in circulating and tissue factors that regulate ICC differentiation and survival contribute to the profound depletion of mature ICC and impair gastric function.

Conclusion

In conclusion, oxidative stress is one of the major contributors to the development of stomach diseases. Recent therapeutic options such as gastroprotective agents including antioxidant properties (e.g., rebamipide) can modulate the level of oxidative stress to enhance anti-inflammatory or antioxidant capacity. The stomach is an organ in direct contact with external pathogens; by presenting a strong acid environment, it has a special biological defense mechanism that eliminates such pathogens. However, *H. pylori* manages to live in the stomach by breaking through this defensive line. In response to the colonization of this bacterium, gastric mucosa can be exposed to severe oxidative stress with considerable levels of inflammatory cell accumulation, which might be related to the development of gastric mucosal as well as neuromuscular disorders.

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Abbreviations

AGEs	advanced glycation end products
GGA	geranylgeranylacetone
GGT	γ-glutamyltranspeptidase
HSF	heat shock factor
ICC	interstitial cells of Cajal
LPO	lipid peroxide
MAPK	mitogen-activated protein kinase
Mtz	metronidazole
nNOS	neuronal nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
PGs	prostaglandins
PHG	portal hypertensive gastropathy
PPI	proton pump inhibitor
RNS	reactive nitrogen species
ROS	reactive oxygen species
RUNX3	Runt domain transcription factor 3
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substance

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ORIGINAL ARTICLE

Enhanced Gastric Ghrelin Production and Secretion in Rats with Gastric Outlet Obstruction

Eisuke Iwasaki · Hidekazu Suzuki · Tatsuhiro Masaoka · Toshihiro Nishizawa · Hiroshi Hosoda · Kenji Kangawa · Toshifumi Hibi

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Abstract
Background and Aim Ghrelin has distinct effects on gastrointestinal motility through the vagus nerve and gastric excitatory neural plexus. The objectives of this study were to investigate the dynamics of ghrelin and expression of neuromuscular markers in a newly established surgically manipulated rat model of gastric outlet obstruction (GOO), akin to the pyloric stricture associated with duodenal ulcer, advanced gastric cancer, and other conditions, in the clinical setting.
Material and Methods The rats were divided into two groups, a control group (sham operation) and the GOO group (proximal duodenal stricture). The animals were sacrificed 2 weeks after the operation. Plasma and gastric ghrelin were measured by radioimmunoassay. mRNA expression in the stomach of neural choline acetyltransferase (ChAT), c-kit, and membrane-bound stem cell factor (SCF) were analyzed by quantitative RT-PCR. In addition, gastric mRNA expression of the aforementioned were also evaluated 60 min after intraperitoneal administration of a synthetic GHS-R1a antagonist ([D-Lys3] GHRP-6 6.0 mg/kg).
Results Mechanical GOO induced increases of fasting plasma ghrelin levels and hyperplasia of the gastric muscle layers, with enhanced expression of the gastric neuromuscular markers. Administration of [D-Lys3] GHRP-6 normalized the enhanced expression of c-kit and SCF.
Conclusion GOO stimulates ghrelin dynamics and then enhances the mechanistic expression of gastric cellular communication network molecules between nerves and smooth muscle cells.

Keywords Ghrelin · Gastric emptying · Motility · Gastric outlet obstruction

E. Iwasaki · H. Suzuki (✉) · T. Masaoka · T. Nishizawa · T. Hibi
Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: hsuzuki@a6.keio.jp

E. Iwasaki
Department of Internal Medicine, Saiseikai Central Hospital, 1-4-17 Mita, Minato-ku, Tokyo 108-0073, Japan

H. Hosoda · K. Kangawa
Department of Biochemistry, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

Introduction

Ghrelin, a 28-amino-acid motilin-related peptide, was first purified from the rat stomach as a natural ligand for the growth hormone secretagogue receptor (GHSR) [1]. It has also been shown to stimulate food intake, induce body weight gain, and enhance gastric motility. Recent animal studies have shown that ghrelin has distinct effects on gastrointestinal motility, which may be mediated through the GHSR expressed on the vagus nerve and enteric nerve endings [2, 3]. Ghrelin has been reported to enhance gastric motility and accelerate gastric emptying in rats and mice and to stimulate small intestinal transit [2, 4]. Fujino et al. reported that ghrelin induces accelerated motor activity of the gastrointestinal tract via ghrelin receptors expressed on vagal afferent nerve terminals and activated neuropeptide Y neurons in the brain [5].

Chasen et al. reported that abnormal electrogastrography diagnosis and increased levels of plasma ghrelin were

found in most patients with advanced cancer [6]. We have previously shown increased fasting plasma levels of ghrelin in patients with functional dyspepsia (FD), especially those with dysmotility-like FD, possibly originating from gastric motility disorders, including delayed gastric emptying [7]. Although the precise molecular mechanisms are not yet clear, such clinical manifestations suggest that ghrelin production might be increased by impaired gastric motility.

The objectives of this study were to investigate the dynamics of ghrelin and expression of neuromuscular markers in a newly established surgically manipulated rat model of gastric outlet obstruction (GOO), akin to the pyloric stricture associated with duodenal ulcer, advanced gastric cancer, and other clinical conditions.

Methods

Animal Procedures

This study was conducted with the approval of Keio University Animal Research Committee (no. 056188). Seven-week-old male specific-pathogen-free (SPF) Sprague–Dawley (SD) rats (Sankyo Laboratory Service, Tokyo, Japan) were used for the study after acclimatization for 7 days in an animal room at a controlled temperature (24 ± 2°C). The rats were fed standard chow and deprived of food for 24 h before the operation. After induction of anesthesia by intraperitoneal injection of 50 mg/kg sodium pentobarbital, the hair was shaved off the upper abdomen of the animals. The abdomen was opened via a 25-mm-long median incision, and the stomach was exposed. The proximal duodenum was then carefully covered with an 18-Fr Nelaton catheter (diameter, 4.0 mm; Nippon Sherwood, Tokyo, Japan) and sutured with a 5–0 nylon thread (Fig. 1). The width of the catheter was 2.0 mm. This surgical duodenal stricture induced incomplete gastric outlet obstruction with gastric retention; this animal group was named the GOO group. After the operation, the animals were deprived of food for 24 h, but allowed free access to water. A sham operation, involving only abdominal incision, was also undertaken on the same number of rats of the control group.

Because weight loss is known to increase circulating levels of ghrelin, we designed a pair-feeding method to evaluate the dynamics of ghrelin without the confounding effect of body weight changes. The results of our preliminary experiments revealed that food intake in the GOO group was lower than that in the control group. We measured daily food intake in the GOO group, and the control group of rats received the same amount of food on the following day. After 2 weeks, after food deprivation for 24 h, the rats were sacrificed under ether anesthesia and the gastric wet weight and intraluminal pH were measured. The thicknesses of the gastric antral mucosal layer and muscular layer were measured by light-microscopic examination of hematoxylin–eosin-stained sections. The average thicknesses 1, 1.5, and 2.0 mm from the pylorus were measured.

Evaluation of Gastric Emptying

Our surgical procedure is the first reported method for establishing an animal model of GOO. We compared the gastric emptying rate in this model with that in another group of 7-week-old SPF male SD rats. The animals were divided into a control group and GOO group as previously described. Two weeks after the operation, the rats were deprived of food, but allowed free access to water for 24 h before the start of the subsequent experiment. The gastric emptying rate was measured by the phenol red (PR) method reported by Ohnishi et al. [8]. One milliliter of PR (100 μg/ml) was administered orally to the rats, and the rats were sacrificed by cervical dislocation either immediately (Control group, $n = 4$) or 15 min (Control group, $n = 4$; GOO group, $n = 6$) after administration of the PR solution. The standard stomachs (0 min) and test stomachs (15 min) were exposed and ligated at the cardia and pylorus. Each stomach was removed and placed in 10 ml 0.1 M Na_2HPO_4 solution, and the contents of the stomach were washed out. The absorbance of the supernatant was measured at 570 nm with a spectrometer (Shimadzu, Kyoto, Japan). The gastric emptying rate for each rat was calculated as described previously [8].

Measurement of the Ghrelin Dynamics

The rats were divided into two groups, the control group ($n = 14$) and the GOO group ($n = 14$). After 2 weeks, following food deprivation for 24 h, the rats were sacrificed under ether anesthesia. The abdomen and chest were opened via a median incision. Blood was collected from the heart and centrifuged at 3,000 rpm for 10 min to obtain plasma and serum samples for assay. After collection of the blood, the stomachs of the animals were excised and the intraluminal pH was examined by use of a pH meter (Horiba Techno Service, Kyoto, Japan). The stomachs were then cut along the greater curvature and rinsed with isotonic saline. The two radioimmunoassays (RIAs) for measurement of the gastric and plasma ghrelin levels were performed as described previously [9, 10]. Two polyclonal rabbit antibodies were raised against the N-terminal [1–11] (Gly1-Lys11) and C-terminal [13–28] (Gln13-Arg28) fragments of rat ghrelin. [Cys12]-rat ghrelin [1–11] (4 mg) and [Cys0]-rat ghrelin [13–28] (10 mg) were separately conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce, Rockford, IL, USA; 6 mg) in conjugation buffer (Pierce). Each conjugate was

emulsified with an equal volume of Freund's complete adjuvant. Two corresponding batches of antiserum were obtained by immunization of New Zealand white rabbits by subcutaneous injection. Using these antibodies, two types of RIAs to measure the plasma and gastric ghrelin levels were performed as described previously [11].

