Gastrin enhances gastric mucosal integrity through cyclooxygenase-2 upregulation in rats

MASATO KOMORI,1* SHINGO TSUJI,1 WEI-HAO SUN,1* MASAKI TSUJII,1 NAOKI KAWAI,1 MASAKAZU YASUMARU,1 YOSHIHI KAKIUCHI,1 ARATA KIMURA,1 YUTAKA SASAKI,1 SHIGEHI HIGASHIYAMA,2,3 SUNAO KAWANO,3 AND MASATSUGU HORI1

Department of Internal Medicine and Therapeutics, 1Department of Biochemistry, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, and 3Department of Clinical Laboratory Science, school of allied Health Sciences, Osaka University Faculty of Medicine, 1-7 Yamadaoka, Suita, 565-0871, Japan

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The aim of the present study was to clarify the effects of exogenous gastrin on gastric mucosal protection in rats and to examine whether HB-EGF, COX-1 and -2, and PGE2 are involved in gastrin-induced gastric mucosal protection.

GASTRIN IS A POLYPEPTIDE HORMONE that is synthesized in gastrin cells and has an important role in modulating various functions in the gastrointestinal tract, including acid secretion, motility, and cell proliferation. Most of these actions are mediated by gastrin/CCKB receptors (21). In rats, gastrin and pentagastrin protect against gastric mucosal damage caused by stress or intragastric ethanol instillation (18, 26, 34). The mechanisms of gastric mucosal protection by gastrin are not yet clear.

PGs and growth factors have important roles in maintaining gastrointestinal mucosal integrity, repair of gastrointestinal mucosal injury, and ulcer healing (1, 15, 24, 35). Synthesis of PGs is governed by PG endoperoxide synthase or cyclooxygenase (COX; EC 1.14.99.1), which is comprised of two isoforms (16, 44). The constitutive isoform (COX-1) is dominantly expressed in platelets, prostate, and stomach. The mitogen-inducible isoform (COX-2) is negligibly expressed in normal stomach (10, 30). COX-2 expression, however, is enhanced in gastric epithelial cells after growth stimulation in vitro (28, 29) and in gastric epithelium after acid-induced damage in vivo (10, 30). We recently demonstrated that COX-2 protein is overexpressed during healing of gastric lesions and that COX-2-specific inhibitors delay the healing in rats, suggesting an important role for this isozyme in gastric ulcer healing (32, 37).

On the other hand, heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a potent growth and proliferation stimulatory polypeptide for a number of cells including gastric epithelial cells. A recent study indicates that gastrin upregulates activity of growth-related proteins including HB-EGF (20). The biological roles of HB-EGF on gastric mucosal protection are not yet clear.

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* M. Komori and W.-H. Sun contributed equally to this work.

Address for reprint requests and other correspondence: S. Tsuji, Dept. of Internal Medicine and Therapeutics, Osaka Univ. Graduate School of Medicine, 2-2 Yamadaoka, Suita, 565-0871, Japan (E-mail: tsuji@medone.med.osaka-u.ac.jp).
MATERIALS AND METHODS

Animals and agents. Specific pathogen-free male Sprague-Dawley rats (Nippon SLC, Shizuoka, Japan), aged 6 wk and weighing ~150 g, were fed standard pellet chow and water ad libitum. The rats were deprived of food for 24 h but allowed free access to water before initiation of the experiments. On the day of experimentation, all animals were randomly assigned to one of the following treatment groups (n = 7/group). All of the experiments were performed according to the guidelines of the Institutional Committee on Experimental Animals.

Synthetic rat gastrin 17 (Sigma Chemical, St. Louis, MO) was dissolved in saline containing 1% BSA (Sigma Chemical). Proton pump inhibitor lansoprazole (a gift from Takeda Chemical, Osaka, Japan) was dissolved in 0.5% carboxymethylcellulose (CMC; Sigma Chemical). Potent and specific gastrin receptor antagonist YM022 (a gift from Yamanouchi Pharmaceutical, Ibaraki, Japan) and specific COX-2 inhibitor NS-398 (Cayman Chemical, Ann Arbor, MI) were dissolved in polyethylene glycol with a molecular weight of 300 (PEG-300). The other agents were purchased from Nacalai Tesque (Kyoto, Japan), unless stated otherwise.

Radioimmunoassay of serum gastrin levels. Before and 1, 3, 6, 12, and 24 h after subcutaneous injection with 100 µg/kg rat gastrin 17 and 1 h after subcutaneous injection with vehicle, saline containing 1% BSA in a volume of 1 ml/kg, or rat gastrin 17 at doses of 1, 10, or 100 µg/kg, respectively, the rats were anesthetized with sevoflurane, and blood was drawn by cardiac puncture. The blood was centrifuged at 2,500 g for 10 min, and serum was collected and stored at −20°C until determination of gastrin (Gastrin-RIA kit II; Dainabot, Tokyo, Japan).

