Induction of Cyclooxygenase-2 in Rat Gastric Mucosa by Rebamipide, a Mucoprotective Agent

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ABSTRACT

Recent studies indicate an expression of mitogen-inducible cyclooxygenase (COX-2) in gastric mucosa. Rebamipide, a mucoprotective agent enhances prostaglandin (PG) synthesis. The present study was designed to clarify the mechanism for rebamipide-induced mucosal protection. Male Sprague-Dawley rats were administered 5, 15, or 50 mg/kg/day rebamipide for 14 days. The expression of constitutive cyclooxygenase (COX-1) and COX-2 in gastric mucosa was determined using Western blot analysis. Another series of rats was used to examine 1) the levels of PGE2 in stomach with and without pretreatment with a COX-2 inhibitor; 2) the protective action of rebamipide against gastric damage caused by 0.6 N HCl; and 3) the effects of a COX-2 inhibitor on rebamipide-induced gastric mucosal protection.

Prostaglandins (PGs) such as PGE2 have potent effects on gastric mucosal protection (Robert, 1979; Miller, 1983; Guth et al., 1984). Endogenous PG synthesis also has an important role in gastric mucosal defense (Robert et al., 1983; Arakawa et al., 1990). Synthesis of PGs is governed by PG endoperoxide synthase, or cyclooxygenase (COX: EC 1.14.99.1), which consists of two isoforms (Masferrer et al., 1996). The constitutive isoform (COX-1) is dominantly expressed in platelets, prostate, and stomach. Expression of the mitogen-inducible isoform (COX-2) is enhanced in gastric epithelial cells after growth stimulation in vitro and in gastric epithelium after acid-induced damage in vivo (Tsui et al., 1996; Sawaoka et al., 1997; Sun et al., 2000). Furthermore, COX-2-specific inhibitors delay healing of acetic acid-induced gastric ulcers in mice (Mizuno et al., 1997; Sun et al., 2000), suggesting an important role for this isozyme in peptic ulcer healing.

Rebamipide, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinone-4-yl]-propionic acid, an antiulcer agent, prevents various acute and chronic gastric mucosal lesions and accelerates the healing of chronic ulcers (Yamasaki et al., 1987, 1989; Kawano et al., 1991; Kleine et al., 1993; Suzuki et al., 1994; Kim and Hong, 1995; Watanabe et al., 1996; Kokura et al., 1997, 1998; Kim et al., 1998). In particular, rebamipide has cytoprotective actions in humans and animals (Yamasaki et al., 1987; Kawano et al., 1991). This compound increases PG levels in gastric tissue and protects gastric mucosa from various insults (Kleine et al., 1993). The mechanism of rebamipide-induced gastric mucosal protection, however, is not known.

Therefore, the present study was designed to clarify the effects of rebamipide on expression of COX-1 and COX-2 in rat gastric mucosa. Because long-term effects of the agent on gastric mucosal protection would be clinically relevant, we focused our attention on the effects of 14-day administration of rebamipide on gastric mucosal expression of COX-1 and COX-2, on gastric mucosal levels of PGE2, and on gastric mucosal protection in rats.

Materials and Methods

Animals and Agents. Specific pathogen-free male Sprague-Dawley rats aged 7 weeks and weighing 200 to 230 g were fed with

ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; CMC, carboxymethylcellulose; EGF, epidermal growth factor; EGF-R, epidermal growth factor-receptor; NF-κB, nuclear factor-κB.
Effects of Rebamipide on Expression of COX-1 and COX-2 in Rat Gastric Mucosa. The rats were randomly divided into four groups, six rats per group. To the control animals (group 1), only 0.5% CMC was given. Groups 2 through 4 rats were administered oral rebamipide at doses of 5, 15, and 50 mg/kg once a day. After 14 days of drug administration, these rats were anesthetized with sevoflurane, sacrificed, and immediately laparatomized. The stomach was harvested and opened along to greater curvature. The oxyntic mucosa was scraped with glass slides, immediately frozen in liquid nitrogen, and stored for Western blotting analysis for COX-1 and COX-2.