Immunohistochemistry for Ghrelin

Stomach samples were fixed in 10% formaldehyde neutral buffer solution for 24 h, then embedded in paraffin. Tissue sections were deparaffinized and hydrated, and endogenous peroxidase was quenched by treatment with 0.3% hydrogen peroxide for 20 min. Nonspecific binding was blocked by use of a blocking reagent (BlockAce; Dainippon Pharmaceuticals, Osaka, Japan). After washing with TBS-T, the tissue slices were incubated for 60 min at 4°C with anti-ghrelin antiserum (final dilution, 1:10,000). Then, after washing again with TBS-T, the slides were incubated with EnVision + Peroxidase rabbit (DAKO Japan, Kyoto, Japan) for 30 min at room temperature, and then visualized after color development with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 1 min. The sections were then counterstained with hematoxylin. The stained sections were examined under high-power magnification (objective lens ×40) by light microscopy equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan). The nuclei were counted using Image-J software (National Institutes of Health, Bethesda, Maryland, USA). The density of the ghrelin-immunoreactive cells was computed by use of the equation: $D_{ghrelin} = (N_g/N_t) \times 100$ (%), where Ng and Nt represent the number of ghrelin-immunoreactive cells and the total cell number, respectively, in the three mucosal regions of the stomach.

Preparation of Total RNA and Quantitative RT-PCR Analysis

Total mRNA was extracted from the stomach tissue by use of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA by use of the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). Cyber green quantitative real-time RT-PCR was performed to detect the mRNA using the Thermal Cycler Dice Real Time System (Takara Bio, Otsu, Japan). The primers used to amplify the target mRNA were: c-kit mRNA 5′-ATC CAG CCC CAC ACC CTG TT-3′, and 5′-TGT AGG CAA GAA CCA TCA CAA TGA-3′, SCF (membrane-bound isoform) mRNA: 5′-TGA GAA AGG GAA AGC CGC-3′, and 5′-TAA GGC TCC AAA AGC AAA GC-3′, choline acetyltransferase (ChAT) mRNA: 5′-CAA CCA TCT TCT GGC ACT GA-3′, and 5′-TAG CAG GCT CCA TAG CCA TT-3′, glyceraldehyde-3-phosphate (GAPDH) mRNA: 5′-GGC ACA GTC AAG GCT GAG AAT G-3′, and 5′-ATG GTG GTG AAG ACG CCA GTA-3′. The target mRNA expression levels were normalized to the GAPDH mRNA expression levels.

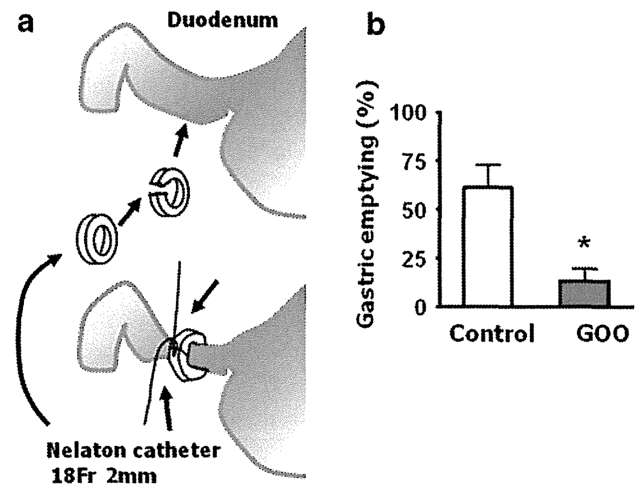

Fig. 1 **a** Surgical manipulation for induction of duodenal stricture. The proximal duodenum was covered with a small piece of an 18Fr-Nelaton catheter. **b** Liquid gastric emptying rates of control rats ($n = 4$, *open bar*) and GOO rats ($n = 6$, *filled bar*) 2 weeks after the operation (mean ± S.E.M. $*P < 0.05$ compared with control)

RT-PCR Analysis for Interstitial Cells of the Cajal (ICC) Network Using a GHSR1a Antagonist

In a separate experiment, to examine the relationship between ghrelin and neuromuscular marker expression, the rats were administered an intraperitoneal injection of [D-Lys3] GHRP-6, a GHSR antagonist, (6.0 mg/kg; Bachem, King of Prussia, PA, USA) in 1 ml saline ($n = 8$), or saline alone ($n = 8$) and then euthanized 60 min after administration. Then, the mRNA expression levels of c-kit, SCF, and ChAT were analyzed by quantitative RT-PCR, as previously described.

Statistical Analysis

All results are expressed as mean ± SE, and the statistical analysis was performed using the Student *t* test (two-tailed test) with Stat Mate III (Atoms, Tokyo, Japan). *P* values less than 0.05 were considered to be statistically significant.

Results

Gastric Emptying Rate in this Rat Model

The gastric emptying rate after 15 min as measured by the PR method was significantly lower in the GOO group than

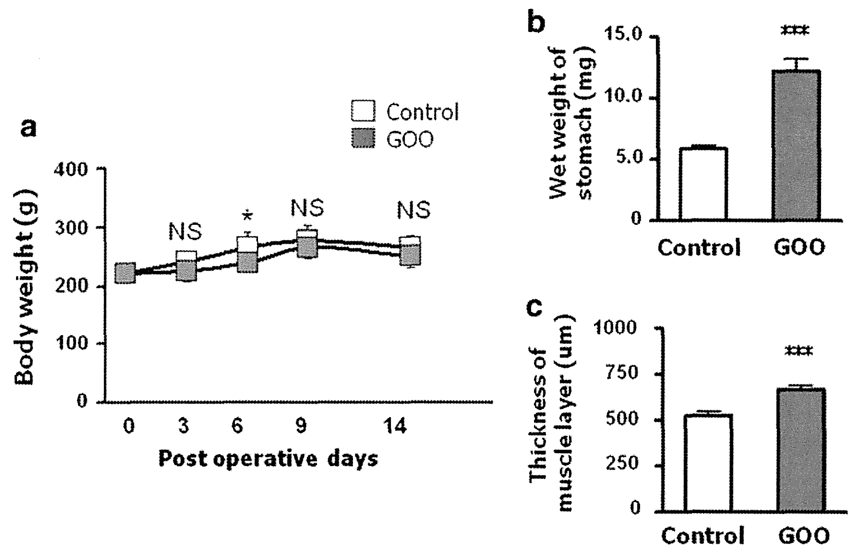

Fig. 2 **a** Body weight was measured 0, 3, 6, 9, and 14 days after the operation. On days 9 and 14 after the operation, no significant differences in the changes of the body weights were observed between the two groups. The mean weight of the rats on the operation day was 220.2 ± 4.5 g in the control group and 218.2 ± 2.0 g in the GOO group. **b** Wet weight of the removed stomach 2 weeks after operation. **c** Thickness of the muscle layer at the antrum using HE stain. (mean ± S.E.M.; $n = 13$ in each group.*$P < 0.05$, ***$P < 0.001$ compared with control.)

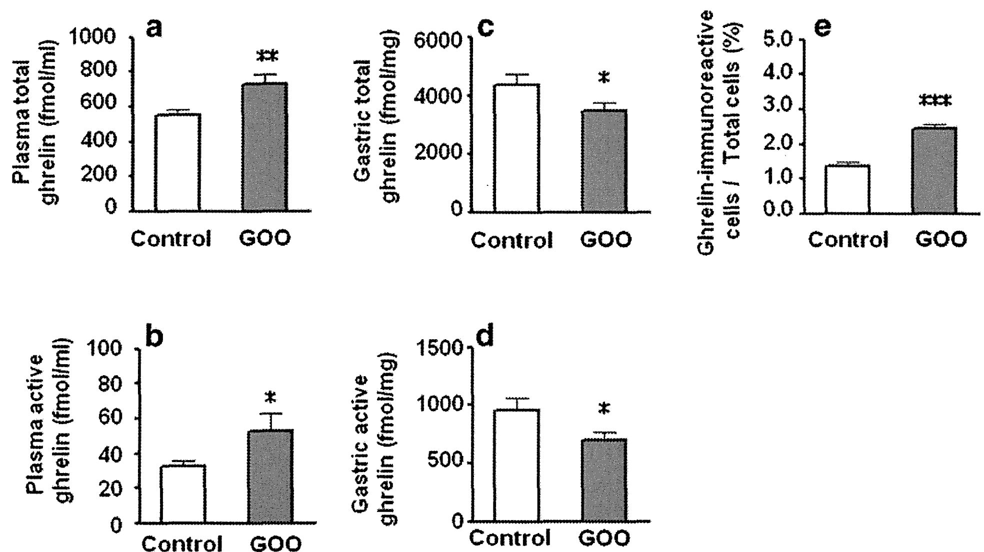

Fig. 3 Fasting levels of total (**a**) and active (**b**) plasma ghrelin concentrations and of the total (**c**) and active (**d**) gastric ghrelin contents were measured (**a–d**). The plasma ghrelin levels were increased in the GOO group, whereas gastric ghrelin levels decreased. **e** Immunohistochemistry for ghrelin. The density of the ghrelin-immunoreactive cells in the gastric corpus (mean ± S.E.M.; $n = 13$ in each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.)

in the control group (21.8 ± 7.8% vs. 61.0 ± 12.0%, $P = 0.011$; Fig. 1b).

