

GHRELIN IMPROVES LPS-INDUCED GASTROINTESTINAL MOTILITY DISTURBANCES: ROLES OF NO AND PROSTAGLANDIN E₂

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Received 2 Mar 2009; first review completed 24 Mar 2009; accepted in final form 13 May 2009

ABSTRACT—Ghrelin, an important orexigenic peptide, exerts gastroprokinetic and anti-inflammatory effects. We investigated the role of ghrelin in LPS-induced gastrointestinal (GI) motility disturbances through NO and prostaglandin E₂ pathways in mice. Ghrelin-containing cells and its receptor, growth hormone secretagogue receptor 1 (GHSR-1), were localized in the stomach and duodenum using an immunohistochemical method. The distribution of ghrelin-containing cells or GHSR-1 immunoreactivity in both the mucosal and the muscle layers was heterogeneous within both tissues. The i.p. administration of ghrelin (1–20 µg/kg) had no effect on gastric emptying but markedly increased the GI transit (GIT) in normal mice. LPS (20 mg/kg i.p.)-treated mice showed significant decreases in the gastric emptying and GIT. Ghrelin attenuated the LPS-induced delay in gastric emptying and GIT. We also performed immunohistochemical experiments on both tissues. Immunohistochemistry showed the presence of iNOS and cyclooxygenase 2 in both tissues of LPS-treated mice. Treatment of LPS-exposed mice with ghrelin (20 µg/kg) diminished the presence of iNOS but not cyclooxygenase 2 in both tissues. The effect of ghrelin on regulating LPS-induced GI motility disturbance was further found to be associated with a reduction in iNOS expression in the GI tract and plasma NO overproduction rather than regulation of neural or endothelial NO synthase expression in the GI tissue. In addition, ghrelin was found to elevate prostaglandin E₂ levels in the GI tissue but showed no significant change in LPS-treated mice. These findings indicate that the action of ghrelin binding to GHSR-1 improves endotoxemia-induced GI motility disturbances mainly through down-regulating the NO pathway in the GI tract.

KEYWORDS—Ghrelin, GHSR-1, LPS, gastrointestinal motility, NO, PGE₂

INTRODUCTION

Ghrelin, the endogenous ligand of the growth hormone secretagogue receptor 1 (GHSR-1), was isolated from the rat stomach (1). Ghrelin can stimulate growth hormone release, plays a role in regulating food intake and in controlling the energy balance (2), and stimulates gastrointestinal (GI) motility (3). In humans, ghrelin enhances gastric emptying in diabetic patients (4) and shows powerful gastroprokinetic activity. Recent evidence points toward a potential role of ghrelin in influencing the immune and the inflammatory systems (5, 6). Ghrelin can reduce proinflammatory cytokines (7) and can correct the myocardial dysfunction and metabolic disturbance of septic shock through preventing systemic inflammatory responses and other inflammatory disorders (8).

LPSs are endotoxins in the cell walls of gram-negative bacteria, which can activate macrophages to secrete at least 100 biologically active substances, including prostaglandins and NO (9, 10). NO formation by NO synthase (NOS) can maintain GI integrity under both physiological and pathophysiological conditions (11, 12). The constitutive isoforms of NOS, endothelial NOS (eNOS) and neural NOS (nNOS), are constitutively expressed, widely distributed, and can synthe-

size NO at low concentrations to regulate numerous physiological functions (13, 14). The third isoform, iNOS, needs transcriptional activation for its expression. Overexpression of NO, generated from iNOS, was previously shown to cause endotoxin-induced motility disturbances. Inhibitors of immune mediators such as iNOS-derived NO can improve GI transit (GIT) disturbances during endotoxemia (15). The inhibition of NO and NOS can ameliorate GI injury by endotoxin.

Prostaglandins can maintain GI integrity (16) and prevent inflammation (17). LPS can induce both cyclooxygenase 2 (COX-2) and iNOS gene expression to synthesize prostaglandins and NO (17, 18). Both NO and prostaglandin E₂ (PGE₂) are important mediators of many inflammatory responses (17, 19). However, the effects of ghrelin on LPS-induced GI dysfunction through regulating the NO and PGE₂ pathways are poorly understood. Therefore, we decided to investigate ghrelin's role in improving GI dysfunction through GHSR-1 and mediation of the NO-NOS and PGE₂-COX-2 pathways in endotoxemic mice. In this study, we also attempted to immunohistochemically characterize the distribution of ghrelin-containing cells their receptors (GHSR-1), iNOS and COX-2, in the GI tract.

MATERIALS AND METHODS

Materials

We dissolved 1 mg of rat ghrelin (Global Peptide Services, LLC, Fort Collins, Colo) in 1 mL of physiological saline containing 20% dimethyl

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DOI: 10.1097/SHK.0b013e3181ae841b

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sulfoxide as the stock solution. LPS (from *Escherichia coli*, serotype 055:B5) was purchased from Sigma-Aldrich Chemical (St Louis, Mo). All drugs were freshly prepared in normal saline on the day of the experiment and were injected at a proportional volume of 0.1 mL/20 g body weight to mice. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich Chemical.

Experimental animals

We housed male ICR mice (National laboratory Animal Center, Taipei, Taiwan) weighing 20 to 25 g at the beginning of the experiment in group cages under conditions of controlled illumination (light cycle, 0600–1800 h), humidity, and temperature ($23 \pm 1^\circ\text{C}$) with free access to a laboratory diet (Lab Diet, Brentwood, Mo) and tap water. All procedures for use of animals in this study were approved by the Animal Care and Use Committee of Taipei Medical University.