The gastric mucosal samples were homogenized in phosphate-buffered saline containing 1% Nonidet-40, 0.5% sodium deoxycholate, and 0.1% SDS. Protein concentration of the homogenate was measured using a protein assay reagent (BCA kit; Pierce, Rockford, IL). The tissue homogenates, 100 μg of protein per lane, were electrophoresed in 10% SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore Corp., Bedford, MA) using a semidry transfer cell (Bio-Rad, Hercules, CA). The blots were pretreated in Tris-buffered saline containing 5% nonfat dry milk, 1% albumin, and 0.1% Tween 20, and incubated with antibodies for COX-1 and COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Filters were washed three times and incubated with a horseradish peroxidase-conjugated secondary antibody against goat IgG (Dako, Glostrup, Denmark), developed using a commercial enhanced chemiluminescence system (Amersham Pharmaacia Biotech Ltd., Buckinghamshire, UK), and exposed to films (Hyperfilm; Amersham Pharmacia Biotech Ltd.). The expression of COX-1 and COX-2 was semiquantified using a densitometric scanner.

For immunohistochemical analysis, gastric corpus tissue from rats treated with vehicle or 50 mg/kg rebamipide was fixed in 10% phosphate-buffered formalin. Four-micrometer-thick sections were cut from paraffin-embedded tissues and were deparaffinized. Sections were then microwaved in citrate buffer, pH 6.1, 95°C, for 10 min for antigen retrieval. Endogenous peroxidase activity was quenched by incubation in 3% H2O2 in methanol for 30 min. Nonspecific binding was blocked with 3% normal rabbit serum in phosphate-buffered saline, and sections were incubated in primary antibodies overnight at 4°C. Primary antibodies raised in goat against COX-1 or COX-2 were used alone (1:200 dilution) or after preincubation with 2 μg/ml pure blocking COX-1 or COX-2 peptide (Santa Cruz Biotechnology) as preabsorption controls. They were stained according to the avidin-biotin-peroxidase complex (ABC) method using a commercial kit (Vectastain kit; Vector Laboratories, Burlingame, CA) and visualized by 3,3′-diaminobenzidine (Vectastain DAB kit; Vector Laboratories). Subsequently, the specimens were counterstained with hematoxylin.

Effects of Rebamipide and NS-398 on PGE2 Levels in Rat Gastric Mucosa. Another series of 36 rats, 6 animals per group, was randomly assigned. Groups 1 through 4 were given either rebamipide (5, 15, or 50 mg/kg) or 0.5% CMC for 14 days. These groups were treated with 0.5% CMC 10 h after the 14-day administration of rebamipide or CMC. Group 5 rats were given 30 mg/kg NS-398 after daily administration of rebamipide (50 mg/kg) for 14 days. Group 6 animals were given 0.5% CMC as a vehicle and then NS-398 (30 mg/kg). One hour later, the rats were anesthetized and administered 1 ml of 0.6 N HCl through orogastric tubing (7 French feeding tube; Top, Tokyo, Japan). One hour later, the rats were sacrificed and laparatomized. The stomach was harvested, opened along the greater curvature, extended on a plastic board, and photographed. The area of the macroscopic hemorrhages and erosions was assessed using planimetry. The ulcer index was expressed as a percentage of the lesion area to the total gastric glandular area.

For histologic assessment, the gastric corpus wall was fixed in phosphate-buffered formalin, sectioned, and paraffin embedded. Semithin sections were deparaffinized, stained with H&E, and examined under a light microscope by a pathologist blind to the group to which the specimen belonged. The specimens were coded and assessed according to the criteria of Whittle et al. (1990). In brief, a 1-cm length of each histologic section was assessed for epithelial cell damage (score 1); glandular disruption, vasocongestion, or edema in the upper mucosa (score 2); hemorrhagic damage in the mid to lower mucosa (score 3); and deep necrosis and ulceration (score 4). Each section was evaluated on a cumulative basis to give the histologic score, the maximum score thus being 10.

Statistical Analyses. Data were shown as mean ± S.E., and were analyzed using one-way ANOVA with Dunnett’s multiple comparison test. A probability value less than .05 was considered statistically significant.

Results

Effects of Rebamipide on Expression of COX-1 and COX-2 in Rat Gastric Mucosa. In the control rats treated with 0.5% CMC, the gastric mucosa expressed COX-1. The mucosa also expressed small amounts of COX-2. In the groups treated with rebamipide (5, 15, or 50 mg/kg) for 14 days, the expression of COX-1 did not change compared with that of the control. Expression of COX-2 was enhanced in the animals after daily administration of rebamipide for 14 days. The level of COX-2 immunoreactivity increased after administration of rebamipide in a dose-dependent manner (Fig. 1). The specificity of the COX-1 and COX-2 antibodies was confirmed by neutralization study with the corresponding blocking peptides (data not shown).