Gastric Wet Weight and Thickness After Outlet Obstruction

In this model of GOO, 2-weeks survival in the GOO group was 92.9% ($n = 13/14$); survival in the control group was 92.9% ($n = 13/14$). The changes in the mean weights of the rats are shown in Fig. 2a. After 14 days of obstruction, the gastric wet weight in the GOO group was increased compared with that in the control group (12.2 ± 3.3 g vs. 5.8 ± 1.1 g, $P < 0.001$; Fig. 2b). The thickness of the gastric antral muscle layer was significantly higher in the GOO group than in the control group (675.4 ± 24.6 μm vs. 558.5 ± 20.8 μm, $P < 0.005$; Fig. 2c). Similarly, the thickness of the gastric antral mucosal layer was also significantly increased in the GOO group compared with that in the control group (322.0 ± 26.4 μm vs. 196.8 ± 7.9 μm, $P < 0.001$). In contrast, there was no significant difference in the fasting intraluminal pH of the stomach between the two groups (control group, pH 1.70 ± 0.13 vs. GOO group, pH 1.70 ± 0.26).

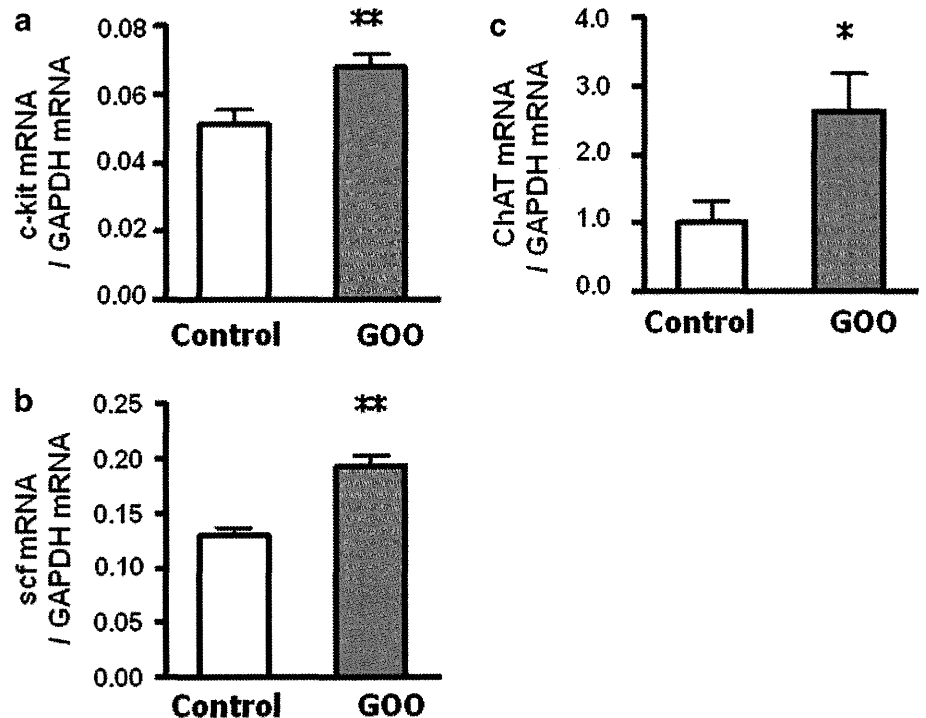

Fig. 4 Gastric c-kit, membrane-bound SCF, ChAT mRNA expression was measured by RT-PCR. **a** gastric c-kit mRNA; **b** membrane-bound SCF mRNA; **c** ChAT mRNA. (mean ± S.E.M.; $n = 13$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control)

Ghrelin Dynamics

The ghrelin dynamics 2 weeks after the operation are shown in Fig. 3. The plasma total and active ghrelin levels were higher in the GOO group than in the control group (plasma total ghrelin; $P = 0.002$, plasma active ghrelin; $P = 0.024$; Fig. 3a, b). In contrast, the gastric total and active ghrelin levels were lower in the GOO group than in the control group (gastric total ghrelin; $P < 0.001$, gastric active ghrelin; $P < 0.001$; Fig. 3c, d). The results of the immunohistochemical analysis to determine the density of the ghrelin-immunoreactive cells in the gastric corpus are shown in Fig. 3e. Increase in the cell count ratio in the GOO group compared with that in the control group was observed ($P < 0.001$).

Gastric Neuromuscular Marker Expression

The mRNA expression levels of these markers as assessed by quantitative RT-PCR analysis are shown in Fig. 4. Significant increases of the expression levels of ChAT mRNA (263.9 ± 54.0% compared with control $P = 0.019$), c-kit mRNA (132.5 ± 7.2% compared with control, $P = 0.008$), and SCF mRNA (149.4 ± 7.8% compared with control, $P < 0.001$) were observed in the GOO group compared with expression levels in the control group.

Effects of [D-Lys3] GHRP-6 on c-Kit and SCF Expression

Significant decreases of expression of c-kit mRNA (54.8 ± 10.3%, $P = 0.024$) and membrane-bound SCF mRNA (51.8 ± 7.5%, $P = 0.009$) were observed in the GOO group after pretreatment with [D-Lys3] GHRP-6 compared with those in the control group (Fig. 5a, b).The expression of ChAT mRNA also tended to decrease to the control level.

Discussion

This study showed experimentally that GOO induced an increase of the fasting plasma ghrelin levels and hyperplasia of the gastric muscle layers. Associated with these pathological processes, expression of ChAT, a marker of vagal efferent fibers in the stomach, c-kit, a marker of the interstitial cells of Cajal (ICC), and SCF, a c-kit ligand on the gastric muscles, were all significantly enhanced.

Two signal transmission pathways from secreted ghrelin to the myenteric plexus in the stomach have been reported [12, 13]. One is a direct route in which ghrelin directly stimulates the GHSR on the surface of the myenteric neurons [12]; the other is an indirect route in which ghrelin signaling stimulates GHSR on the vagal afferent fibers, with the vagal signal traveling through the central nervous system and then to the vagal efferent nerve fibers, finally

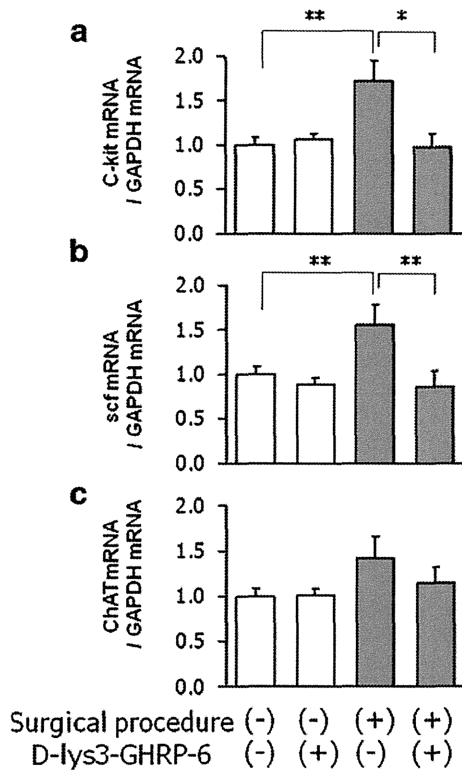

Fig. 5 Effect of synthetic GHS-R1a antagonist ([D-Lys3]-GHRP-6 6.0 mg/kg) on mRNA expression of gastric c-kit (**a**), membrane-bound SCF (**b**) and ChAT (**c**) at 2 weeks after the operation. (mean ± S.E.M.; $n = 8$ in each group. $*P < 0.05$, $**P < 0.01$ compared with control)

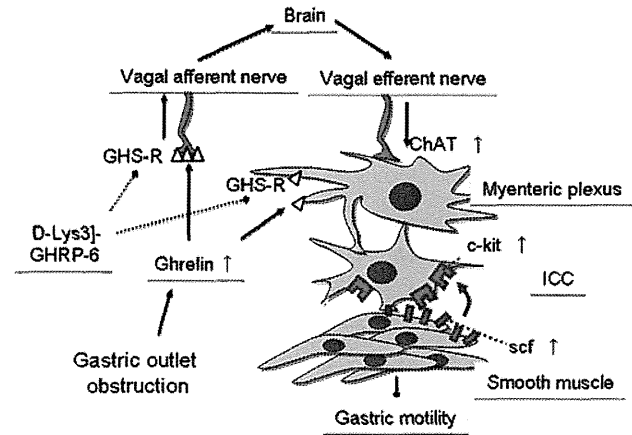

Fig. 6 Model of the association of ghrelin with the ICC network in the present GOO model. Signal of enhanced levels of plasma ghrelin in the GOO model is transmitted to the brain via vagal afferent nerves. Enhancement of ChAT mRNA might be induced via vagal efferent nerves from the central nervous system. Sustained enhanced ghrelin secretion might be associated with the activated ICC network in this animal model. The compensative ghrelin secretion and production are enhanced by gastric outlet obstruction, and enhanced ghrelin activates the ICC network either through the vagal nerve or the direct effect of ghrelin

activating the myenteric plexus [13]. In this study, because not only c-kit and SCF expression but also expression of ChAT were enhanced (Fig. 4), the ghrelin signal might be transmitted not only through the direct route, but also via the indirect route (Fig. 6).