Western blot analysis. The rats were divided into four groups. The first group was subcutaneously injected with the vehicle, saline containing 1% BSA in a volume of 1 ml/kg. Groups 2–4 were subcutaneously injected with rat gastrin 17 at doses of 1, 10, or 100 µg/kg, respectively. One hour later, they were killed by cervical dislocation under anesthesia and laparotomized. The stomach was harvested and opened along the greater curvature. The oxyntic mucosa was scraped with glass slides and immediately frozen in liquid nitrogen and stored at −80°C for Western blot analysis for hepatocyte growth factor (HGF), HB-EGF, COX-1, and COX-2 expression. The gastric mucosal samples were homogenized in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. Protein concentrations of the homogenized samples were measured using a protein assay reagent (BCA kit, Pierce, Rockford, IL). These homogenized tissues, 100 µg protein per lane, were electrophoresed in 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, 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 incubated with antibodies for HGF, HB-EGF, COX-1, and COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes 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 (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK), and exposed to films (Hyperfilm, Amersham Pharmacia Biotech). HB-EGF and COX-2 expression was semiquantified using a densitometric scanner.

For the time sequence study, the rats received subcutaneous injection with 100 µg/kg rat gastrin 17 and were killed 0, 1, 3, 6, 12, or 24 h after the injection. The stomachs were quickly removed, and HGF, HB-EGF, COX-1, and COX-2 expression in gastric mucosa was examined by Western blot analysis. HB-EGF and COX-2 expression were semiquantified using a densitometric scanner.

The rats were given subcutaneous rat gastrin 17 (100 µg/kg) along with intraperitoneal pretreatment of the selective gastrin/CCK-B receptor antagonist YM022 (10, 30, or 100 mg/kg) or equal volume (5 ml/kg) of the vehicle (PEG 300) or saline containing 1% BSA in a volume of 1 ml/kg with pretreatment of the vehicle (PEG 300). One hour later, the stomachs were quickly removed, and HGF, HB-EGF, COX-1, and COX-2 expression in the gastric mucosa was examined by Western blot analysis. HB-EGF and COX-2 expression was semiquantified using a densitometric scanner.

Immunohistochemistry. To determine the effects of a specific HB-EGF-neutralizing antibody on gastrin-induced COX-2 expression, a rat was killed by cervical dislocation under anesthesia and laparotomized. The stomach was harvested, opened along the greater curvature, and cut into small pieces. The pieces were incubated at 37°C with or without 10−8 g/ml rat gastrin 17 and 10−10 g/ml rat gastrin 17 and 10 µg/ml rat HB-EGF-neutralizing antibody in DMEM (GIBCO-BRL, Grand Island, NY) containing 0.1% BSA in Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY). One hour later, the specimens were fixed in 10% buffered formalin. Four-micrometer-thick sections cut from paraffin-embedded tissues were deparaffinized. Sections were then microwave in citrate buffer (pH 6.1, 95°C) for 10 min for antigen retrieval. Endogenous peroxidase activity was quenched by incubation in 3.0% H2O2 in methanol for 30 min. Nonspecific binding was blocked with 3% normal rabbit serum in PBS, and the tissues were incubated with primary goat polyclonal antibodies against COX-2 (1:200 dilution) overnight at 4°C in 1.5% normal rabbit serum. They were stained using the avidin-biotin-peroxide complex method with a commercial kit (Vestecin kit; Vector Laboratories, Burlingame, CA) and visualized using 3,3′-diaminobenzidine (DAB; Vestecin DAB kit, Vector Laboratories). Subsequently, the specimens were counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

Furthermore, the pieces were incubated at 37°C with or without 10−8 g/ml human recombinant HB-EGF (Genzyme/Teche, Minneapolis, MN) or with 10−8 g/ml human recombinant HB-EGF and pretreatment with 1 µM specific inhibitor of EGF receptor (BIOMOL Research Laboratories, Plymouth Meeting, PA) in DMEM containing 0.1% BSA in chamber slides. One hour later, the specimens were fixed and COX-2 expression was examined as described above.

