansoprazole 90 (μmol/kg)/day for 8 days. Each column indicates the mean value obtained from six to eight animals ± SEM (vertical lines). Significant difference from ethanol–HCl alone: $^*P < 0.05$.

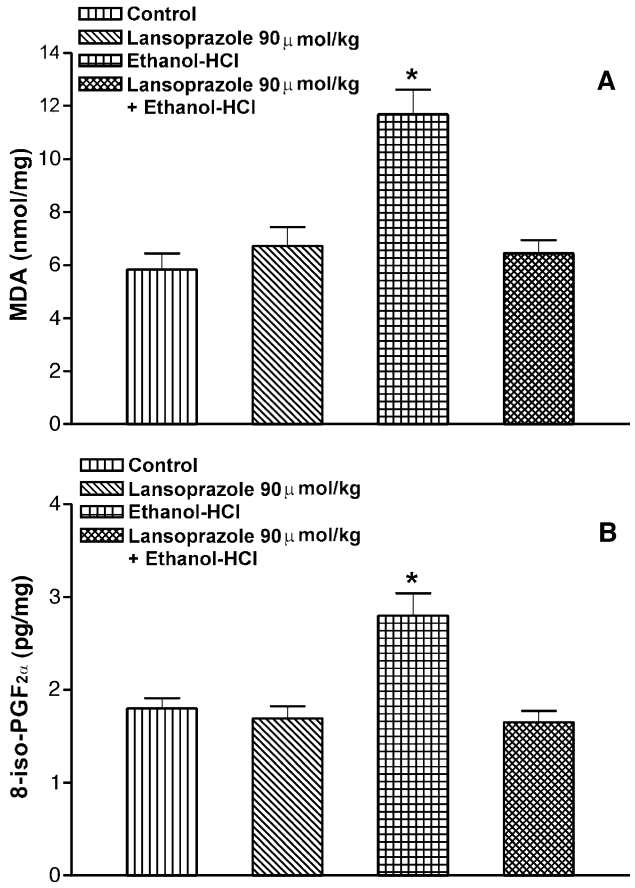

Fig. 3. Rats subjected to intragastric administration of ethanol–HCl. Effects of a single intraduodenal dose of lansoprazole 90 μmol/kg on gastric mucosal levels of MDA (A) and 8-*iso*-$PGF_{2\alpha}$ (B). Each column indicates the mean value obtained from 8 to 10 animals ± SEM (vertical lines). Significant difference from control rats: $^*P < 0.05$.

evaluated by Student's *t* test for unpaired data or one-way analysis of variance (ANOVA) followed by post hoc analysis by Dunnett's test, and *P* values lower than 0.05 were considered significant; "*n*" indicates the number of experiments. All statistical procedures were performed by personal computer programs.

Drugs. The following drugs and reagents were used: lansoprazole (Takeda Italia Farmaceutici, Rome, Italy); urethane ethyl carbamate, indomethacin, *N*-(1-naphthyl)ethylenediamine, sulfanilamide, NADPH, nitrate reductase (from *Aspergillus*), lipopolysaccharide (from *Escherichia coli* 0111:B4), HEPES, ethylenediaminetetraacetic acid, phenylmethylsulphonyl fluoride, sodium orthovanadate, glycerol, leupeptin, aprotinin, Triton-X 100, Tween-20, sodium dodecylsulfate, human native low-density lipoproteins, buthyldihydroxytoluene (Sigma Co., St. Louis, MO, USA); polyacrylamide (Bio-Rad); goat anti-rat COX-1 and COX-2 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); diethyl ether (Mallinckrodt Baker BV, Deventer, The Netherlands). Other reagents were of analytical grade. Lansoprazole was suspended in 1% methocel and injected in a volume of 0.5 ml for intragastric or intraduodenal administration.