Immunohistochemical procedure

The immunohistochemical procedure was similar to that described elsewhere (20). Briefly, mice were starved for 24 h, then deeply anesthetized with ether and perfused transcardially with 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1 M of a phosphate-buffered solution (PBS) at pH 7.4. The stomach and the duodenum were removed, postfixed for 4 h in 4% paraformaldehyde, and then transferred to 20% sucrose in PBS (wt/vol) overnight. The tissue was placed in an embedding medium in cryomatrix (Thermo Electron Corporation, Pittsburgh, Pa) and immediately frozen. Tissue sections were cut with a cryostat at 30 μm thick and mounted onto gelatinized slides. We rinsed sections with 0.1 M PBS and incubated them in 0.3% hydrogen peroxide in PBS for 10 min at room temperature. Afterward, sections were rinsed in 0.1 M Tris-buffered saline (TBS; pH 7.4) and blocked by incubation in TBS containing 0.05% Tween 20 (TBST) and 1.0% BSA for 1 to 2 h, and then the rabbit primary antibody (ghrelin, GHSR-1, iNOS, and COX-2; a dilution in TBST containing 1% BSA) was added for 24 h at 4°C in the same buffer at the dilutions indicated above. After intermittent rinsing in TBS, we incubated sections for 2 h with the secondary antibody, biotinylated rabbit antgoat (1:1,000 dilution in TBST; Santa Cruz Biotechnology, Santa Cruz, Calif), at room temperature. After rinsing with TBST three times, we conducted pilot experiments with a tyramide-based amplification technique (NEL700; Perkin-Elmer Life Sciences, Boston, Mass) following the manufacturer's recommendations. Sections were stained using 0.05% diaminoben-

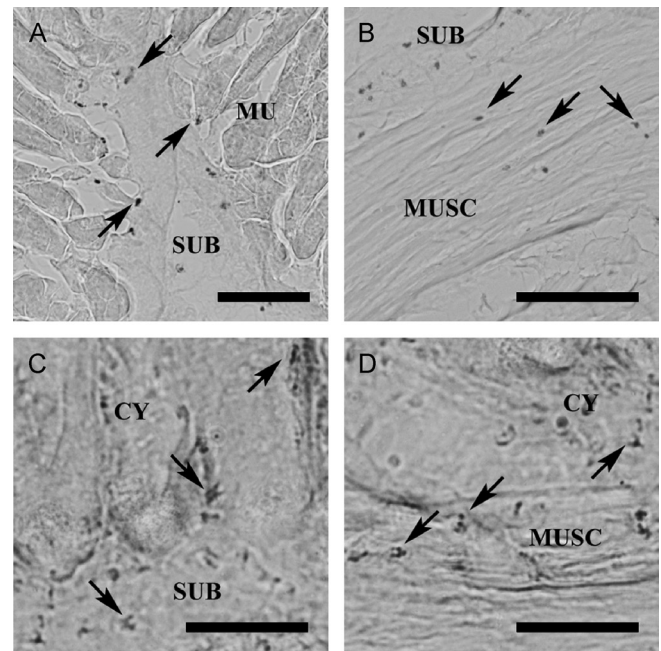


FIG. 2. Immunohistochemical localization of the GHSR receptor subunit, GHSR-1, in the stomach (A, B) and duodenum (C, D). Light microscopy of a cross-section shows GHSR-1 immunoreactivity in the gastric muscularis mucosa, submucosal, and myenteric plexus layers (A, B). The GHSR-1 immunoreactivity was recognized and mainly distributed near the crypts (C) and the myenteric plexus layers (D) in the duodenum. MU, mucosa; SUB, submucosa; MUSC, muscle layer; CY, crypt. Scale bars, 50 μm .

zidine in diaminobenzidine buffer (0.05 M Tris-HCl and 0.1 M imidazole; pH 7.6) as a chromogen in the presence of Ni ions, and the reaction was terminated by PBS once it reached the optimal intensity. Finally, we air-dried the slides, dehydrated them in alcohol, cleared them in xylene mounted on a slide with Permount (Fisher), and covered them with slips for light microscopic examination. Anti-ghrelin (1:150) and anti-GHSR-1 (1:150) were purchased from Santa Cruz Biotechnology. Anti-iNOS (1:200) and anti-COX-2 (1:100) were purchased from BD Transduction Laboratories (Lexington, UK) and Cayman (Ann Arbor, Mich), respectively.

LPS-treated mice

In animal models, endotoxemia is often induced by an injection of LPS. However, there are considerable differences between species and doses of LPS. The first set of experiments was designed to determine the dosage of LPS-induced mice as described by De Winter et al. (21). On the day of experimentation, the mice were starved but given free access to water and were randomly divided into several groups: the control group received an i.p. injection of saline and the other group received LPS (5–20 mg/kg). We measured leukocyte numbers and rectal temperature as indicators of endotoxemia severity. Preliminary results indicated that LPS at a dose of 20 mg/kg in mice decreased the rectal temperature, leukocyte number, gastric emptying, and GIT after 24 h without inducing diarrhea and with an acceptable mortality rate of less than 10%. All LPS-treated mice showed severe conjunctivitis, immobility with less explorative behavior and ruffled fur. In accordance with the abovementioned symptoms and characteristics, LPS (20 mg/kg) was used in the further study.

Gastric emptying rates of phenol red meals in conscious mice

Gastric emptying was determined by a modified phenol red recovery technique described elsewhere (22) with slight modifications. The mice received an i.p. injection of LPS (20 mg/kg) or saline and then were starved for 24 h. Afterward, the mice received an i.p. injection of saline or ghrelin, and 15 min later, the mice received 150 μL of a 1.5% methylcellulose solution containing phenol red by gavage. Fifteen minutes later, we killed the mice by cervical dislocation. The stomach was separated from the abdomen by a laparotomy, quickly ligated at the pylorus and cardia, and then placed in individual tubes. We used phenol red, recovered from animals killed immediately after administration of the methylcellulose solution, as the standard (0% emptying).