Enzyme immunoassay of PGE2 levels in gastric mucosa. To determine whether gastrin influences PGE2 synthesis in rat gastric mucosa, the rats were divided into five groups. The first group was subcutaneously injected with the vehicle, saline containing 1% BSA in a volume of 1 ml/kg. Groups 2, 3, and 4 were subcutaneously injected with rat gastrin 17 at doses of 1, 10, or 100 µg/kg, respectively. The last group was given subcutaneous gastrin 17 (100 µg/kg) along with intraperitoneal pretreatment of YM022 (100 mg/kg). One hour later, all the rats were anesthetized with sevoflurane, intragastrically administered with saline containing 100 µM indomethacin and 10 mM EDTA to block excess PG production in the gastric mucosa and immediately laparotomized. The stomachs were harvested. The oxyntic mucosa was scraped with glass slides and immediately frozen in liquid nitrogen. The tissue was weighed and homogenized at 4°C in cold ethanol. The homogenate was acidified to pH 4 using dilute HCl and centrifuged. PGE2 in the supernatant was purified
using C-18 solid-phase extraction cartridges (Sep-Pak, Waters, Millford, MA) and eluted with ethyl acetate containing 1% methanol. PGE₂ was solidified using a rotary evaporator reconstituted in an enzyme immunoassay (EIA) buffer and measured by EIA (Cayman Chemical, Ann Arbor, MI). PGE₂ levels in the gastric mucosa were expressed as picograms PGE₂ per gram wet tissue.

Further studies were conducted to confirm that a specific COX-2 inhibitor influences PG synthesis in rat gastric mucosa. NS-398 (10 mg/kg) was given intraperitoneally 1 h before saline containing 1% BSA or gastrin 17 (100 μg/kg) treatment, whereas controls received an equal volume of the vehicle (PEG 300). Six hours later, the gastric mucosa was harvested and homogenized for determination of PGE₂ levels as described above.

Ulcer index and histological score. To ascertain whether rat gastrin 17 prevents or attenuates acidified ethanol-induced gastric mucosal injury, the rats were divided into four pretreatment groups. The first group received the vehicle, saline containing 1% BSA. Groups 2, 3, and 4 were treated with rat gastrin 17 at doses of 1, 10, or 100 μg/kg, respectively, and all agents were administered subcutaneously in a volume of 1 ml/kg. One hour after each pretreatment, all animals were orally administered 1 ml of acidified ethanol (60% ethanol in 150 mM HCl) by esophageal intubation and then killed 1 h later (29). The stomachs were harvested, opened along the greater curvature, extended on a plastic board, and photographed. The area of the macroscopic hemorrhages and erosions was assessed by planimetry. The ulcer index was expressed as a percentage of the lesion area to the total gastric glandular area. For histological assessment, the gastric corpus wall was fixed in phosphate-buffered formalin, sectioned, and paraffin embedded. Semithin sections were stained with hematoxylin and eosin, and examined under a light microscope by a pathologist without knowledge of the group to which the specimen belonged. The specimens were coded and assessed according to the criteria of Whittle et al. (42). In brief, a 1-cm length of each histological section was assessed for epithelial cell damage (score of 1); glandular disruption, vasocoagulation or edema in the upper mucosa (score of 2); hemorrhagic damage in the middle-lower mucosa (score of 3); and deep necrosis and ulceration (score of 4). Each section was evaluated on a cumulative basis to give the histological score, the maximum score being 10.

To address whether the gastroprotective effect was due to gastrin itself or to PGE₂ production due to increased luminal hydrochloric acid by gastrin, the rats were divided into four pretreatment groups. The first group received the vehicle, saline containing 1% BSA with pretreatment of 50 mg/kg lansoprazole via a gastric tube for 0.5 and 12 h. Groups 2, 3, and 4 were treated with rat gastrin 17 at doses of 1, 10, or 100 μg/kg with pretreatment of 50 mg/kg lansoprazole for 0.5 and 12 h, respectively. One hour after each pretreatment, gastric mucosal injury was induced with acidified ethanol. The macroscopic injury and the histological score were calculated as described previously.

The mechanism underlying the protective effect of gastrin 17 against acidified ethanol-induced injury was assessed by evaluating the effect of a selective gastrin/CCK B receptor antagonist during gastrin 17 (100 μg/kg) pretreatment. YM022 (10, 30, or 100 mg/kg) was given intraperitoneally in a volume of 5 ml/kg 30 min before saline containing 1% BSA or gastrin 17 subcutaneous pretreatment, whereas controls received an equal volume of the vehicle (PEG 300). After the usual 1 h pretreatment with saline containing 1% BSA or gastrin 17, gastric mucosal injury was induced with acidified ethanol. The macroscopic injury and the histological score were calculated as described previously.

To ascertain whether the protective activity of gastrin 17 against acidified ethanol-induced gastric injury depended on COX-2-mediated PG synthesis, additional study was undertaken using the specific COX-2 inhibitor NS-398. NS-398 (10 mg/kg) was given intraperitoneally 1 h before saline containing 1% BSA or gastrin 17 (100 μg/kg) subcutaneous pretreatment, whereas controls received vehicle (PEG 300). After the usual 1-h pretreatment with saline containing 1% BSA or gastrin 17, gastric mucosal injury was induced with acidified ethanol. The macroscopic injury and the histological score were calculated as described previously.