Results

Animals subjected to gastric mucosal injury

Effects of lansoprazole on gastric mucosal damage induced by ethanol–HCl

In single-dose experiments, control animals as well as those receiving lansoprazole alone displayed a negligible gastric injury, with a total necrotic damage of mucosa accounting for 0.97 ± 0.7% and 0.82 ± 0.2%, respectively. Under these conditions, the intragastric instillation of ethanol–HCl elicited gross lesions in the glandular part of the stomach, and the histological examination of mucosa showed necrotic lesions of various extent and depth (not shown). Histomorphometric analysis of gastric sections revealed that the total necrotic damage affected 10.37 ±

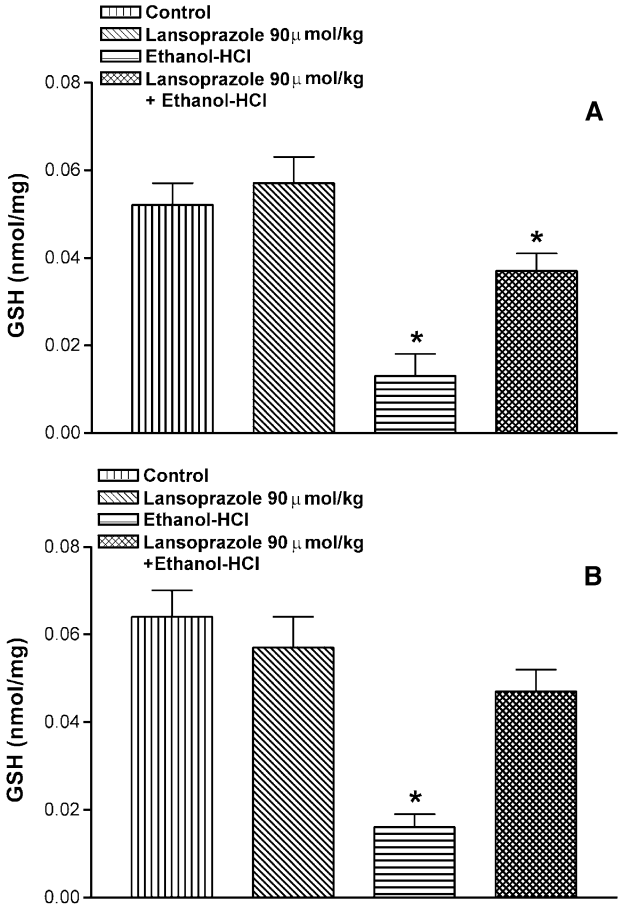

Fig. 4. Rats subjected to intragastric administration of ethanol–HCl. Effects of a single intraduodenal dose of lansoprazole 90 μmol/kg (A), or intragastric lansoprazole 90 (μmol/kg)/day for 8 days (B) on gastric mucosal concentration of GSH. Each column indicates the mean value obtained from six to nine animals ± SEM (vertical lines). Significant difference from control rats: $^*P < 0.05$.

Table 1
Effects of lansoprazole on PGE_2, bicyclo-PGE_2, and NO_x concentrations in the gastric juice from pylorus-ligated rats

	Lansoprazole administration	
	Single dose	Repeated dose
PGE_2 (ng/h)		
Control	1.24 ± 0.37	1.87 ± 0.54
Lansoprazole	1.42 ± 0.31	2.03 ± 0.61
Bicyclo-PGE_2 (ng/h)		
Control	0.47 ± 0.09	0.76 ± 0.15
Lansoprazole	0.72 ± 0.14	1.10 ± 0.22
NO_x (nmol/h)		
Control	84.4 ± 9.9	76.1 ± 8.7
Lansoprazole	99.5 ± 10.5	106.9 ± 10.4

Data are presented as means ± standard error of the mean (SEM) and represent the mean values obtained from six to eight experiments.

1.2% of gastric mucosa, with type I, type II, and type III lesions accounting for 2.45 ± 0.7%, 2.77 ± 9%, and 5.15 ± 1.1%, respectively (Fig. 2A). Pretreatment with lansoprazole 90 μmol/kg as single dose caused a significant reduction of mucosal damage, with a degree of protection more pronounced toward types II and III than type I lesions (Fig. 2A).

In experiments performed after repeated administrations of lansoprazole, a negligible damage of mucosa was detected in both control and lansoprazole-treated animals (not shown). Following intragastric injection of ethanol–HCl, the histomorphometric analysis showed a total necrotic damage covering 8.83 ± 0.9% of mucosa. Treatment with lansoprazole [90 (μmol/kg)/day for 8 days] significantly prevented the mucosal injury elicited by ethanol–HCl, and as already observed in single-dose experiments, the drug afforded a higher degree of protection against types II and III than type I lesions (Fig. 2B).