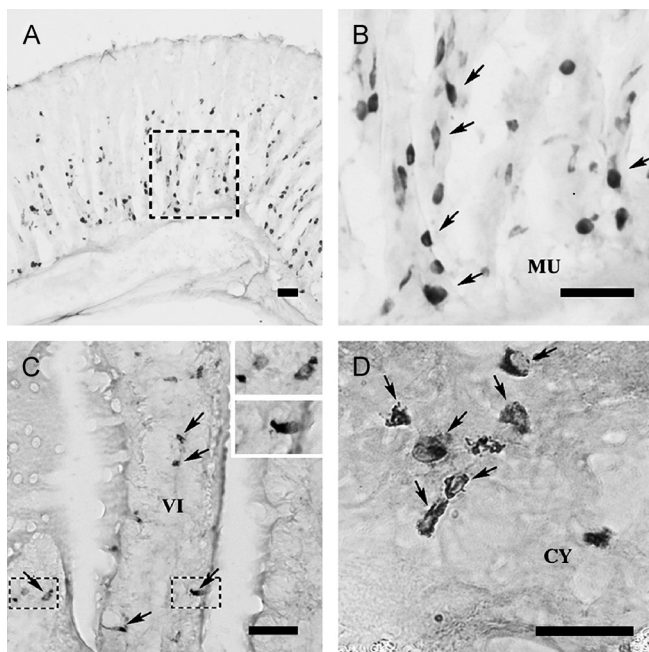


FIG. 1. Ghrelin-immunoreactive cells in the stomach (A, B) and duodenum (C, D). Ghrelin-containing cells were found in the gastric mucosa. Ghrelin-containing cells were found to be most abundant in the glandular base and to decrease in number toward the glandular neck. B, High-magnification micrograph of a ghrelin-containing cell in the mucosal layer (arrows). C, Ghrelin-containing cells in the duodenum were found in the villi. High-magnification micrograph ($\times 2$) showing immunoreactive cells in the villi. D, Crypt. MU, mucosa; VI, villus; CY, crypt. Scale bars, 50 μm .

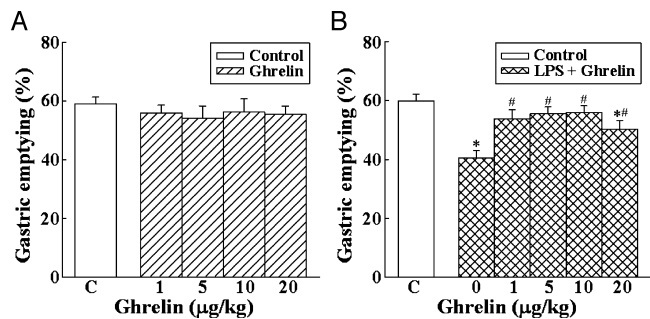


FIG. 3. Effects of different doses of ghrelin on gastric emptying of liquids in conscious mice. A, Ghrelin's effect on gastric emptying; B, ghrelin's effect on LPS-induced gastric emptying. Mice were starved and received an i.p. injection of normal saline or 20 mg/kg LPS 24 h before the experiment. On the experimental day, mice received an i.p. injection of normal saline (control group) or ghrelin (1–20 µg/kg) 15 min before gastric intubation of a phenol red meal. Fifteen minutes later, the mice were decapitated. Gastric emptying was determined using a spectrophotometer at 560 nm. * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with LPS treatment. Each column represents the mean $\pm$ SEM ($n = 12 - 16$).

Each stomach was subsequently homogenized for 30 s in 10 mL of 0.1 N NaOH, and the homogenate was placed at room temperature for 1 h. We added 0.5 mL of 20% trichloroacetic acid to 5 mL of the homogeneous solution to precipitate the proteins. After centrifugation at 10,000 g for 10 min, 2.5 mL of the supernatant was removed to a new tube, and 2 mL of 0.5 N NaOH was added. We measured the absorbance of the samples with a spectrophotometer at 560 nm. The calculation was as follows: gastric emptying (%) = $[1 - (\text{absorbance of a test sample} / \text{average absorbance of a standard stomach})] \times 100$.

GIT of a charcoal meal in conscious mice

We assessed the effect of ghrelin on intestinal propulsion by measuring the GIT of a charcoal meal, as described by Liang et al. (17) with slight modifications. Briefly, the mice received an i.p. injection of LPS (20 mg/kg) or saline and were starved for 24 h. Afterward, the mice received an i.p. injection of saline or ghrelin, and 15 min later, the mice received 0.5 mL of an activated charcoal meal (0.5 mL of 10% charcoal suspended in 10% gum acacia) by oral gavage. After 15 min, we killed the mice by cervical dislocation. We separated the small intestine from the omentum without stretching it and measured the length of the small intestine from the pyloric sphincter to the ileocecal junction and the distance traveled by the charcoal meal. The GIT was calculated as the percent of the distance traveled by the charcoal meal (percent of GIT), relative to the total length of the small intestine.

Gel electrophoresis and Western blotting

We carried out the Western blot procedure as described previously (17). Briefly, 30 min after ghrelin administration (1 and 20 µg/kg i.p.), we killed the mice. We obtained the stomach and small intestine from the abdomen and washed the tissues twice in PBS. Portions (0.5 g) of the stomach or small

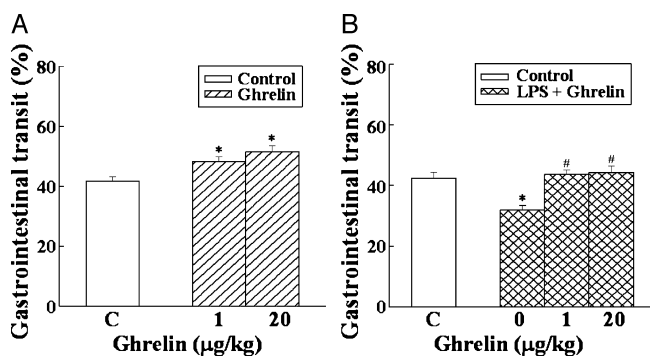


FIG. 4. Effects of various doses of ghrelin on GIT of a charcoal meal in conscious mice. A, Ghrelin's effect on GIT; B, ghrelin's effect on LPS-induced GIT. The conditions were the same as those described in Fig. 3, except that the phenol red meal was replaced by a charcoal meal. * $P < 0.05$ compared with the control group. # $P < 0.05$ compared with LPS treatment. Each column represents the mean $\pm$ SEM ($n = 12 - 18$).