Statistical analysis. Data were expressed as means ± SE. Parametric data were analyzed using analysis of variance with the Bonferroni’s multiple-comparison test or the Student’s two-tailed t-test for unpaired observations. Nonparametric data were analyzed with the Mann-Whitney U-test. Probability values of <0.05 were considered to be statistically significant.

RESULTS

Serum gastrin levels. The fasting serum gastrin levels of 0, 1, 3, 6, 12, and 24 h after subcutaneous injection with 100 μg/kg rat gastrin 17 were 140, 3,260, 370, 330, 310, and 290 pg/ml, respectively. Gastrin significantly increased serum gastrin levels at the peak of 3,260 pg/ml 1 h after subcutaneous injection (P < 0.0001 vs. 0 h; Fig. 1A). One hour after subcutaneous injection with the vehicle, saline containing 1% BSA in
a volume of 1 ml/kg, or rat gastrin 17 at doses of 1, 10, or 100 μg/kg, the serum gastrin levels were 150, 290 (P < 0.05 vs. saline), 700 (P < 0.0001 vs. saline), and 2,880 (P < 0.0001 vs. saline) pg/ml, respectively. Subcutaneous gastrin injection significantly increased serum gastrin levels in a dose-dependent manner (Fig. 1B).

Effects of gastrin 17 on HGF, HB-EGF, COX-1, and COX-2 expression. HGF and COX-1 expression in rat gastric mucosa subcutaneously injected with rat gastrin 17 for 1 h at doses of 1, 10, or 100 μg/kg did not significantly change compared with that of the vehicle, saline containing 1% BSA in a volume of 1 ml/kg. On the other hand, HB-EGF and COX-2 expression were significantly enhanced by gastrin 17 in a dose-dependent manner (P < 0.05 vs. saline; Fig. 2, A and B).

For the time-sequence study, HGF and COX-1 expression in rats subcutaneously injected with 100 μg/kg rat gastrin 17 did not change significantly 0, 1, 3, 6, 12, or 24 h after the injection. On the other hand, HB-EGF and COX-2 expression significantly peaked 1 h after the injection (P < 0.05 vs. 0 h; Fig. 2, C and D).

HB-EGF and COX-2 expression induced by gastrin 17 was decreased by pretreatment with a selective gastrin/CCKB receptor antagonist YM022 (10, 30, or 100 μg/kg) in a dose-dependent manner (P < 0.05 vs. PEG), whereas that of HGF and COX-1 was not altered significantly (Fig. 2, E and F).

Effects of a specific HB-EGF-neutralizing antibody on gastrin-induced COX-2 expression. Influences of the HB-EGF-neutralizing antibody on gastrin-induced COX-2 expression were examined by immunohistochemical staining using sliced gastric mucosal samples. Immunostaining for COX-2 was negligible in the control slice (Fig. 3A). After gastrin administration, however, COX-2 immunoreactivity was strongly detected in almost all types of gastric mucosal epithelial cells (Fig. 3B). On the other hand, after administration of gastrin and a specific HB-EGF-neutralizing antibody, COX-2 immunoreactivity was detected in almost all types of gastric mucosal epithelial cells (Fig. 3C). When the antibody preincubated with blocking COX-2 peptide was applied to the sections, no immunoreactive signals appeared (Fig. 3D).

Furthermore, COX-2 expression induced by HB-EGF was examined. Immunostaining for COX-2 was negligible in the control slice (Fig. 4A). After HB-EGF administration, however, COX-2 immunoreactivity was detected in almost all types of gastric mucosal epithelial cells (Fig. 4B). On the other hand, after administration of HB-EGF and pretreatment with a specific inhibitor of EGF receptor, COX-2 immunoreactivity was not detected (Fig. 4C). When the antibody preincubated with blocking COX-2 peptide was applied to the sections, no immunoreactive signals appeared (Fig. 4D).

In conclusion, a specific HB-EGF-neutralizing antibody decreased gastrin-induced COX-2 expression. Furthermore, COX-2 expression was induced by HB-EGF but blocked by pretreatment with a specific inhibitor of EGF receptor activation. Thus gastrin-induced COX-2 expression is partially mediated by HB-EGF. Microscopic examination (hematoxylin and eosin) indicated that gastric mucosa was not damaged after gastrin administration (data not shown).

PGE2 levels in gastric mucosa. One hour after subcutaneous injection with the vehicle, saline containing 1% BSA in a volume of 1 ml/kg, rat gastrin 17 at a dose of 1, 10, or 100 μg/kg, or 100 μg/kg gastrin 17 with pretreatment of 100 mg/kg YM022, gastric mucosal PGE2 levels were 670 (P < 0.05 vs. saline), 1,200 (P < 0.05 vs. saline), 1,930 (P < 0.0001 vs. saline), and 510 (P < 0.0001 vs. gastrin 100 μg/kg) pg/g wet tissue, respectively. Subcutaneous gastrin injection significantly increased gastric mucosal PGE2 levels in a dose-dependent manner (Fig. 5A).