Effects of lansoprazole on MDA, 8-iso-$PGF_{2\alpha}$, and GSH levels in the gastric mucosa

Concentrations of MDA and 8-*iso*-$PGF_{2\alpha}$ were determined in the gastric mucosa of animals scheduled to undergo treatment with lansoprazole as single dose. In control rats, mucosal levels of MDA and 8-*iso*-$PGF_{2\alpha}$ were 5.83 ± 0.61 nmol/mg and 1.80 ± 0.11 pg/mg, respectively. These values were not significantly modified by administration of lansoprazole 90 μmol/kg (Fig. 3). Both MDA and 8-*iso*-$PGF_{2\alpha}$ mucosal concentrations increased significantly following the induction of gastric damage. However, such increments were no longer detected in injured animals pretreated with lansoprazole (Fig. 3).

Measurements of mucosal GSH concentrations were carried out in stomachs obtained from animals receiving lansoprazole as single dose or repeated administrations. In

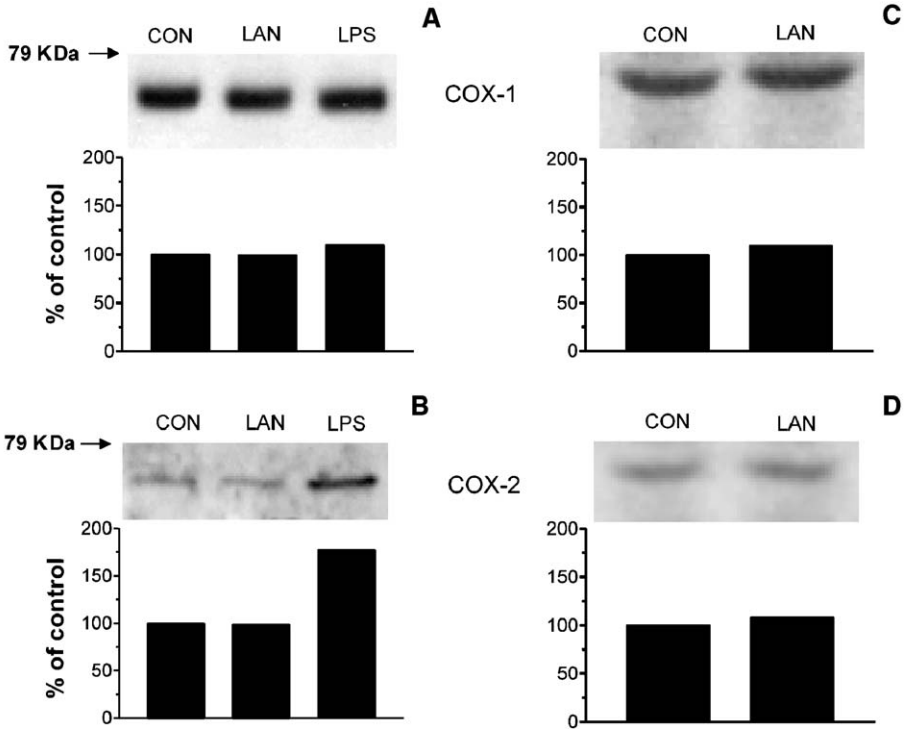

Fig. 5. Rats subjected to pylorus ligation for 2 h. Western blot analysis of COX-1 and COX-2 protein expression in gastric mucosal lysates obtained from animals treated with single intraduodenal dose of lansoprazole 90 μmol/kg (LAN), single intragastric dose of lipopolysaccharide 5 mg/kg (LPS) (A, B), or intragastric LAN for 8 days (C, D). Each panel displays a representative blot and a column graph referring to the densitometric analysis of immunoreactive bands. CON = control.

control animals of the former group, mucosal GSH levels were 0.052 ± 0.005 nmol/mg. Under these conditions, lansoprazole did not significantly affect the mucosal concentration of endogenous sulfhydryl compounds. However, the intraluminal instillation of ethanol–HCl was associated with a significant decrease in mucosal GSH levels that was partly counteracted in animals pretreated with lansoprazole (Fig. 4A). Similar results were obtained when assaying GSH concentrations in the gastric mucosa of animals exposed to lansoprazole for 8 days (Fig. 4B).