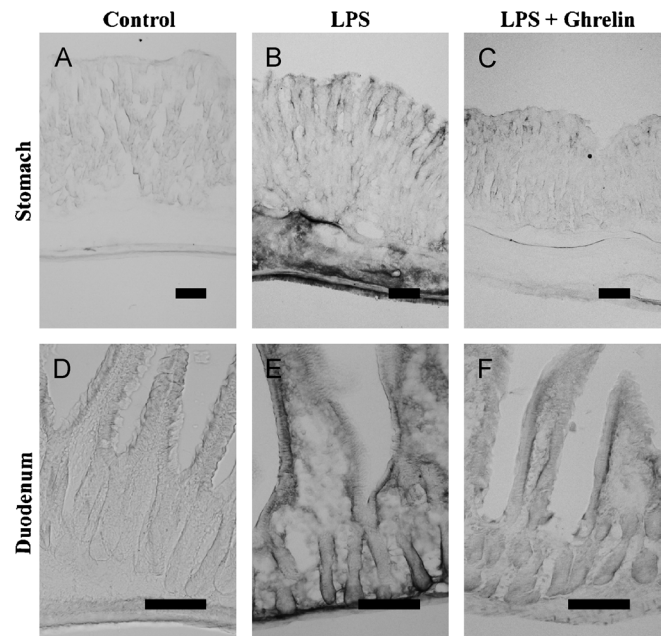


FIG. 5. Immunohistochemical staining of iNOS protein in the stomach (A–C) and duodenum (D–F). A and D, control mice; B and E, revealed LPS-induced mice; and C and F, ghrelin on LPS-induced mice. After the mice had received an i.p. injection with LPS (20 mg/kg) or saline and were starved for 24 h, they received an i.p. injection of saline or ghrelin (20 µg/kg). Thirty minutes later, the mice were staining as described in the Materials and Methods section. Scale bars, 50 µm.

intestine were homogenized in 1 mL of chilled RIPA buffer (0.15 M NaCl, 50 mM Tris, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, and 0.1% sodium dodecyl sulfate [SDS]), including protease inhibitors. We placed this mixture on ice for 1 h and then centrifuged it at 10,000g for 30 min at 4°C. The supernatant was stored at -80°C until being assayed. The protein concentrations of lysates were determined using the DC protein assay (Bio-Rad, Hercules, Calif). Equal amounts (100 µg/well) of total protein from the lysates were mixed with 5× SDS–polyacrylamide gel electrophoresis sample buffer

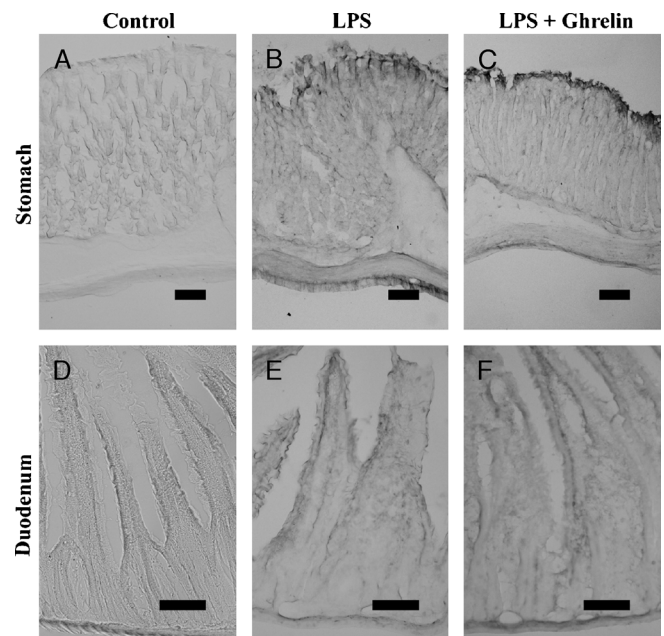


FIG. 6. Immunohistochemical staining of COX-2 protein in the stomach (A–C) and duodenum (D–F). (A and D) control mice, (B and E) revealed LPS-induced mice and (C and F) ghrelin on LPS-induced mice. The conditions were the same as those described in Fig. 5. Scale bars, 50 µm.

and boiled for 10 min. Lysates were loaded on a 10% SDS-polyacrylamide gel electrophoresis. We carried out electrophoresis according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Science, Birmingham, UK) using an electroblotting minitransfer apparatus as per the manufacturer's protocol. Membranes were blocked for 1 h in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dried milk at room temperature for at least 1 h, washed twice with TBST, and incubated overnight with a monoclonal antibody against nNOS (1:2,500), iNOS (1:10,000), COX-2 (1:500; Cayman), or GAPDH (1:10,000; Biogenesis, Poole, UK). After washing three times with TBST for 10 min, each membrane was incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody. We also visualized the protein bands with an enhanced chemiluminescence method to detect immunoreactive proteins.