Six hours after the subcutaneous injection with saline containing 1% BSA along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10 mg/kg NS-398 or 100 μg/kg gastrin 17 along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10 mg/kg NS-398, gastric mucosal PGE2 levels were 570, 630, 1,500, and 820 pg/g wet tissue, respectively. Gastric mucosal PGE2 levels induced by gastrin 17 were significantly decreased by intraperitoneal NS-398 pretreatment (P < 0.05 vs. PEG 300; Fig. 5B).

Effects of gastrin 17 on acidified ethanol-induced gastric mucosal injury. One hour after acidified ethanol administration with subcutaneous pretreatment of the vehicle, saline containing 1% BSA or 1, 10, or 100 μg/kg gastrin 17, the ulcer index was 6.3, 2.4 (P = 0.0004 vs. saline), 0.4 (P < 0.0001 vs. saline), and 0.2% (P < 0.0001 vs. saline; Fig. 6A), and the histological score was 8.3, 4.7 (P < 0.0001 vs. saline), 2.9 (P < 0.0001 vs. saline), and 0.7 (P < 0.0001 vs. saline; Fig. 6B), respectively. Gastrin 17 significantly prevented or attenuated acidified ethanol-induced gastric mucosal injury in a dose-dependent manner.

One hour after saline containing 1% BSA or 1, 10, or 100 μg/kg gastrin 17 subcutaneous treatment with pretreatment of 50 mg/kg lansoprazole for 0.5 and 12 h, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index was 6.5, 2.3 (P < 0.0001 vs. saline), 0.3 (P < 0.0001 vs. saline), and 0.2% (P < 0.0001 vs. saline; Fig. 6C), and the histological score was 8.3, 4.3 (P < 0.0001 vs. saline), 2.7 (P < 0.0001 vs. saline), and 0.7 (P < 0.0001 vs. saline; Fig. 6D), respectively. In the presence of complete acid suppression, gastrin 17 significantly prevented or attenuated acidified ethanol-induced gastric mucosal injury in a dose-dependent manner.

To assess the mechanism underlying the protective effect of gastrin 17 against acidified ethanol-induced injury, the effect of a selective gastrin/CCKB receptor antagonist, YM022, during 100 μg/kg gastrin 17 pretreatment was evaluated. One hour after subcutaneous injection with saline containing 1% BSA subcutaneous treatment along with intraperitoneal pretreatment with the vehicle (PEG 300) or 10, 30, or 100 mg/kg YM022 for 30 min, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index...
Fig. 2. The expression of hepatocyte growth factor (HGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), and cyclooxygenases (COX-1 and COX-2) in rat gastric mucosa after subcutaneous rat gastrin 17 (G) injection. One hour after subcutaneous injection with saline containing 1% BSA in a volume of 1 ml/kg or rat G at a dose of 1, 10, or 100 μg/kg, the expression of HGF, HB-EGF, COX-1, and COX-2 in rat gastric mucosa was examined by Western blot analysis (A). HB-EGF and COX-2 expression in A were semiquantified using a densitometric scanner. Error bars indicate means ± SE (n = 4; B). C: 0, 1, 3, 6, 12, or 24 h after subcutaneous injection with 100 μg/kg rat G, the expression of HGF, HB-EGF, COX-1, and COX-2 in rat gastric mucosa was investigated. HB-EGF and COX-2 expression in C was semiquantified using a densitometric scanner. Error bars indicate means ± SE (n = 4; D). One hour after subcutaneous injection with 100 μg/kg rat G along with intraperitoneal pretreatment of a selective gastrin/CCK<sub>B</sub> receptor antagonist YM022 (YM; 10, 30, or 100 mg/kg) or an equal volume (5 ml/kg) of the vehicle (PEG 300) or saline containing 1% BSA in a volume of 1 ml/kg with pretreatment of the vehicle (PEG 300), the expression of HGF, HB-EGF, COX-1, and COX-2 in rat gastric mucosa was investigated (E). HB-EGF and COX-2 expression in E was semiquantified using a densitometric scanner. Error bars indicate means ± SE (n = 4; F).
was 4.5, 5.8, 5.4, and 7.4% (Fig. 7A), and the histological score was 7.2, 7.8, 7.8, and 8.3 (Fig. 7B), respectively. On the other hand, 1 h after subcutaneous treatment with 100 μg/kg gastrin 17 along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10, 30, or 100 mg/kg YM022 for 30 min, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index was 0.5 (P < 0.001 vs. PEG 300