Animals subjected to pylorus ligation

Effects of lansoprazole on the concentrations of PGE_2, bicyclo-PGE_2, and NO_x in the gastric juice

The concentrations of PGE_2, bicyclo-PGE_2, and NO_x were determined in samples of gastric juice collected from animals treated with lansoprazole as single dose or repeated administrations ($n = 6–8$). In the former group, lansoprazole administration did not significantly modify PGE_2 and bicyclo-PGE_2 release into the gastric juice (Table 1). Similar results were obtained for the assay of PGE_2 and bicyclo-PGE_2 in the gastric juice of animals treated with lansoprazole for 8 days (Table 1). In both single-dose and repeated treatment groups, lansoprazole did not significantly affect the release of NO breakdown products into the gastric lumen (Table 1).

Effects of lansoprazole on the expression of COX-1 and COX-2 in the gastric mucosa

The protein expression of COX isoforms was evaluated by Western blot analysis of gastric mucosal samples obtained from animals subjected to either single or repeated administration of lansoprazole. After pylorus ligation for 2 h, Western blot analysis revealed the expression of both COX-1 and COX-2 in the gastric mucosa of control animals as well as in rats treated with single-dose lansoprazole (Figs. 5A, B). The densitometric analysis of immunoreactive bands revealed that the relative expression of COX-1 or COX-2, in the presence of lansoprazole, did not differ from that estimated in control animals. However, separate experiments showed that the intraluminal injection of bacterial lipopolysaccharide was followed by a marked enhancement of COX-2, but not COX-1, expression in the gastric mucosa (Figs. 5A, B), indicating that in rat stomachs the gene encoding for COX-2 can be rapidly induced upon application of appropriate stimuli. Similar results were obtained when examining the effects of lansoprazole on gastric COX-1 and COX-2 expression after a treatment course of 8 days (Figs. 5C, D).

In vitro assay of antioxidant activity of lansoprazole

In control experiments, 8-*iso*-$PGF_{2\alpha}$ concentration was 1200.03 ± 73.64 pg/ml ($n = 5$; Table 2). In the presence of lansoprazole 10 or 100 μM, 8-*iso*-$PGF_{2\alpha}$ production underwent a significant decrease, with percent reduction of 26.9% and 84.6%, respectively (Table 2).

Table 2
Antioxidant activity of lansoprazole

	8-*iso*-$PGF_{2\alpha}$ (pg/ml)
Control	1200.03 ± 73.64
Lansoprazole 10 μM	876.83 ± 61.23*
Lansoprazole 100 μM	185.19 ± 27.26*

Data are presented as means ± standard error of the mean (SEM) and represent the mean values obtained from five experiments.
*$P < 0.05$: significant difference from control value.

Discussion

Several lines of evidence indicate that, beside the marked inhibition of gastric acid secretion, the antiulcer effects of benzimidazole derivatives may depend on acid-independent protective actions (Blandizzi et al., 1999a; Kawano et al., 1992; Takahashi et al., 1997). In the present study, the gastroprotective properties of lansoprazole were assayed in a model of gastric injury elicited by intraluminal application of ethanol and HCl, and the histomorphometric analysis of gastric mucosa clearly demonstrated that this drug significantly prevent the occurrence of the most severe types II and III lesions. These findings are in keeping with data obtained in a previous investigation where lansoprazole counteracted the gastric mucosal damage evoked by ethanol–HCl or hemorrhagic shock at mean effective doses that differed from those ensuring a marked blockade of acid secretion (Blandizzi et al., 1999a).