Plasma NO assay

After the mice had received an i.p. injection with LPS (20 mg/kg) or saline and were starved for 24 h, they received an i.p. injection of saline or ghrelin (1–20 µg/kg). Thirty minutes later, mice were anesthetized with ether. Blood samples obtained from the heart of each group of mice were centrifuged at 3,000g for 15 min at 4°C, and the stomach and the small intestine were removed, weighed, and immediately stored at –80°C. We collected plasma samples and stored them at –80°C until the assay. NO undergoes a series of biological reactions and transforms into nitrite and nitrate as the final products *in vivo*. The relative proportion of nitrite and nitrate is variable; hence, the best reflection of the total NO level is the sum of both nitrite and nitrate. We

determined plasma levels of NO with a nitrate/nitrite fluorometric assay kit (Cayman). Briefly, plasma samples were pipetted into a 96-well plate, and nitrate reductase and enzyme cofactors supplied with the fluorometric assay kit were added. We protected the plate from light and incubated it at room temperature for 2 h. Afterward, we incubated each well with 10 µL 2,3-diaminonaphthalene (a fluorometric reagent) for 10 min followed by 20 µL NaOH to enhance the detection of the fluorescent product, 1(*H*)-naphthotriazole. We analyzed the plate with fluorescence spectrometry (BMG FluoStar, Offenburg, Germany) at an excitation wavelength of 370 nm and at an emission wavelength of 450 nm and expressed the levels of NO in nanomoles per milliliter of plasma.

PGE₂ Assay

Tissue samples were homogenized in 0.1 M phosphate (pH 7.4, containing 1 mM EDTA and 10 µM indomethacin) and precipitated the proteins with ethanol, then centrifuged them at 3,000g for 10 min to remove the precipitated proteins. We acidified and purified the samples according to a protocol provided with the enzyme immunoassay using a PGE₂ kit (Cayman). Briefly, we acidified the sample with 1 M formic acid to pH 3.5 and PGE₂ and extracted it with a C-18 solid-phase extraction column, eluted it with ethyl acetate, dried it under nitrogen, and stored it at –80°C. Samples were re-suspended in 500 µL of assay buffer supplied with the enzyme-linked immunosorbent assay (ELISA) kit before the PGE₂ ELISA was run. We measured the absorbance of samples with a spectrophotometer at 405 nm. The capacities of the stomach and small intestine to generate PGE₂ were expressed in picograms per microgram wet tissue.

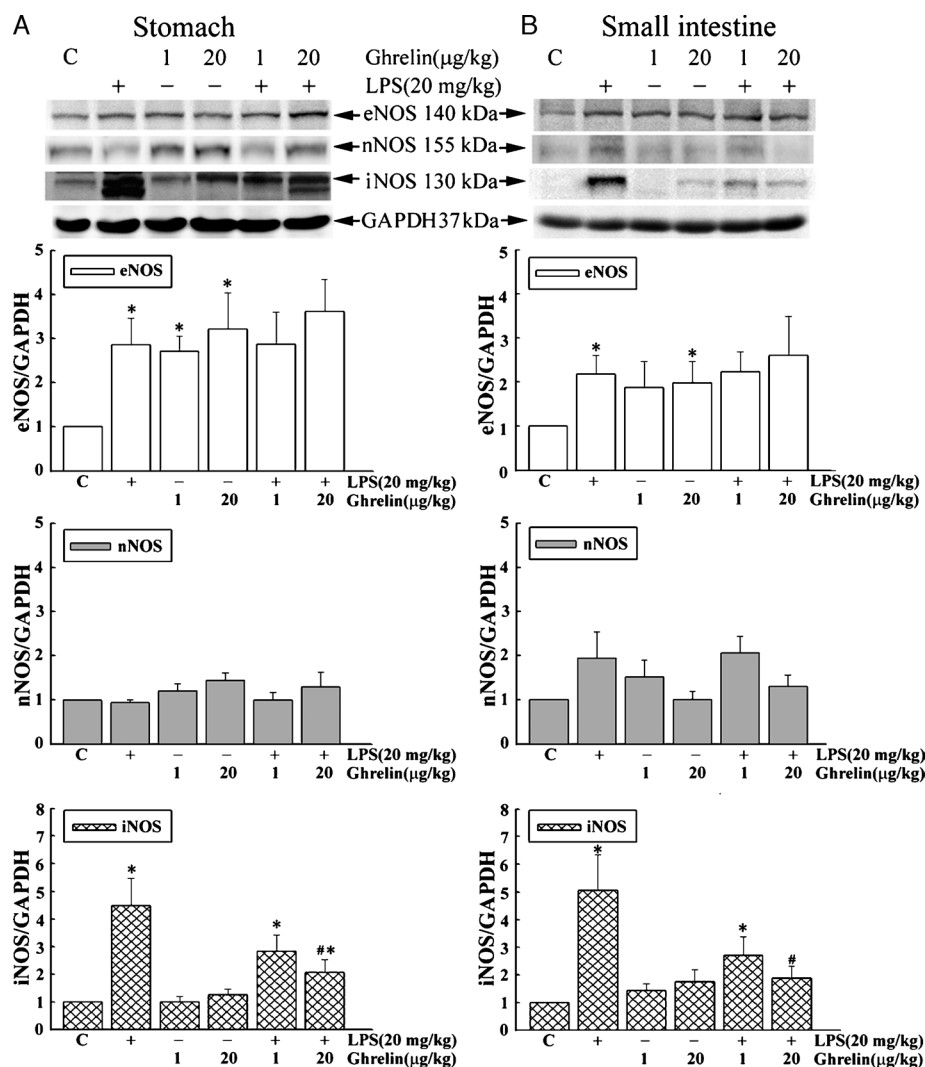


FIG. 7. Effects of ghrelin on LPS-induced nNOS, eNOS, and iNOS protein expressions in the stomach and small intestine. Saline or ghrelin was i.p. injected at 24 h after having injected saline or LPS (20 mg/kg i.p.). Tissue was collected at 30 min after the saline or ghrelin injection. Comparisons between the groups were made by determining the respective ratios of nNOS, eNOS, and iNOS to GAPDH by densitometry. **P* < 0.05 compared with the control group; #*P* < 0.05 compared with LPS treatment. Each column represents the mean ± SEM (*n* = 6).