Furthermore, we determined the cellular localization of COX-1 and COX-2 protein by immunohistochemical staining. COX-1 immunostaining was seen in the gastric epithelial cells and mucous neck cells in the control rats (Fig. 2A). Rebamipide administration did not cause dramatic changes in the location of COX-1 immunoreactivity (Fig. 2B). Specific
Histologic assessment demonstrated that hemorrhagic damage to mid to lower mucosa was infrequently associated with deep necrosis in rats treated with CMC and then HCl. In contrast, in the groups treated with rebamipide and then HCl, the gastric mucosal injury was restricted to glandular disruption and damage within the upper part of the mucosa. These results indicate that rebamipide protects gastric mucosa from acid-induced injury. On the other hand, there were no significant differences in the histologic score between the group given CMC and the group given CMC and NS-398. Therefore, NS-398 did not aggravate the HCl-induced microscopic injury of stomach. The histologic score was significantly larger in the group given rebamipide and NS-398 than in the group given rebamipide and CMC (Fig. 5). Consequently, treatment with NS-398 inhibited gastric mucosal protection by rebamipide.

Discussion

PGs have long been implicated in protection of gastric mucosa from various insults and acceleration of gastric wound healing (Miller, 1983). PGE$_2$ is one of the major prostanoids in gastric tissue. Rebamipide enhances endogenous production of PGs, particularly that of PGE$_2$ in the stomach, and protects gastric mucosa from injury. The precise mechanism for PG-synthesis stimulated by this mucoprotective agent is not yet known. Because COX is the rate-limiting enzyme for production of PGs from arachidonate, we investigated the influence of rebamipide on expression of the two isoforms of COX. The present study demonstrated that rebamipide, which was administered to the animals for 14 days, enhanced expression of mitogen-inducible COX-2, but not that of constitutive COX-1. Rebamipide also increased PGE$_2$ in gastric mucosa in a dose-dependent manner. The rebamipide-induced increase in gastric mucosal PGE$_2$ was blocked by a COX-2-specific inhibitor. Therefore, 14-day administration with rebamipide increases gastric mucosal PGE$_2$ by enhancing COX-2 expression.

After the discovery of the mitogen-inducible COX-2 (Fletcher et al., 1992; Kujubu and Herschman, 1992), Seibert et al. (1994) examined mRNA levels of COX-1 and COX-2 in normal rat tissue using RNAse protection assay. COX-1 mRNA was readily detectable in all normal tissue examined, whereas levels of COX-2 mRNA were substantially lower with the exception of brain. Our previous study also demonstrated that unstimulated gastric mucosa expresses smaller amount of COX-2, whereas gastric mucosal epithelium expresses this isoform after various stimuli (Sawaoka et al., 1997; Sun et al., 2000). The present study confirmed that gastric mucosa expresses both COX-1 and COX-2 in control rats treated with vehicle. Our result is in accord with previous findings demonstrating COX-2 expression in the stomach (Iseki, 1995; Zimmermann et al., 1998).

On the other hand, daily administration of rebamipide for 14 days enhanced COX-2 expression in gastric mucosa and significantly reduced both macroscopic injury and histologic damage caused by HCl in a dose-dependent manner. We did not examine the protective effects of rebamipide against the other different types of gastric injury. However, previous studies have shown that rebamipide reduces or prevents experimental acute gastric mucosal injury caused by nonsteroidal anti-inflammatory drugs, restraint and water-immer-

Fig. 1. Expression of COX-1 and COX-2 in rat gastric mucosa after intraluminal administration of 0.5% CMC and rebamipide (5, 15, or 50 mg/kg/day) for 14 days. Numbers in parentheses after R indicate doses of rebamipide in milligrams per kilogram. A, Western blotting analysis for COX-1 and COX-2. B, quantified data using densitometry. Data are expressed as the percentages of the densities of the CMC-treated control, shown as mean ± S.E., n = 6 per group, and analyzed using ANOVA with Dunnett’s multiple comparison test of the means. *P < .05 versus the control group treated with CMC.

immunostaining for COX-2 was observed in the gastric epithelial cells at the bottom of the fundic glands in the control rats (Fig. 2D). After 14 days administration of rebamipide; however, COX-2 immunoreactivity was strongly detected at the bottom of the fundic glands and mucous neck cells (Fig. 2E). When the antibody preincubated with blocking COX-1 peptide (Fig. 2C) or COX-2 peptide (Fig. 2F) was applied to the sections, no immunoreactive signals appeared.