Fig. 3. COX-2 immunolocalization in rat stomach after gastrin stimulation and influence of immunoneutralization of HB-EGF on gastrin-induced COX-2 expression. Small gastric pieces were incubated in chamber slides at 37°C in DMEM containing 0.1% BSA with (B) and without (A) 10^{-8} g/ml rat G and with 10^{-8} g/ml rat G and 10 μg/ml rat HB-EGF-neutralizing antibody (C). Specificity of COX-2 immunoreactivity is confirmed using blocking COX-2 peptide in gastric specimen stimulated with 10^{-8} g/ml rat G (D). One hour later, COX-2 expression was investigated by immunohistochemical staining.

Fig. 4. COX-2 immunolocalization in rat stomach after HB-EGF stimulation and influence of an EGF receptor blocker on HB-EGF-induced COX-2 expression. Small gastric pieces were incubated in chamber slides at 37°C in DMEM containing 0.1% BSA with (B) and without 10^{-8} g/ml human recombinant HB-EGF (A). In Fig. 4C, a gastric tissue was pretreated with 1 μM specific inhibitor of EGF receptor and then stimulated with 10^{-8} g/ml human recombinant HB-EGF. Specificity of COX-2 immunoreactivity is confirmed in gastric specimen stimulated with 10^{-8} g/ml human recombinant HB-EGF, to which the COX-2 antibody is preincubated with blocking COX-2 peptide (D). One hour later, COX-2 expression was investigated by immunohistochemical staining.
saline), 1.4, 2.5 \((P < 0.01 \text{ vs. PEG 300 gastrin})\), and 5.7\% \((P < 0.001 \text{ vs. PEG 300 gastrin; Fig. 7A})\), and the histological score was 1.1 \((P < 0.001 \text{ vs. PEG 300 saline})\), 3.6 \((P < 0.01 \text{ vs. PEG 300-gastrin})\), 5.3 \((P < 0.001 \text{ vs. PEG 300 gastrin})\), and 7.4 \((P < 0.0001 \text{ vs. PEG 300 gastrin; Fig. 7B})\), respectively. In conclusion, pretreatment with YM022 significantly abolished the protective effect of gastrin 17 against acidified ethanol-induced injury in a dose-dependent manner.

One hour after subcutaneous injection with saline containing 1\% BSA along with the intraperitoneal pretreatment of the vehicle (PEG 300) or 10 mg/kg NS-398, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index was 4.6 and 5.0\% (Fig. 8A), and the histological score was 7.1 and 7.7 (Fig. 8B), respectively. On the other hand, 1 h after subcutaneous injection with 100 \(\mu\)g/kg gastrin 17 along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10 mg/kg NS-398 for 1 h, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index was 0.5 \((P < 0.0001 \text{ vs. PEG 300 saline})\) and 4.4\% \((P < 0.0001 \text{ vs. PEG 300 gastrin; Fig. 8A})\), and the histological score was 1.1 \((P < 0.0001 \text{ vs. PEG 300 saline})\) and 6.7 \((P < 0.0001 \text{ vs. PEG 300 gastrin; Fig. 8B})\), respectively. In conclusion, NS-398 significantly abolished the mucosal protection afforded by gastrin against acidified ethanol-induced injury.

**DISCUSSION**

Gastrin and pentagastrin protect against gastric lesions (18, 26, 34). The present findings also indicate that subcutaneous gastrin injection prevents and attenuates acidified ethanol-induced gastric mucosal injury in a dose-dependent manner. The mechanism of gastrin-induced gastric mucosal protection has not yet been clarified. The present study clearly demonstrates that gastrin upregulates HB-EGF, not HGF, and COX-2, not COX-1, and protects gastric mucosa from ethanol-induced damage.

The mucosal protection induced by gastrin was abolished by YM022, a specific antagonist of gastrin/CCKB receptor. These results confirm that the gastrin-induced mucosal protection is mediated by the gastrin/CCKB receptor. Gastric mucosal PGE2 increased after gastrin administration, which was also suppressed by the gastrin/CCKB receptor antagonist. These results suggest that gastrin increases mucosal PGE2 and maintains mucosal integrity, both of which are dependent on the gastrin/CCKB receptor. However, an earlier in vitro study indicates that gastrin does not have a direct stimulatory role in gastric epithelial cells (17) that cover the luminal side of gastric mucosa. Consequently, gastrin may indirectly stimulate gastric PG synthesis in mucosal epithelium.