Statistical analysis

Quantitative data are presented as the mean $\pm$ SEM. Results were analyzed for statistically significant differences using ANOVA, followed by Student or Bonferroni *t* test. Differences between groups were considered significant for *P* values of <0.05 .

RESULTS

Expression of ghrelin and its receptor, GHSR-1, in the stomach and duodenum

Numerous ghrelin-containing cells were observed from the base to the neck region of the glands in the gastric mucosa in the mouse stomach (Fig. 1, A and B). In the duodenum, ghrelin-containing cells were distributed in the villi and the crypt (Fig. 1, C and D). Ghrelin-containing cells were distributed in the columnar epithelia, which had a spindly shape and a high cytoplasmic density, and were in contact with the lumen (Fig. 1C). Both tapered and rounded ghrelin cells with high cytoplasmic densities were situated in the lamina propria (Fig. 1C) and crypt (Fig. 1D). The GHSR-1 immunoreactivity was observed in the stomach and duodenum (Fig. 2, A and B). The GHSR-1 immunoreactivity was present in the gastric submucosa, muscularis mucosa, and myenteric plexus layers of the stomach (Fig. 2, A and B) and was also detected around the crypts and submucosa and in the myenteric plexus layers of the duodenum (Fig. 2, C and D).

Effects of ghrelin on gastric emptying

The vehicle had no significant effect on gastric emptying in control or ghrelin-treated mice. Ghrelin (1–20 $\mu\text{g/kg}$ i.p.) had no significant effect on gastric emptying in control mice (Fig. 3A). LPS-treated mice showed significantly decreased gastric emptying from 57.1% to 40.4% (Fig. 3B). But in LPS-treated mice, ghrelin (1–20 $\mu\text{g/kg}$) was found to reverse the endotoxin-induced delay in gastric emptying (Fig. 3B).

Effects of ghrelin on GIT

Ghrelin (1 and 20 $\mu\text{g/kg}$) significantly increased GIT of a charcoal meal in vehicle-treated control mice (Fig. 4A). LPS-treated mice showed a significant decrease in GIT from 42.4% to 31.9%. Ghrelin significantly reversed the endotoxin-induced GIT delay in LPS-induced mice (Fig. 4B).

The localization of iNOS and COX-2 in the stomach and duodenum

The expression of iNOS and COX-2 was examined in the mice stomach and duodenum with immunohistochemical assay (Figs. 5 and 6). There were no observable iNOS and COX-2 expressions in the tissue of saline-treated control mice (Fig. 5, A and D, and Fig. 6, A and D). At 24 h after LPS administration, high expression of iNOS was found in the stomach and duodenum (Fig. 5, B and E). Ghrelin (20 $\mu\text{g/kg}$ i.p.) dramatically reduced iNOS expression in submucosal and muscle layers of both tissues of LPS-induced mice (Fig. 5, C and F).

LPS-increased COX-2 expression was predominantly observed in epithelial cells of gastric mucosa (Fig. 6B) and expressed in the epithelial cells of the villi and the muscular layer of the duodenum (Fig. 6E). After ghrelin administration

in LPS-treated mice, there was no distinguishable change of COX-2 expression in the stomach and duodenum (Fig. 6, C and F).

Effects of ghrelin on nNOS, eNOS, and iNOS expressions in the stomach and small intestine of endotoxemic mice

To determine whether the production of NOS in GI tissues resulted from the induction of nNOS, eNOS, and iNOS in endotoxemic mice, Western blot analysis was performed. Ghrelin increased eNOS protein expression in GI tissues. In endotoxemic mice, ghrelin did not significantly reduce the LPS-induced increase in eNOS protein expression (Fig. 7).

As shown in Fig. 7, in control mice, there were only slightly detectable levels of nNOS and iNOS expression in both tissues. Ghrelin did not affect nNOS or iNOS protein expression in either tissue. The protein expression of iNOS but not nNOS markedly increased in both tissues of endotoxemic mice. Ghrelin (20 $\mu\text{g/kg}$) significantly decreased the LPS-induced iNOS protein overexpression but did not alter nNOS protein expression in either tissues of endotoxemic mice (Fig. 7).

Effects of ghrelin on plasma NO levels

Ghrelin (1 $\mu\text{g/kg}$) or vehicle, but not the 20- $\mu\text{g/kg}$ treatment, in mice did not affect plasma NO basal levels (Fig. 8). LPS induced a significant increase in plasma NO levels. Ghrelin (20 $\mu\text{g/kg}$) markedly reduced the LPS-induced increase in plasma NO production from 2,420 to 1,737 nmol/mL (Fig. 8).

Effects of ghrelin on COX-2 expression and the levels of PGE₂ in the stomach and small intestine in endotoxemic mice

There was only a slightly detectable level of COX-2 expression in GI tissues. In endotoxemic mice, the protein expressions of COX-2 significantly increased in GI tissues (Fig. 9A).

Ghrelin (1 and 20 $\mu\text{g/kg}$ i.p.) showed no effect on LPS-induced COX-2 protein expression in GI tissues. Ghrelin (20 $\mu\text{g/kg}$) significantly induced PGE₂ content to the extents of

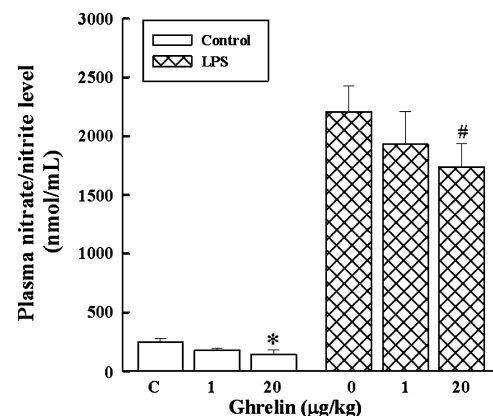


FIG. 8. Effects of ghrelin on LPS-induced plasma NO production. The conditions were the same as those described in Fig. 7. Blood samples were collected by puncturing the heart in mice anesthetized with ether. **P* < 0.05 compared with the control group; #*P* < 0.05 compared with LPS treatment. Each column represents the mean $\pm$ SEM (*n* = 7 or 8).