Because [D-Lys3] GHRP-6, a receptor antagonist of GHSR1a, normalized the enhanced expression levels of c-kit and SCF (Fig. 5), it seems that ghrelin signaling might be an upstream event in relation to other neuromuscular activation markers, for example vagal efferent choline acetyl transferase (ChAT), c-kit (ICC), and SCF (gastric smooth muscle).

Both the increase in the plasma ghrelin levels and in the number of ghrelin-immunoreactive cells in the gastric corpus clearly indicates the activation of ghrelin production under the state of GOO (Fig. 3a, b, e). On the other hand, the decreased gastric ghrelin content in GOO (Fig. 3c, d) might be because of emptying (degranulation) of ghrelin from the A-like cells of the stomach in response to fasting. A similar phenomenon has already been reported in the Mongolian gerbil model of *Helicobacter pylori* infection [14].

Whereas the rat model of diabetic gastroparesis induced by STZ showed vagal denervation [15] and the rat model of ischemia–reperfusion induced transient gastroparesis showed vagal and c-kit damage [16], this method of induction of mechanical GOO was superior to the above-mentioned methods, because it involved simple obstruction of gastric outflow without vagal denervation or drug administration. Therefore, we could observe the neurological or hormonal feedback in simple outlet obstruction under the condition of intact gastric mucosa, vagal nerve, and gastric nerve plexus.

In conclusion, this study provides the first evidence to suggest that the production and secretion of gastric ghrelin is increased in rats with GOO, implying that dysregulation of gastric motility may alter the ghrelin dynamics, as reported in clinical settings [7, 17]. This experimental rat model is not only useful for study of GOO, but also for that of chronic gastric emptying disorders, for example gastric paresis or FD, especially postprandial distress syndrome.

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Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion

Sachiko Mogami,[1,2] Hidekazu Suzuki,[1] Seiichiro Fukuhara,[1] Juntaro Matsuzaki,[1] Kenji Kangawa,[3] and Toshifumi Hibi[1]

[1]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan;* [2]*Tsumura Research Laboratories, Tsumura & Co., Ibaraki, Japan;* [3]*National Cardiovascular Center Institute, Osaka, Japan*

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Mogami S, Suzuki H, Fukuhara S, Matsuzaki J, Kangawa K, Hibi T. Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion. *Am J Physiol Gastrointest Liver Physiol* 302: G359–G364, 2012. First published December 8, 2011; doi:10.1152/ajpgi.00297.2011.—The gastrointestinal (GI) tract is one of the most susceptible organs to ischemia. We previously reported altered gastric motility after gastric ischemia and reperfusion (I/R). However, there have also been few reports of alterations in the eating behavior after gastric I/R. Ghrelin is a GI peptide that stimulates food intake and GI motility. Although ghrelin itself has been demonstrated to attenuate the mucosal injuries induced by gastric I/R, the endogenous ghrelin dynamics after I/R has not yet been elucidated. The present study was designed to investigate the relationship between food intake and the ghrelin dynamics after gastric I/R. Wistar rats were exposed to 80-min gastric ischemia, followed by 12-h or 48-h reperfusion. The food intake, plasma ghrelin levels, gastric preproghrelin mRNA expression levels, and the histological localization of ghrelin-immunoreactive cells were evaluated. The effect of exogenous ghrelin on the food intake after I/R was also examined. Food intake, the plasma ghrelin levels, the count of ghrelin-immunoreactive cells corrected by the percentage areas of the remaining mucosa, and the expression levels of preproghrelin mRNA in the stomach were significantly reduced at 12 h and 48 h after I/R compared with the levels in the sham-operated rats. Intraperitoneal administration of ghrelin significantly reversed the decrease of food intake after I/R. These data show that gastric I/R evoked anorexia with decreased plasma ghrelin levels and ghrelin production, which appears to be attributable to the I/R-induced gastric mucosal injuries. The decrease in the plasma ghrelin levels may have been responsible for the decreased food intake after gastric I/R.

food intake; ghrelin; mucosal injury

GASTROINTESTINAL (GI) TRACT is one of the most susceptible organ systems to ischemia. Various investigations have demonstrated that ischemia and reperfusion (I/R) contribute significantly to the gastric mucosal injuries caused by stress, such as burn stress (17) or hemorrhagic shock (35), nonsteroidal anti-inflammatory drugs (30), and *Helicobacter pylori* (*H. pylori.*) infection (26, 27). We previously demonstrated, not only postischemic mucosal injury, but also transient delay in gastric emptying in a rat model of gastric I/R (28). These changes were found to be associated with disruption of the network of the interstitial cells of Cajal and decrease in neuronal nitric oxide synthase-positive neurons in the smooth muscle layer. On the other hand, there have been no reports on alterations in eating behavior after gastric I/R, at least to our knowledge.

Ghrelin, a 28-residue octanoylated peptide, is an endogenous ligand of the growth hormone secretagogue receptor (18) and is produced and secreted from the A-like cells found mainly in the oxyntic glands of the gastric fundus (8). Gastric ghrelin accounts for the major part of circulating ghrelin, as an ~80% reduction in the circulating levels of ghrelin has been demonstrated after gastrectomy or fundectomy (10). Ghrelin is now known to play a role, not only in growth-hormone release, but also in stimulating gastric motility and food intake (1, 21, 32). Recent studies have also reported the gastroprotective effect of ghrelin; ghrelin has been demonstrated to reduce ethanol-induced gastric ulceration (23), acetic acid-induced chronic gastric and duodenal ulceration (6), and I/R-induced gastric ulceration (11) in rats. Although changes in the plasma ghrelin levels and association with various GI diseases have been reported such as in functional dyspepsia (22), chronic gastritis and gastric ulcer (14), the ghrelin dynamics after gastric I/R has yet to be elucidated.

The present study was designed to investigate the influences of gastric I/R injuries on the food intake and ghrelin dynamics in a rat model of gastric I/R injury.

MATERIALS AND METHODS

I/R. Six-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). All rats were handled according to the guidelines of the Keio University Animal Research Committee (approved protocol No. 078086) and the Experimental Animal Ethics Committee of Tsumura & Co. (approved protocol No. 09–155, 09–157, 10–096, 10–110, 10–156). All rats were used after acclimation for 1 wk and denied access to food for 22–24 h (but allowed free access to water) before the operation. The rats were anesthetized with pentobarbital sodium (50 mg/ kg ip) during the surgery. The abdomen was opened by a midline incision, and the celiac artery was occluded with a small clamp for 80 min. Reperfusion was established for 12 h or 48 h by removal of the clamp. For comparison, some rats were subjected to a sham operation (surgery, but no clamping). Rats were supplied with food after the surgery (returned to normal feeding). Food intake was measured at 12 h after I/R (when gastric emptying was delayed compared with sham-operated rats) and at 48 h after I/R (when gastric emptying was restored) (Fig. 1*A*). In the fasting condition, food deprivation was continued after the surgery when reperfusion was established for 12 h. When reperfusion was established for 48 h, the rats were fed after the operation (normal feeding), but were again deprived of food for 24 h before euthanasia to establish the fasted condition (Fig. 1*B*). To measure plasma ghrelin levels in the fed condition at 48 h after I/R, I/R rats were fed ad libitum after the surgery. Sham-operated rats were given the same amount of food as

Address for reprint requests and other correspondence: H. Suzuki, Div. of Gastroenterology and Hepatology, Dept. of Internal Medicine, Keio Univ. School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, JAPAN (e-mail: hsuzuki@a6.keio.jp).

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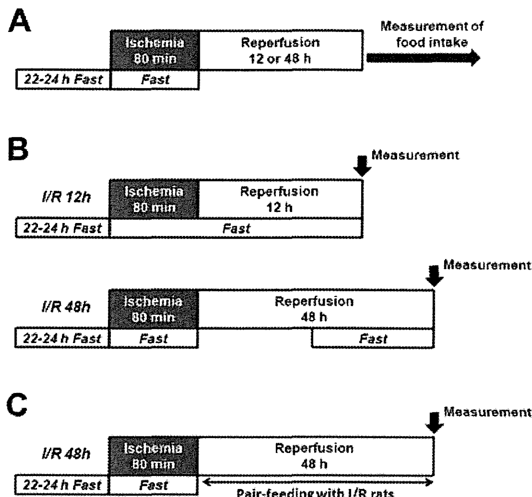

Fig. 1. Experimental protocol. *A*: measurement of cumulative food intake at 12 h or 48 h after gastric ischemia and reperfusion (I/R) in fed condition. *B*: measurement of gastric emptying rates or plasma ghrelin levels at 12 h or 48 h after I/R in fasted condition. *C*: measurement of plasma ghrelin levels at 48 h after I/R in the fed condition.

the I/R rats to eliminate the effect of the difference in the food intake (Fig. 1*C*).