The intragastric instillation of ethanol–HCl, like other gastric-injurying agents, is known to evoke acute tissue edema, subepithelial hemorrhages, cellular exfoliation, and time-dependent infiltration by inflammatory cells that may contribute to the induction of mucosal injury through the generation of reactive oxygen species (Kountouras et al., 2001; Kwicien et al., 2002). Ethanol is also able to induce a direct oxidative damage against gastric mucosal tissues (Terano et al., 1989). In the present study, the luminal application of ethanol–HCl was followed by a significant increment in the mucosal content of both MDA and 8-*iso*-$PGF_{2\alpha}$, two endogenous compounds that were assayed as indices of tissue oxidative damage. MDA is one of the end products resulting from peroxidation of polyunsaturated fatty acids and related esters within cell membranes, and the measurement of this substance represents a suitable index of lipid peroxidation (Kwicien et al., 2002). 8-*Iso*-$PGF_{2\alpha}$ is also regarded as a specific index of lipid peroxidation, because prostaglandin-related compounds, termed isoprostanes, are produced by direct free radical-mediated peroxidation of arachidonic acid or prostaglandins (Morrow and Roberts, 1997). Among isoprostanes, tissue accumulation of 8-*iso*-$PGF_{2\alpha}$ has been proposed as a reliable marker of endogenous antioxidant deficiency or oxidative stress. At

the same time, this eicosanoid displays relevant biological activities, including vasoconstriction and platelet aggregation, that might contribute to enhance the ulcerogenic actions of necrotizing agents able to promote tissue oxidation, such as ethanol–HCl. Although the putative influence of 8-*iso*-$PGF_{2\alpha}$ on gastric mucosal integrity has not been currently clarified, it is noteworthy that this isoprostane stimulates the contractile activity of isolated gastric fundus through the activation of TP receptor subtypes (Sametz et al., 2000). Overall, because in our experiments lansoprazole counteracted the generation of MDA and 8-*iso*-$PGF_{2\alpha}$ promoted by ethanol–HCl within gastric mucosa, it is conceivable that this proton pump blocker is endowed with antioxidant properties that may play a relevant role in mediating its gastroprotective action.

Two major mechanisms might support the antioxidant effects displayed by lansoprazole in the present study, since this drug could exert a direct scavenging activity against reactive oxygen species or inhibit the oxidative metabolism of activated inflammatory cells. To address this issue, a series of specific experiments was performed in an in vitro system, and evidence was obtained that similarly to in vivo findings, lansoprazole was able to directly counteract the formation of 8-*iso*-$PGF_{2\alpha}$ originated by oxidation of native LDLs. These results are in line with the report by Lapenna et al. (1996) where omeprazole, another proton pump blocker, was shown to markedly scavenge hypochlorous acid as well as to inhibit iron- and copper-driven oxidative reactions in in vitro systems. As far as the indirect antioxidant mechanisms are concerned, it is known that besides parietal cells, a proton pump-driven acidification occurs also in phagolysosomes of activated polymorphonuclear neutrophils that are then responsible for an increased generation of free oxygen radicals at inflammatory sites (Segal and Geisow, 1981). This process appears to be sensitive to the pharmacological blockade by proton pump inhibitors (Agastya et al., 2000; Suzuki et al., 1996). Indeed, omeprazole inhibited the oxidative burst of polymorphonuclear neutrophils in vitro (Suzuki et al., 1996; Wandall, 1992). Moreover, lansoprazole blocked the oxygen-derived free radical output from neutrophils activated by *Helicobacter pylori* (Suzuki et al., 1995) and reverted the increase in plasma levels of peroxidated lipids in patients with duodenal ulcer (Manjari and Das, 1998). However, polymorphonuclear infiltration does not seem to occur early after ethanol administration (Robert et al., 1992), and in the present study, the examination of gastric mucosa, 90 min after intraluminal ethanol–HCl injection, did not reveal a significant number of infiltrating polymorphonuclear cells. Overall, in our experimental model, the antioxidant action of lansoprazole against oxygen radicals generated during inflammatory processes (Suzuki et al., 1995) does not appear to be the major mechanism accounting for gastroprotection. It can be rather suggested that lansoprazole behaves as an antioxidant agent, exerting a direct inhibitory influence on cellular oxidative injury elicited by intragastric injection of ethanol (Kwicien et al., 2002; Matsumoto et al., 1993).