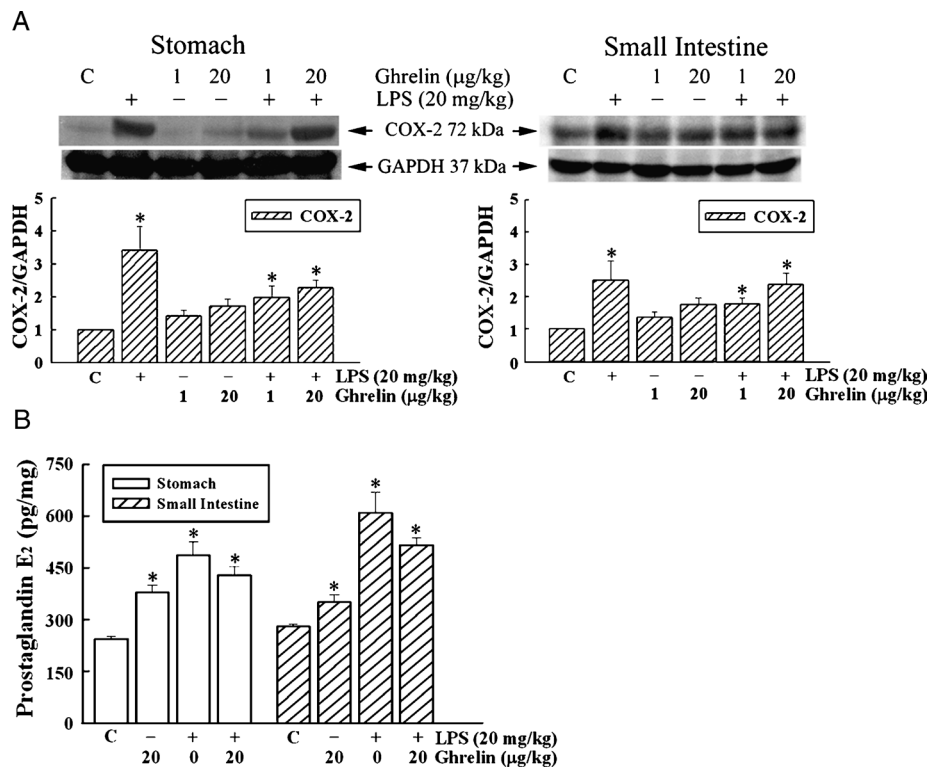


FIG. 9. Effects of ghrelin on LPS-induced COX-2 protein expression and PGE₂ production in the stomach and small intestine. A, LPS-induced COX-2 protein expression; B, LPS-induced PGE₂ production. The conditions were the same as those described in Fig. 7. * $P < 0.05$, significantly different from the individual control. Each column represents the mean $\pm$ SEM ($n = 6$).

244 to 379 and 280 to 351 pg/mg in the stomach and small intestine, respectively. We observed a slight but insignificant reduction in PGE₂ levels by ghrelin in LPS-treated mice, and PGE₂ levels went from 487 to 429 pg/mg ($P = 0.24$) in the stomach and from 609 to 516 pg/mg ($P = 0.17$) in the small intestine (Fig. 9B) according to the ELISA assay.

DISCUSSION

In this communication, we have shown that ghrelin-producing cells are distributed through mucosal layers in the GI tract, although the stomach seems to be the richest source of cells producing ghrelin (Fig. 1). Ghrelin-containing cells abundantly exist mainly on the gastric mucosa and are widespread in the villi and the crypt of the duodenum (23, 24). Ghrelin can stimulate GI myoelectrical activity through both the vagal cholinergic muscarinic and the noncholinergic intrinsic local pathway in the enteric nervous system (25). The present study provides immunohistochemical evidence for the presence of ghrelin and its functional receptor, GHSR-1, in the GI tract of the mice. The GHSR-1 immunoreactivity was present in the submucosa, muscularis mucosa, and myenteric plexus layers of the stomach and was also detected around the crypts and submucosa and in the myenteric plexus layers of the duodenum (Fig. 2). Therefore, we suggest that GHSR-1 may be activated by ghrelin, which is directly released from the stomach or intestine as a circulating hormone influencing GI motility.

Proinflammatory and oxidative conditions can cause LPS-induced endotoxemia in mice, and ghrelin has been suggested to have therapeutic potential for LPS-induced endotoxemia (21, 26). In the present study, we showed that ghrelin im-

proved LPS-induced GI motility disturbances (Figs. 3 and 4). A low dose of ghrelin accelerated the GIT of a charcoal meal but did not influence gastric emptying of liquids in normal mice. LPS at 20 mg/kg induced endotoxemia, which triggers clinical features of gram-negative bacterial infection, including reduced gastric emptying and GIT. Ghrelin reversed the LPS-induced reduction of gastric emptying and GI motility (Figs. 3 and 4) and decreased localization of iNOS in the GI tract of mice with LPS-induced endotoxemia. The current data also showed that ghrelin modulated NO and PGE₂ levels in endotoxemic mice, suggesting that ghrelin may improve the inflammatory response to endotoxemia. However, how ghrelin protects the GI tract through NO and PGE₂ from LPS-induced GI motility disturbances remains unclear and requires further investigation.