Measurement of food intake. All rats were housed in individual hanging-wire cages. After reperfusion for 12 h or 48 h, the rats were supplied with preweighed food, and the cumulative food intake of each rat was calculated as the difference between the food weights before and after the feeding period. In the experiment to determine the effect of exogenous ghrelin, rat ghrelin (30 nmol/0.5 ml saline per rat; Peptide Institute, Osaka, Japan) or 0.5 ml saline was administered intraperitoneally, immediately before the supply of the preweighed food (Fig. 1*A*).

Evaluation of gastric emptying of solid food. Solid gastric emptying was evaluated using powdered food (13) and glass beads (24, 34). After 24-h food deprivation (Fig. 1*B*), 1 ml of the test meal containing powdered food and glass beads (0.2-mm diameter, BZ-02; AS One, Osaka, Japan) was orally administered to the rats through a Teflon tube (AWG-14) attached to a 1-ml syringe, using a 10Fr Nelaton's catheter. The test meal contained 32 g of ground meal, 40 g of glass beads, and 80 ml of distilled water. Rats were then killed by decapitation 2.5 h after the test meal administration, except for the animals that were killed immediately after the injection to recover the entire dose of the test meal. The gastric contents were then recovered from the stomach, dried, and weighed. The gastric emptying of solid food was calculated as follows: Gastric emptying (%) = [1 − (dried weight of food recovered from stomach / dried weight of food recovered from the stomach immediately after the test meal administration)] × 100.

Measurement of the plasma ghrelin levels. After 24-h food deprivation (Fig. 1*B*) or after 48-h pair feeding (Fig. 1*C*), whole blood samples were obtained from the right ventricle under ether anesthesia in tubes containing EDTA-2Na (1 mg/ ml) and aprotinin (500 kIU/ ml). Samples were promptly centrifuged at 4°C, and the supernatant was acidified with 1 mol/l HCl (1/10 volume) and stored at −80°C until use. The ghrelin level was determined using the Active Ghrelin ELISA Kit, and the desacylghrelin (ghrelin without octanoyl acid modification) level was determined using the Desacyl Ghrelin ELISA Kit (Mitsubishi Chemical Medience, Tokyo, Japan).

Immunohistochemistry. Stomach tissue specimens were fixed in 10% neutralized formalin and embedded in paraffin. After deparaffinization and hydration, the antigens were retrieved by heating for 20 min at 97°C in Dako REAL Target Retrieval Solution (DAKO Japan, Tokyo, Japan). Nonspecific binding was blocked by Protein Block (DAKO Japan). All sections were incubated overnight at 4°C with anti-ghrelin (13–28) antiserum (7) (1:10,000). After being washed with TBS-T, the slides were incubated with peroxidase-labeled dextran polymer conjugated anti-rabbit IgG in Tris-HCl (EnVision/HRP; Dako Japan) for 30 min at room temperature and then visualized after color development using 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 3 min. Counterstaining was performed with hematoxylin. The stained sections were observed under a light microscope equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan), and the photomicrographs were obtained in areas without gastric I/R-induced mucosal injuries. DAB-stained ghrelin immunoreactive cells were counted by visual inspection, and hematoxylin-stained nuclei were counted using the ImageJ program (National Institutes of Health, Bethesda, MD). The numbers of ghrelin-IR cells were normalized by dividing by the total number of cells counterstained with hematoxylin. The numbers of ghrelin-IR cells were further corrected by the percentages of the remaining mucosal areas without erosive lesions, which were quantified using the image analysis software. The erosive lesions are indicated by dashed lines in Fig. 4*A*. Corrected IR cells = % of number of ghrelin-IR cells × [(mucosal area without the erosive lesion)/(total area)]. Hematoxylin-eosin (HE) staining was also conducted to evaluate the severity of the injuries induced by I/R.

Preparation of total RNA and quantitative RT-PCR analysis. Total RNA was extracted from the stomach tissue using RNeasy Mini kit (Qiagen, Valencia, CA), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). Quantitative RT-PCR analysis was performed using Dice (Takara) with SYBR Premix Ex TaqII (Takara). The primer sequences used were as follows; preproghrelin mRNA: 5′-GGA ATC CAA GAA GCC ACC AGC′ and 5′-GCT CCT GAC AGC TTG ATG CCA-3′; GAPDH mRNA: 5′- GGC ACA GTC AAG GCT GAG AAT G -3′, 5′- ATG GTG GTG AAG ACG CCA GTA -3′. The mRNA expression levels were normalized using the GAPDH mRNA expression levels.

Statistical analysis. All values were expressed as means ± SD. The statistical significance of any differences between two groups was evaluated using unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$, unless otherwise indicated.

RESULTS

Food intake after gastric I/R. Cumulative food intakes were significantly reduced at 12 h after gastric I/R compared with that in the sham-operated rats in the fed condition (Fig. 2*A*). No significant difference was observed in the cumulative food intakes of shorter period, probably because 12 h was not sufficient for recovery from the surgical stress, and the food intake was very small even in the sham-operated rats. Cumulative food intakes (2, 4, 6, and 24 h) were also significantly reduced at 48 h after gastric I/R compared with those in the sham-operated rats in the fed condition (Fig. 2*B*). Decreased food intakes were also observed in the fasting condition after I/R (data not shown).

Gastric emptying of solids after gastric I/R. Gastric emptying rates were investigated using powdered food and glass beads at 48 h after I/R because decreased gastric emptying of liquids at 12 h after I/R was restored at 48 h although food intake was reduced in the I/R rats compared with that in the sham-operated rats. Figure 2*C* shows that the gastric emptying rates of solids in the I/R rats (50.1 ± 15.5%) were comparable with those in the sham-operated rats (57.0 ± 16.9%).

Plasma ghrelin levels. Plasma ghrelin and desacylghrelin levels were measured at 12 and 48 h after gastric I/R in the fasting (Fig. 3, *A* and *B*) and pair-fed (Fig. 3*C*) conditions to eliminate the effect of food intake. As shown in Fig. 3*A*, fasting

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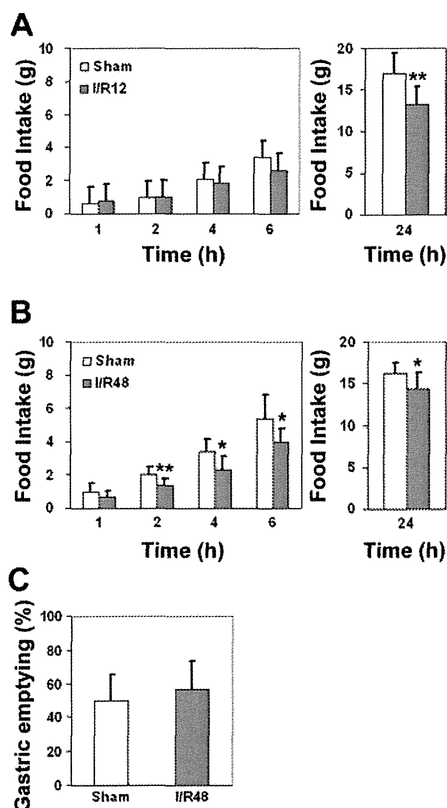


Fig. 2. *A*: effect of gastric I/R on the cumulative food intakes at 12 h after I/R in the fed condition. Sham-operated rats, open bar (n = 8); I/R rats, solid bar (n = 10). *B*: effect of gastric I/R on the cumulative food intakes at 48 h after I/R in the fed condition. Sham-operated rats, open bar (n = 9); I/R rats, solid bar (n = 10). *C*: gastric emptying rates of solids in the sham-operated rats (open bar) and I/R rats (solid bar) at 48 h (Sham, n = 6; I/R, n = 7) after I/R. Data are means ± SD. *P < 0.05, **P < 0.01 compared with the sham-operated rats by Student's t-test.

plasma ghrelin levels at 12 h and 48 h after I/R were significantly lower than those in the sham-operated rats at the corresponding time-points (sham 12 h, 64.5 ± 13.5 fmol/ml; I/R 12 h, 46.3 ± 9.25 fmol/ml; sham 48 h, 97.2 ± 30.3 fmol/ml; I/R 48 h, 70.9 ± 18.4 fmol/ml). Fasting plasma desacylghrelin levels at 12 h and 48 h after I/R were also significantly lower than those in the sham-operated rats at the corresponding time-points (sham 12 h, 834 ± 137 fmol/ml; I/R 12 h, 663 ± 113 fmol/ml; sham 48 h, 1,092 ± 150 fmol/ml; I/R 48 h, 835 ± 187 fmol/ml), as shown in Fig. 3*B*. Plasma ghrelin (sham, 115 ± 30.0 fmol/ml; I/R, 45.4 ± 23.7 fmol/ml) and desacylghrelin (sham, 1,311 ± 118 fmol/ml; I/R, 577 ± 201 fmol/ml) levels in the fed condition were also significantly decreased compared with those in the pair-fed sham-operated rats at 48 h after I/R (Fig. 3*C*).