Because COX is the rate-limiting enzyme in PGE2 synthesis, expression of the COX isozymes in gastric mucosa before and after the gastrin administration was investigated using Western blotting and immunohistochemistry. COX-2 was upregulated, whereas COX-1 was unchanged, in gastric mucosa after stimulation with gastrin. NS-398, the COX-2-specific inhibitor, reduced gastric mucosal PGE2 level and abolished mucosal integrity induced by gastrin. These results suggest that COX-2 is induced in gastric mucosa by gastrin and is responsible for gastrin-induced gastric PGE2 production and gastric mucosal protection. Interestingly, the immunohistochemical analysis showed that COX-2 immunoreactivity existed not only in enterochromaffin cell-like (ECL) cells and parietal cells that express gastrin/CCKB receptors. COX-2 occurred in a wide range of gastric mucosal epithelium, suggesting that gastrin might upregulate COX-2 indirectly in gastric mucosa in vivo.

Therefore, in the following studies, possible inducers of gastrin-dependent COX-2 expression in gastric mucosa were examined. Among numerous inducers reported (43), we focused our attention particularly on two growth factors. HGF is one of the most potent growth factors and morphogenic factors in gastric epithelial cells (33, 38) and is able to induce COX-2 in gastric mucosa in vitro (9, 27). Another candidate is HB-EGF, a member of EGF-like growth factors that is membrane bound is released by activation of G protein-coupling receptors.
Several investigators have reported that gastric epithelial cells produce EGF-like growth factors that might modulate the growth and differentiation of these cells in a paracrine and/or autocrine manner (2, 3, 5, 12, 20, 25). These growth factors, including HB-EGF, activate EGF receptors and initiate intracellular signaling pathways that require tyrosine kinase activity of the EGF receptor. On the other hand, Miyazaki et al. (20) reported that HB-EGF at least partially mediates the proliferative effects of gastrin on gastric epithelial cells. Western blotting analysis in the present study clearly demonstrated that gastrin induced the expression of HB-EGF, not HGF, in gastric mucosa. Immunohistochemistry also confirmed that both gastrin and HB-EGF were able to upregulate COX-2 expression. Furthermore, prior administration with neutralizing antibody against HB-EGF abolished COX-2 immunoreactivity in gastric mucosa induced by gastrin. The

![Figure 6](http://ajpgi.physiology.org/)

**Fig. 6.** Effects of G with and without prior acid suppression with proton pump inhibitor lansoprazole on gastric mucosal injury induced by acidified ethanol. One hour after the acidified ethanol administration with subcutaneous pretreatment of the vehicle, saline containing 1% BSA or 1, 10, or 100 μg/kg G, the ulcer index (A) and the histological score (B) were calculated. One hour after saline containing 1% BSA or 1, 10, or 100 μg/kg G subcutaneous treatment with pretreatment of 50 mg/kg lansoprazole for 0.5 and 12 h, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index (C) and the histological score (D) were calculated.

![Figure 7](http://ajpgi.physiology.org/)

**Fig. 7.** Effects of YM022 on gastric mucosal protection induced by gastrin. Open bars: 1 h after saline containing 1% BSA subcutaneous treatment along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10, 30, or 100 mg/kg YM022 for 30 min, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index (A) and the histological score (B) were calculated. Closed bars: 1 h after 100 μg/kg G subcutaneous treatment along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10, 30, or 100 mg/kg YM022 for 30 min, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index (A) and the histological score (B) were calculated.

![Graph A](http://ajpgi.physiology.org/)

![Graph B](http://ajpgi.physiology.org/)

![Graph C](http://ajpgi.physiology.org/)

![Graph D](http://ajpgi.physiology.org/)
GASTRIN UPREGULATES COX-2

EGF receptor specific tyrosine-kinase inhibitor abolished this HB-EGF-induced COX-2 expression, indicating that the EGF receptor is responsible in COX-2 expression in the present study. Therefore, gastrin induces HB-EGF and subsequent activation of the EGF receptor in rat gastric mucosa, which results in COX-2 upregulation and enhanced PGE\(_2\) release and protects gastric mucosa from an insult with acidified ethanol in rats.

In the present study, subcutaneous injection with gastrin significantly enhanced serum gastrin levels, the expression of HB-EGF and COX-2, and PGE\(_2\) levels in rat gastric mucosa in a dose-dependent manner. Furthermore, gastrin injection prevented and attenuated acidified ethanol-induced gastric mucosal injury.