Effects of Rebamipide and NS-398 on Acid-Induced Gastric Mucosal Injury. In rats given 0.5% CMC, significant hemorrhagic streaks and erosions developed mainly in the gastric corpus mucosa 1 h after the intragastric administration with 0.6 N HCl. In the groups given rebamipide (5, 15, or 50 mg/kg), however, the ulcer index was significantly smaller than in the control group treated with CMC. On the other hand, there were no significant differences in the ulcer index between the group given CMC and the group given CMC and NS-398. Therefore, NS-398 did not aggravate the HCl-induced macroscopic injury of the stomach. The ulcer index was significantly higher in the group given rebamipide and NS-398 than the group given rebamipide and CMC (Fig. 4).
sion stress, histamine (Yamasaki et al., 1989), ischemia-reperfusion (Kim and Hong, 1995) and necrotizing agents such as absolute ethanol and 0.2 N NaOH (Yamasaki et al., 1987). The present study clearly shows that rebamipide-induced gastric mucosal protection was associated with an increase in gastric mucosal PGE\(_2\). NS-398 (30 mg/kg), a specific inhibitor of COX-2, significantly blocked the rebamipide-induced gastric mucosal protection against HCl, as well as the rebamipide-induced increase in gastric mucosal PGE\(_2\). Several studies (Futaki et al., 1993; Masferrer et al., 1994; Ogino et al., 1997) have confirmed that NS-398 inhibited COX-2 with an IC\(_{50}\) of 0.32 \(\mu\)M, but never affected COX-1 activity, even at 100 \(\mu\)M. Futaki et al. (1993, 1997) reported that NS-398 even at 100 mg/kg did not significantly suppress COX-1 activity in rats. In the previous studies we confirmed that NS-398 specifically inhibits COX-2 in the doses ranging from 0.4 to 40 mg/kg in rats (Sun et al., 2000) and from 10 to 100 mg/kg in mice (Sawaoka et al. 1998). Therefore, at the dose used in this study, NS-398 is sufficient to inhibit COX-2 without significant influences to COX-1 activity. The protective effects of rebamipide appear to be mediated by PGs, such

![Fig. 2. Immunohistochemical staining of COX-1 (A–C) and COX-2 (D and F) in rat gastric tissues. Photographs A and D were taken from mucosa of rats treated with CMC; the others were taken from mucosa of rats treated with rebamipide (50 mg/kg). COX-1 immunoreactivity was detected in surface epithelial cells (A and B). COX-2 immunoreactivity was sparse in vehicle-treated rats (D). Fourteen days of rebamipide administration resulted in a marked increase in COX-2 immunoreactivity, particularly in epithelial cells lining the middle to deep portions of the gastric glands (E). When the antibody preincubated with blocking COX-1 (C) or COX-2 (F) peptide was applied to the rebamipide-treated gastric tissue, no immunoreactive signals appeared at these cells. Original magnification, 100×.](image)

![Fig. 3. Effects of 0.5% CMC, rebamipide (5, 15, or 50 mg/kg/day for 14 days), and NS-398 (COX-2 inhibitor; 30 mg/kg) after CMC or rebamipide (50 mg/kg/day for 14 days) on gastric mucosal PGE\(_2\). The PGE\(_2\) level in the gastric mucosa was expressed as picograms of PGE\(_2\) per gram tissue. *P < .05, **P < .01 versus the control group treated with 0.5% CMC, and †P < .01 versus the group treated with rebamipide (50 mg/kg/day for 14 days) and then 0.5% CMC. Data are shown as mean ± S.E., n = 6 per group, and analyzed using ANOVA with Dunnett’s multiple comparison test of the means.](image)
progression, and nuclear factor-κB (NF-κB) (Reddy et al., 2000; Wadleigh et al., 2000). We did not examine which transcription factor(s) contributes to the up-regulation of COX-2 expression in the present study. However, a number of studies have suggested that rebamipide is an oxygen radical scavenger and thereby inhibits lipid peroxidation (Naito et al., 1995; Hahm et al., 1997) and oxidant-mediated activation of NF-κB (Kim et al., 2000). Therefore, it is likely that transcriptional regulatory elements other than NF-κB play an important role in the rebamipide-induced expression of COX-2. The precise mechanism for COX-2 induction caused by long-term administration of rebamipide remains to be investigated in future studies.

In conclusion, the present results clearly demonstrate that intragastric administration of rebamipide induces COX-2 expression in gastric mucosa, increases gastric mucosal PGE₂ levels, and protects gastric mucosa from necrotizing agents in rats. Treatment with NS-398, a specific COX-2 inhibitor, abolishes the rebamipide-induced increase in gastric mucosal PGE₂ and protection of gastric mucosa from acute injury. In this experimental ulcer model of ulcers in rats, the mucoprotective effects of rebamipide depend on COX-2-induced gastric mucosal defense by increasing COX-2-dependent production of PGs in gastric mucosa.

References


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