Immunohistochemical staining for ghrelin-producing cells. Ghrelin-immunoreactive (IR) cells were counted in the mucosal layer of the fundic gland region (Fig. 4*A*). In case of counting in the mucosal layers of I/R group, the places without the mucosal injuries induced by gastric I/R were selected. The count of ghrelin-IR cells was decreased at 12 h after I/R (Sham, 0.92 ± 0.18%; I/R, 0.58 ± 0.11%, P = 0.0017) but recovered by 48 h (Sham, 0.86 ± 0.17%; I/R, 0.92 ± 0.20%) (Fig. 4*B*). However, because erosive lesion areas were observed at 12 h and 48 h after I/R (Fig. 4*A*, *right*), we corrected the numbers of ghrelin-IR cells by the percentages of the remaining mucosal areas not showing erosive lesions (Fig. 4*C*). The corrected numbers of ghrelin-IR cells were significantly decreased throughout the observation period (44.7 ± 11.1% at 12 h and 78.4 ± 18.6% at 48 h after I/R relative to the value in the sham-operated rats).

Ghrelin production after gastric I/R. The expression levels of preproghrelin mRNA were significantly reduced at 12 h and 48 h (53.4 ± 22.7% and 42.3 ± 16.8% relative to the value in the sham-operated rats) after I/R compared with the levels in the sham-operated rats at the corresponding time points (Fig. 5*A*). Mucosal injuries in the fundic gland regions, where ghrelin-IR cells are mainly distributed, persisted throughout the observation period, as visualized in the HE-stained sections (Fig. 5*B*).

Restoration of decreased food intake by exogenous ghrelin administration. In Fig. 6, ghrelin was administered intraperitoneally (30 nmol/rat) to sham-operated and I/R rats to investigate the effect of exogenous ghrelin on the decreased food intake at 48 h after I/R. In sham-operated rats, food intake was enhanced for 1 h, but not at 2- and 3-h cumulative food intake (Fig. 6*A*). However, administration of ghrelin significantly restored the decreased cumulative food intake (2 and 3 h) in I/R rats (Fig. 6*B*). The effect of decreased food intake restoration by exogenous ghrelin waned 4 h after administration. Administration of 10 nmol ghrelin per rat failed to increase food intake in both sham-operated and I/R rats (data not shown).

DISCUSSION

In the present study, we demonstrated that anorexia was induced after gastric I/R associated with decreased plasma ghrelin levels in rats. Not only the plasma ghrelin level but also ghrelin production was reduced by continuous mucosal injuries. Exogenous ghrelin administration significantly restored the food intake, indicating that it was the decrease in the levels of the orexigenic hormone that induced the anorexia after gastric I/R.

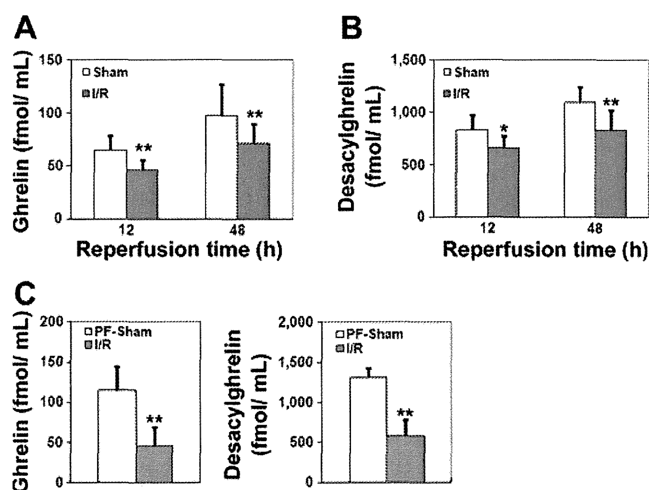


Fig. 3. Fasting plasma ghrelin (*A*) and desacylghrelin (*B*) levels in the sham-operated rats (open bar) and I/R rats (solid bar) at 12 h (Sham, n = 18; I/R, n = 16) and 48 h (Sham, n = 12; I/R, n = 13) after I/R. *C*: plasma ghrelin levels of I/R rats in the fed condition (solid bar, n = 9) and of sham-operated rats in the pair-fed condition (open bar, n = 6) at 48 h after I/R. Data are means ± SD. *P < 0.05, **P < 0.01 compared with the sham-operated rats by Student's t-test.

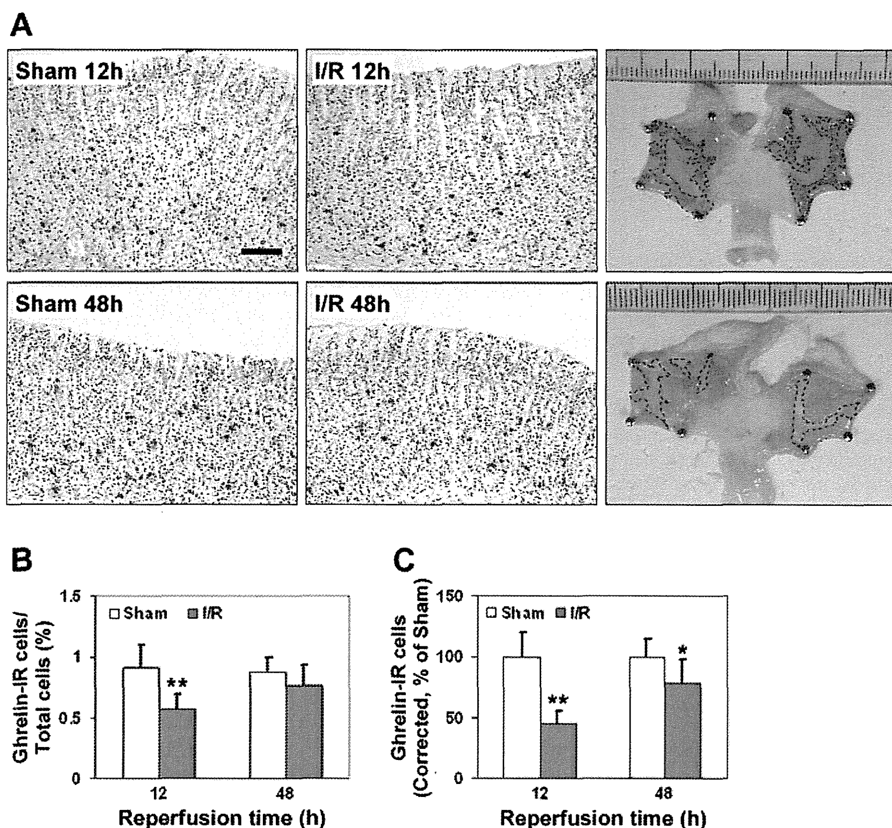


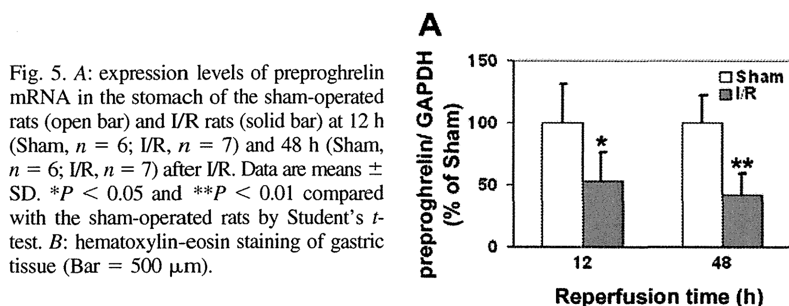
Fig. 4. *A*: representative photomicrograph of ghrelin-immunoreactive (IR) cells in the gastric fundic mucosa of the sham-operated rats and I/R rats at 12 h and 48 h after I/R. The brown-colored cells represent the ghrelin-IR cells. Bar = 100 μm. *Right* photographs of the gastric mucosa obtained from the I/R rats at 12 h and 48 h after I/R. The erosive lesion areas are shown by the dashed lines. *B*: numbers of ghrelin-IR cells in the images from the sham-operated rats (open bar) and I/R rats (solid bar) were counted and normalized by the total number of cells counterstained with hematoxylin, which was quantified using the image analysis software. *C*: numbers of ghrelin-IR cells in the images were corrected by the percentages of the remaining mucosal areas without the erosive lesions, which was quantified using the image analysis software. Sham, $n = 6$; I/R; $n = 7$. Data are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sham-operated rats by Student's *t*-test.

Thermal injuries (3) have been reported to induce decreased food intake, and aspirin treatment led to a further and significant decrease of food intake compared with that in the controls (16). A previous study reported that the restoration of gastric ghrelin production was associated with ulcer healing and improvement of the appetite in patients with *H. pylori*-associated active duodenal or gastric ulcer (15). Although these events are reported to induce gastric I/R (17, 26, 27, 30, 35), whether anorexia can be induced by gastric I/R alone remains unclear. The present study is the first report documenting decreased food intake associated with reduced production of ghrelin after gastric I/R.