One of the major issues that should be addressed when examining mucosal injury with secretagogues such as gastrin is whether aggressive factors were controlled, because gastrin is a potent stimulant of gastric secretion of hydrochloric acid. We applied acidified ethanol as a necrotizing agent in the present study to adjust intraluminal pH during gastric damage induction. However, several investigators report that increased luminal acid induces PGE\(_2\) synthesis (6) or release of gastroprotective peptides such as gastrin and CCK (13, 19). We previously showed that 0.6 M hydrochloric acid induced gastric COX-2 expression in rats (39). Therefore, gastrin may protect gastric mucosa from necrotizing agent indirectly via stimulating gastric acid secretion. To exclude the effects of increased luminal acid by gastrin, we also examined the influences of gastrin on gastric mucosal protection with the proton pump inhibitor lansoprazole. In the presence of lansoprazole-induced acid suppression, gastrin significantly attenuated acidified ethanol-induced gastric mucosal injury in a dose-dependent manner. The results show that the gastroprotective effect was not due to increased luminal acid induced by gastrin.

In general, gastric surface epithelia are interfaces of stomach and luminal contents including a necrotizing agent. However, it is known that gastrin mainly activates cells expressing gastrin/CCK\(_B\) receptors (i.e., ECL cells, parietal cells, etc.) (4, 8, 11, 14, 23, 31). We also investigated the COX-2 immunolocalization in rat gastric mucosa with or without gastrin administration. Interestingly, COX-2 immunoreactivity was present not only in cells expressing gastrin/CCK\(_B\) receptors (i.e., ECL cells, parietal cells, etc.), but in various types of gastric mucosal epithelial cells. Consequently, gastrin might indirectly enhance COX-2 expression in rat gastric mucosa. A specific HB-EGF-neutralizing antibody decreased gastrin-induced COX-2 expression. The results suggest that HB-EGF is one of the mediators enhancing gastric mucosal COX-2 expression. Previous studies demonstrated that the EGF receptor, the receptor for HB-EGF and other EGF-like ligands, exists in almost all types of gastric epithelial cells including parietal cells, chief cells, mucous neck cells of the proliferative zone, and surface epithelial cells (3, 22, 36). Thus it is possible that gastrin protects all types of gastric epithelial cells, not only parietal and ECL cells expressing gastrin/CCK\(_B\) receptors, via indirect stimulation of COX-2 in rat gastric mucosa.

As a clinical aside, we assume that this COX-2-PGE\(_2\)-dependent mechanism is normally present in the postprandial state. Although earlier studies indicate that COX-2 is minimally expressed in rodents, Ehrlich et al. (7) reported COX-2-PGE\(_2\)-dependent gastroprotection after peptone-induced physiological gastrin release in the presence of ulcerogens. Furthermore, recent human studies clearly showed that human gastric mucosa is able to express two isoforms of COX: constitutive COX-1 and inducible COX-2. COX-1 is detected in most normal tissues (10), whereas COX-2 is induced in vivo by proinflammatory stimuli and \(H.\) pylori infection. We assume that PGs generated via the COX-1 pathway are essential for physiological functions such as maintenance of gastrointestinal mucosal integrity, whereas those via the COX-2 pathway have a role in pathophysiological events such as hypergastrinemia and gastric inflammation. Consequently, NSAIDs that block both COX-1 and COX-2 cause severe gastric

Fig. 8. Effects of NS-398 on gastric mucosal protection induced by gastrin. Open bars: 1 h after saline containing 1% BSA subcutaneous treatment along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10 mg/kg NS-398, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index (A) and the histological score (B) were calculated. Closed bars: 1 h after 100 µg/kg G subcutaneous treatment along with intraperitoneal pretreatment of the vehicle (PEG 300) or 10 mg/kg NS-398 for 1 h, gastric mucosal injury was induced with acidified ethanol. One hour later, the ulcer index (A) and the histological score (B) were calculated.

* P < 0.0001 vs. vehicle-saline; P < 0.0001 vs. vehicle-gastrin

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damage, whereas selective COX-2 inhibitor causes little
table damage.

In addition to its function as a stimulant of gastric acid secretion, gastrin is implicated to protect the gastric mucosa (18, 26, 34). The present study clearly demonstrated that gastrin protects gastric mucosa from acidified ethanol. Furthermore, gastrin is also related to gastric mucosal proliferation and increased risk for gastric carcinogenesis. Indeed, gastric mucosal hypertrophy has been reported in patients with hypergastrinemia, such as the Zollinger-Ellison syndrome. Gastrin receptor-deficient mice display fundic mucosal atrophy. On the other hand, COX-2 upregulation suppresses epithelial cell senescence and programmed cell death in the gastrointestinal tract and is related to gastrointestinal carcinogenesis (40, 41). Consequently, gastrin-induced COX-2 expression might be involved in gastric hypertrophy and gastric carcinogenesis over the long term.

In conclusion, the present study demonstrates that subcutaneous injection with gastrin increases serum gastrin levels and gastric mucosal COX-2 expression, which is partially mediated by HB-EGF, and gastric mucosal PGE2 levels and protects gastric mucosa from a necrotizing agent in rats. Thus gastrin has important roles not only in gastric secretion, but also in COX-2-dependent gastric mucosal protection.

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