The effects of ghrelin on gastric emptying and GIT have been studied with solid and liquid meals, but the appropriate dosages are still undefined in different species of animals (21, 27). In our study, we also observed that low doses of ghrelin (1–20 μg/kg i.p.) did not significantly change gastric emptying in normal mice. This finding agrees with results of other rodent studies (21, 27). We found that ghrelin at 1 to 20 μg/kg significantly accelerated GIT but not gastric emptying in normal mice (Fig. 4). Our findings on gastric emptying, but not GIT, agree with those in a previous study (21). Our observations are the first to show that an i.p. injection of 1 to 20 μg/kg ghrelin improved the LPS-induced reduction in gastric emptying and GI motility within 15 min of onset. At 5 h after an LPS injection, 30 and 300 μg/kg ghrelin had significantly reversed the gastric emptying of a liquid meal in

LPS-treated rats (27), but LPS at a low dose (100 µg/kg i.p.) reduced food intake and gastric emptying without endotoxemia in their study. The present results agree with previous findings, suggesting its potential as a therapeutic agent for the LPS-induced delay in GIT even at low doses. Our results agree with previous reports on ghrelin's ability to improve GI motility (27) and to accelerate gastric emptying in LPS-treated mice at 1 h after a ghrelin injection at 20 µg/kg (21). Taken together, we suggest that ghrelin has a beneficial effect on LPS-induced GI motility disturbances in mice.

NO is a well-known inhibitory neurotransmitter of GI motility. It was shown that NOS is present in the enteric nervous system (28, 29). Activation of any isoforms of NOS, that is, nNOS, was demonstrated in the myenteric plexus, which indicates that ghrelin's enhancement of GI motility may be mediated by modulating nNOS-derived NO. In this study, we found no significant changes in nNOS expression in ghrelin-treated mice compared with control or LPS-treated mice. Atropine administration blocking the effect of ghrelin suggests that ghrelin receptors are present in the intestinal neuromuscular tissue through a cholinergic mechanism (30). Ghrelin can affect noncholinergic vagal neurons' modulation of gastric myoelectrical activity and the gastric emptying rate, but that is not completely blocked by atropine (25). In addition, few ghrelin-positive cells stained for nNOS were observed in the longitudinal muscle/myenteric plexus of the guinea pig ileum. Ghrelin mainly colocalized with choline acetyl transferase and may therefore regulate locally excitatory inputs to enteric smooth muscle cells (31). Our study results indicated that ghrelin increased GIT of a charcoal meal without affecting nNOS-derived NO synthesis (Figs. 4 and 7). Taken together, we suggest that ghrelin can modulate GI contractions through activating a cholinergic excitatory pathway instead of the nNOS-NO pathway.

Although oxidative stress influences GI motility is not clear. The muscularis macrophage network plays a major role in mediating smooth muscle dysfunction such as the ileus by the inflammatory response (32). The role of NO in disturbed motor activity related to endotoxin-induced inflammatory GI diseases has traditionally focused on iNOS (15, 17, 33). Inhibiting iNOS-NO can accelerate gastric emptying and improve the delay in small intestinal transit in septic rodents (15, 17, 21). Although ghrelin produces no mediatory effect on nNOS-NO expression, our study showed that ghrelin had a beneficial effect on LPS-induced GI disturbances associated with the down-regulation of iNOS-NO expression (Figs. 5, 7, and 8). Relaxation of the GI tract by NO produced by iNOS delays gastric emptying and GIT in LPS-treated rats (15, 17, 21). In the control group, ghrelin (1 and 20 µg/kg) had no significant effect on iNOS protein expression in the stomach or small intestine. At 24 h after LPS administration, iNOS protein and NO levels had increased, and this is consistent with the findings of Liang et al. (17). Ghrelin administration at 20 µg/kg significantly inhibited the plasma NO level and iNOS protein in both the stomach and small intestine in LPS-treated mice. Exogenous ghrelin was also shown to decrease the proinflammatory cytokines, IL-1β, and TNF-α in LPS-stimulated murine macrophages (5) or in a rat model of

polymicrobial sepsis induced by cecal ligation and puncture (34). On the basis of these results, we suggest that ghrelin's antioxidative effect and its beneficial effect on LPS-induced GI motility disturbances are associated with down-regulation of NO expression.

It is well known that eNOS affects gastric mucosal integrity via actions on the microcirculation and the surface mucous-bicarbonate barrier (13, 14). We observed that ghrelin increased eNOS expression in the stomach and small intestine of conscious mice. These results provide evidence that ghrelin increases cytoprotection and gastric blood flow by eNOS to regulate gastric function and to provide gastroprotection and GI motility.

In normal physiological conditions, the amount of free intracellular arachidonic acid available is small, but the activation of phospholipases (mainly phospholipase A2) can induce the release of arachidonic acid from membrane phospholipids. Free intracellular arachidonic acid can then be metabolized through COX pathways. Thus, this fatty acid is the precursor for the further metabolism of a large number of biologically active products, named eicosanoids (35). Although both isoforms of COX (COX-1 and COX-2) are involved in PGE₂ formation, they are likely to have fundamentally different biological roles. Cyclooxygenase 1 is a housekeeping enzyme that is constitutively expressed in most mammalian tissues to maintain physiological functions. In contrast, COX-2 is barely detectable under normal physiological conditions, but it can be rapidly and transiently induced by proinflammatory mediators. In this study, ghrelin was found to slightly but insignificantly down-regulate PGE₂ and COX-2 expressions elicited by LPS (Figs. 6 and 9). This finding suggests that ghrelin treatment does not block PGE₂/COX-2 activation stimulated by LPS. However, the detailed mechanism needs to be further elucidated.

In conclusion, our study shows that ghrelin maintained GI homeostasis through differential modulation of NO and PGE₂ production in control and LPS-treated mice. Expression of GHSR-1 in the GI tract suggests that ghrelin may participate in stabilizing GI function during an inflammatory response. Therefore, the study findings suggest that ghrelin can protect against endotoxemia-induced GI imbalances.

ACKNOWLEDGMENTS

We would like to thank Dr Yen-Chou Chen (Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University) for her critical reading of manuscript. This study was sponsored by the Taipei Medical University-Wan Fang Hospital (96TMU-WFH-17).

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