We previously reported transient delay in gastric emptying of liquids at 12 h after I/R (28), which may be considered as inducing early satiety and contribute to the anorexia. However, the delayed gastric emptying of liquids was normalized at 48 h after I/R. The gastric emptying rates of solids at 48 h were also not significantly different between the sham-operated rats and I/R rats in the present study. The normalized gastric emptying rates do not explain the decrease of food intake at 48 h after I/R; therefore, other factors may also be associated with the anorexia.

Significant decrease in the plasma levels of ghrelin, an orexigenic hormone, in the fasting condition were observed at 12 h and 48 h after I/R in this study. Decreased plasma ghrelin levels are reported to induce anorexia, such as in the lipopolysaccharide-induced food intake and gastric emptying-altered model (31) and cisplatin-induced anorexia model (29). Therefore, we assumed that the decrease in the plasma ghrelin levels may have contributed to the persistent decrease of food intake after I/R in this study. This is also supported by our observation that intraperitoneal administration of exogenous ghrelin restored the food intake at 48 h after I/R.

Ghrelin has been reported to attenuate mucosal injuries induced by gastric I/R (11) and intestinal I/R (33). In the present study, single ghrelin administration, after the formation of mucosal injuries (mucosal injuries were already present after 1-h reperfusion, and exogenous ghrelin was administered at 48



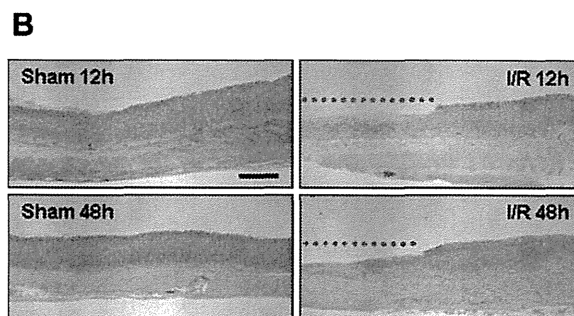


Fig. 5. *A*: expression levels of preproghrelin mRNA in the stomach of the sham-operated rats (open bar) and I/R rats (solid bar) at 12 h (Sham, $n = 6$; I/R, $n = 7$) and 48 h (Sham, $n = 6$; I/R, $n = 7$) after I/R. Data are means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the sham-operated rats by Student's *t*-test. *B*: hematoxylin-eosin staining of gastric tissue (Bar = 500 μm).

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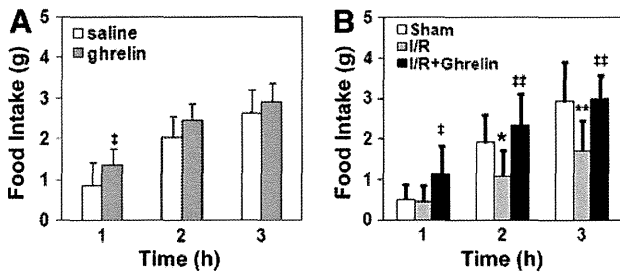

Fig. 6. *A*: effect of exogenous ghrelin (30 nmol/rat ip) in sham-operated rats in the fed condition. Saline-injected sham-operated rats, open bar (n = 10); ghrelin-injected sham-operated rats, shaded bar (n = 9). Data are means ± SD. ‡P < 0.05 compared with the saline group by Student's t-test. *B*: effect of exogenous ghrelin (30 nmol/rat ip) in I/R rats in the fed condition. Sham-operated rats, open bar (n = 9); I/R rats, shaded bar (n = 9); ghrelin-administered I/R rats, solid bar (n = 7). Data are means ± SD. *P < 0.05; **P < 0.01 compared with the sham-operated rats by Student's t-test. ‡‡P < 0.01 compared with the I/R rats by Student's t-test.

h after I/R in this study) restored the gastric I/R-induced decreased food intake; however, the restoration effect lasted only 3 h. Although ghrelin has the potential to attenuate mucosal injuries, it is unlikely that ghrelin can regenerate the gastric mucosa in 3 h. Also, if the restoration effect of exogenous ghrelin in this study is attributable to the attenuation of gastric mucosal injuries, the restoration effect should continue and should not wane after 3 h. Therefore, in this study, we considered that ghrelin administration restored the decreased food intake without attenuating mucosal injuries, indicating that decreased plasma ghrelin level, rather than mucosal injury itself, induces anorexia. However, because ghrelin is expressed in the gastric mucosa, gastric mucosal injury may induce decreased ghrelin production and subsequently induce anorexia. In a previous study, it was reported that lower concentrations of ethanol (but not absolute ethanol) induced increased plasma ghrelin levels despite the increase in the area of hemorrhagic erosions. This may represent the phenomenon of adaptive cytoprotection mediated by mild irritants, and 1 h after ethanol administration was not enough to decrease the ghrelin production in this model (5). Another report showed that the plasma total and active ghrelin levels were significantly higher in cysteamine-treated duodenal ulcer model rats probably attributable to the inhibition of somatostatin secretion, not to the formation of ulcers (12).

We cannot deny the possibility that gastric I/R-induced damages in central ghrelin production and peripherally administered ghrelin might have penetrated the blood-brain barrier and restored the decreased central ghrelin production. However, it is unlikely that a single administration of ghrelin abrogated central injury in 3 h. Ghrelin has an orexigenic effect by activating neuropeptide Y/AgRP (agouti-related protein) neurons through vagal afferent nerves. This signaling pathway is believed to be retained after gastric I/R because ghrelin administration increased food intake in I/R rats in this study although some damages are undeniable.

The ghrelin-IR cells were significantly decreased in number compared with that in the sham-operated rats at 12 h after gastric I/R in this study. According to previous studies, the number of gastric A-like cells is decreased by gastric mucosal injury induced by *H. pylori* infection (25, 29), probably attributable to the large amounts of reactive oxygen species produced during the process of colonization of the host by the bacteria (2, 9). Oxidative stress produced by the xanthine-xanthine oxidase system after gastric I/R may damage the A-like cells. The number of ghrelin-IR cells was restored in the remaining mucosa at 48 h, and we did not detect any cell death by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling in the mucosal layers, except in the areas adjacent to the erosive lesion area (data not shown). Therefore, it is unlikely that the A-like cells were destroyed after I/R and regenerated within 48 h. The expression of ghrelin in the A-like cells might be transiently decreased and the stores of ghrelin in each cell reduced, thereby making the number of ghrelin-immunoreactive cells appear to be decreased, although the precise mechanisms remain to be elucidated. The percentages of ghrelin-immunoreactive cells relative to the total number of cells were restored at 48 h after I/R. However, the numbers of ghrelin-IR cells corrected by the percentage areas not showing the erosive lesions were significantly decreased compared with those in the sham-operated rats at 48 h after I/R. This decrease might have induced the decreased ghrelin production and consequently, decreased plasma ghrelin levels.

The expressions of preproghrelin mRNA in the total stomach were significantly downregulated after I/R, which may explain the decreased plasma ghrelin levels throughout the observation period. We investigated the expression of preproghrelin mRNA in the total stomach, including the mucosal layer and muscle layer, which would reflect the total gastric production. Ghrelin is expressed only in the mucosal layer, thus mucosal injuries alone may decrease the mRNA expression of ghrelin. Erosive legion areas were observed predominantly in the fundic gland region, which is the region in which ghrelin-producing cells are predominantly identified. Thus mucosal injuries might induce decreased ghrelin production and, consequently, decreased plasma ghrelin levels, after gastric I/R.

Expressions of preproghrelin mRNA in the gastric mucosa were previously reported to be increased in response to mucosal injuries, such as those induced by gastric I/R (reperfusion 3 h) (20) and 1 h after ethanol exposure (19), and 3.5-h water-restraint stress (4). Not only preproghrelin mRNA, but also ghrelin protein expression was demonstrated to be increased at 1 h after the ethanol exposure. The expressions were investigated within a short time after the occurrence of the mucosal injuries. In this study, we examined the preproghrelin mRNA expression at 12 h and 48 h after I/R, which could have yielded different results. If the ghrelin protein expression continued to increase after I/R, the number of ghrelin-IR cells in the remaining mucosa would be unlikely to decrease, which was observed in this study. The density of the ghrelin-IR cells did not appear to differ between the sham-operated rats and I/R rats. Therefore, the expression of preproghrelin mRNA might have transiently increased at 3 h but decreased at 12 h after I/R. Also, the expression was determined only in the remaining mucosal layer in previous studies, different from the case in our present study, because we used total stomach to evaluate the total gastric ghrelin production.

In conclusion, gastric I/R caused anorexia associated with a significant decrease of the plasma ghrelin levels, which is attributed to the gastric mucosal injuries induced by I/R. The decrease in the plasma ghrelin levels may have been responsible for the decrease in the food intake after gastric I/R, as it

was restored by exogenous ghrelin administration. The results of this study show that ghrelin can stimulate food intake in rats with mucosal injuries induced by gastric I/R, suggesting that ghrelin or its analogs may also prove useful for attenuating I/R-induced dysfunctions.

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DISCLOSURES

S. Mogami is employed by Tsumura & Co. H. Suzuki had received grant support from Tsumura & Co. from 2007 to 2009. S. Fukuhara, J. Matsuzaki, K. Kangawa, and T. Hibi have no conflicts of interest to declare.