Sulfhydryl compounds have been significantly implicated in the maintenance of gastric integrity, particularly when reactive oxygen species are involved in the pathophysiology of tissue damage (Kimura et al., 2001; Tepperman and Jacobson, 1994). Indeed, the tripeptide GSH participates in many aspects of oxidative metabolism, including the removal of hydroperoxides, the protection from ionizing radiations, the maintenance of the physiological sulfhydryl status of proteins, and the condensation with xenobiotics or endogenous reactive compounds, to aid their detoxification and excretion (Hayes and McLellan, 1999; Loguercio and Di Pierro, 1999). In this respect, the present decrease in mucosal GSH content, evoked by ethanol–HCl, was not unexpected because in previous studies, ethanol-induced gastric damage was associated with a significant reduction of mucosal GSH levels in both humans (Loguercio et al., 1991) and experimental animals (Takeuchi et al., 1989; Victor et al., 1991). Moreover, the administration of GSH prevented the ethanol-induced gastric injury in humans (Loguercio et al., 1991), and treatments with sulfhydryl donor drugs protected the gastric mucosa against injuries evoked by necrotizing agents, stress, or ischemia (Tepperman and Jacobson, 1994). On this basis, because the present antioxidant effects of lansoprazole might result in a preservation of mucosal GSH levels, it is likely that an increased bioavailability of endogenous sulfhydryl compounds play a significant role in the gastroprotective properties of this proton pump inhibitor. In support of this view, *N*-ethylmaleimide, a potent blocker of sulfhydryl compounds, fully prevented the protective effects of lansoprazole against ethanol–HCl (Blandizzi et al., 1999a). Furthermore, it has been reported that the GSH redox cycle is specifically involved in the protection of gastric epithelial cells against ethanol (Hiraishi et al., 1999).

The involvement of prostaglandins in the antiulcer action of proton pump inhibitors is currently controversial. For instance, lansoprazole did not seem to affect the prostaglandin synthetic activity in rat gastric mucosa (Fukuda et al., 1995), but its protective effect against ethanol–HCl was partly prevented by COX inhibition (Blandizzi et al., 1999a). To address this issue, we performed a series of experiments on rats subjected to pylorus ligation for 2 h, a procedure that allows a certain degree of mucosal oxidation (Rastogi et al., 1998), without the occurrence of relevant mucosal ulceration and intraluminal blood leakage. Evidence was thus obtained that lansoprazole did not significantly increase the intraluminal release of both PGE_2 and bicyclo-PGE_2, a stable compound resulting from the conversion of all PGE_2 metabolites. In addition, lansoprazole did not modulate the expression of COX-1 or COX-2 at level of the gastric mucosa, as indicated by Western blot analysis. Therefore, lansoprazole does not seem to exert a positive influence on gastric PGE_2 levels, at least in the present experimental model. In support of this view, omeprazole failed to affect the in vitro production of prostaglandins when applied to cultured gastric mucosal cells (Ota

et al., 1993). More recently, Tsuji et al. (2002) reported that lansoprazole can induce both COX-2 expression and PGE_2 production in the gastric mucosa via an increase in gastrin secretion. Differences in the experimental conditions might explain the discrepancies between this report and our findings, because in the study by Tsuji et al. (2002), lansoprazole was consecutively administered for 14 days at very high doses [up to 150 μ mol/kg/day).

NO is an important endogenous transmitter produced from L-arginine by either constitutive or inducible NO synthase, and both enzymes have been detected in the rat gastric mucosa by histochemical (Price et al., 1996) and biochemical evaluations (Brown et al., 1992). There is evidence that NO may exert either protective or detrimental effects on the pathophysiology of gastroprotection (Lopez et al., 1993). The present results show that lansoprazole does not affect the concentration of NO_x in the secretory fluids collected from gastric lumen, suggesting that the NO pathway does not play a relevant role in the protective actions of this proton pump blocker. In line with this finding, the NO synthase inhibitor N^G-nitro-L-arginine was not able to counteract the protective effect of lansoprazole against ethanol–HCl in a previous study (Blandizzi et al., 1999a).

In conclusion, the present results suggest that the protective effects of lansoprazole can be ascribed to a reduction of gastric oxidative injury, which results also in an increased bioavailability of mucosal sulfhydryl compounds. It is also proposed that lansoprazole does not exert modulator effects on the gastric expression and enzyme function of COX isoforms as well as on the activity of NO pathways.

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