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Original Article

Proton pump inhibitor-amoxicillin-clarithromycin versus proton pump inhibitor-amoxicillin-metronidazole as first-line *Helicobacter pylori* eradication therapy

Toshihiro Nishizawa,[1,2] Hidekazu Suzuki,[2,*] Masayuki Suzuki,[1] Masahiko Takahashi[1] and Toshifumi Hibi[2]

[1]Division of Gastroenterology, National Hospital Organization Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan
[2]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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The aim of this study was to compare the efficacy and tolerability of the first-line *Helicobacter pylori* (*H. pylori*) eradication regimen composed of proton pump inhibitor, clarithromycin, and amoxicillin, with those of a regimen composed of proton pump inhibitor, metronidazole, and amoxicillin. Data of patients, who were administered the first-line *H. pylori* eradication regimen at Tokyo Medical Center between 2008 and 2011, were reviewed. All patients had *H. pylori* gastritis without peptic ulcer disease. The 7-day triple regimen composed of lansoprazole, clarithromycin, and amoxicillin was administered to 55 patients, and that composed of omeprazole, metronidazole, and amoxicillin was administered to 55 patients. Intention-to-treat and per-protocol eradication rates were 74.5 and 80.4%, respectively, for the regimen of lansoprazole, clarithromycin, and amoxicillin, whereas the corresponding rates were 96.4 and 100%, respectively, for the regimen of omeprazole, metronidazole, and amoxicillin. In conclusion, first-line *H. pylori* eradication therapy composed of omeprazole, metronidazole, and amoxicillin was significantly more effective than that composed of lansoprazole, clarithromycin, and amoxicillin, without differences in tolerability.

Key Words: ***Helicobacter pylori***, **eradication, antibiotics**

Eradication of *Helicobacter pylori* (*H. pylori*) infection has been reported as an effective strategy in the treatment of peptic ulcers and gastric mucosa-associated lymphoid tissue lymphoma, in addition to the prevention of recurrence of gastric cancer after endoscopic resection.[1–6] The first-line regimen for the treatment of *H. pylori* infection in Japan is triple therapy with a proton pump inhibitor (PPI), amoxicillin (AMX), and clarithromycin (CLR), administered for 7 days. Failure of this first-line therapy against *H. pylori* infection has been reported in approximately 20% of infected patients.[7,8] At the 2008 meeting of the Japanese Society for Helicobacter Research, the mean national CLR resistance rates from 2002 to 2006 were reported to be 18.9, 21.2, 27.7, 29.0, and 27.2%. The mean nationwide CLR resistance rate determined by the Japanese Society of Chemotherapy was 7.0% (21/302) in 2000, indicating an increase of resistance by approximately 20% over several years.[9] Furthermore, it appears that the prevalence of CLR-resistant *H. pylori* is increasing rapidly, and therefore, a resultant decrease in eradication achieved by the therapy, currently available under the national health insurance scheme, is a concern.

Failure of therapy with PPI-AMX-metronidazole (MNZ), administered for 1 week as a second-line regimen after failure of the first-line regimen, has been reported in approximately 10% of infected patients. Although the prevalence of *H. pylori* resistant to MNZ has been reported to be 8–80% in different countries, that in Japan has been reported to be 5–12%.[10,11]

These findings suggest that the first-line therapy with PPI-AMX-MNZ may be recommended in Japan. The aim of our retrospective study was to compare the efficacy and tolerability of the 7-day first-line *H. pylori* eradication regimen composed of PPI, CLR, and AMX, with those of a regimen composed of PPI, MNZ, and AMX.

Patients and Methods

Data of patients, who were administered first-line *H. pylori* eradication therapy at the Tokyo Medical Center between April 2008 and November 2011, were reviewed. Patients who had received a previous eradication therapy and had known peptic ulcer diseases and had used nonsteroidal anti-inflammatory drug, aspirin or clopidogrel were excluded from the study. Endoscopic examinations were conducted before treatment for all the patients, and *H. pylori* positivity was confirmed by the results of the ^{13}C-urea breath test or the presence of *H. pylori*-specific IgG antibodies in the serum.

The 7-day triple regimen composed of lansoprazole (30 mg, b.d.), CLR (400 mg, b.d.), and AMX (750 mg, b.d.) was administered to 55 patients, and that composed of omeprazole (20 mg, b.d.), MNZ (250 mg, b.d.), and AMX (750 mg, b.d.) was administered to 55 patients. The choice of regimen for *H. pylori*-associated gastritis was random. If the day of the week of patient's first visit was Tuesday, Wednesday, or Thursday, the regimen composed of lansoprazole, CLR, and AMX was chosen. If the day of the week of patient's first visit was Monday or Friday, the regimen composed of omeprazole, MNZ, and AMX was chosen. Eradication was confirmed by the results of the ^{13}C-urea breath test at 12 weeks after completion of the therapy. The ^{13}C-urea used was 100 mg ^{13}C-labelled urea produced by Otsuka Pharmaceutical Co. Ltd., Japan. The procedure was modified from the European standard protocol for detection of *H. pylori*. The study was approved by the Ethics Committee of National Hospital Organization Tokyo Medical Center, and informed consent was given by all the patients prior to the treatments.

Statistical analysis. Statistical analyses were performed

*To whom correspondence should be addressed.
E-mail: hsuzuki@a6.keio.jp

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Table 1. Comparison of first-line *H. pylori* eradication regimen composed of lansoprazole, amoxicillin, and clarithromycin (LAC) with that composed of omeprazole, amoxicillin, and metronidazole (OAM)

Characteristics	LAC (n = 55)	OAM (n = 55)	p value
Age (mean ± SD)	57.9 ± 14.9	60.1 ± 12.2	0.396
Sex (male/female)	28/27	28/27	0.849
Adverse effects	13.7% (7/51)	11.3% (6/53)	0.711
Eradication rate (ITT)	74.5% (41/55)	96.4% (53/55)	0.001
Eradication rate (PP)	80.4% (41/51)	100% (53/53)	0.001

using the chi-square, Fisher's exact, and Student's t tests, as appropriate. p values of less than 0.05 were considered to indicate statistical significance.

Results

One hundred ten patients were enrolled; of these 6 patients dropped out of the study. Table 1 shows the demographic data for these patients. The baseline characteristics were not statistically different between the 2 groups. The combination of lansoprazole, CLR, and AMX resulted in eradication rates of 74.5% (intention-to-treat; ITT) and 80.4% (per protocol; PP). The combination of omeprazole, MNZ, and AMX resulted in eradication rates of 96.4% (ITT) and 100% (PP). The regimen composed of omeprazole, MNZ, and AMX was significantly more effective than that composed of lansoprazole, CLR, and AMX ($p<0.05$ for ITT, $p<0.01$ for PP, Table 1).

The compliance of the patients with the prescribed treatment was excellent. Adverse events were observed in 7 of 51 patients (13.7%) in the PPI + CLR + AMX group and 6 of 53 patients (11.3%) in the PPI + MNZ + AMX group. In all the cases, the side effects were mild and mainly included mild diarrhea, taste disturbance, or stomatitis.

Discussion

One week of triple therapy using a PPI combined with AMX and CLR is recommended as the first-line treatment choice for the eradication of *H. pylori* in Japan. The resistance to CLR is easily acquired and widespread prescription of CLR over the years lead to the spread of resistance of *H. pylori* to CLR. Sasaki *et al.*[12] reported the eradication rates of first-line eradication therapy with CLR from 1995 to 2008 (divided into four terms; 1997–2000, 2001–2003, 2004–2006, 2007–2008), and the eradication rate decreased significantly from 90.6 to 80.2%, 76.0, and 74.8%. The study demonstrated the evident decline in eradication rates for the triple therapy using a PPI, AMX, and CLR in Japan.

The present study showed an excellent eradication rate of 96.4% (ITT) and 100% (PP) with a 7-day regimen of MNZ, AMX, and PPI as the first-line treatment. This regimen composed of PPI, MNZ, and AMX was significantly more effective than that composed of PPI, CLR, and AMX, without differences in tolerability. The first-line regimen with CLR should be changed into another regimen such as the regimen with MNZ in order to improve the eradication rate, because recent prevalence of CLR resistance is estimated as more than 30% in Japan.

We previously reported the resistant rates of *H. pylori* to AMX after unsuccessful eradication. The resistance rates to AMX (MIC≥0.06 μg/ml) in groups with no history of eradication treatment, a history of 1 treatment failure, and a history of 2 treatment failures were 13.6, 26.5, and 49.5%, respectively. The MIC_{90} of AMX increased by 2-fold after every eradication failure, and accumulation of *PBP1* mutations were associated with a gradual increase in the resistance to AMX.[13] These results suggest that the first-line therapy with less chances of failure should be recommended to prevent further spread of AMX-resistant *H. pylori* strains.

Although lansoprazole does not have a significant advantage over omeprazole in *H. pylori* eradication,[14] drug costs of lansoprazole are more expensive than those of omeprazole. Niv also reported that there was no statistically significant difference between omeprazole and lansoprazole as part of a PPI-based triple therapy for eradication of *H. pylori*.[15] The costs of CLR for 7 days are five times more expensive than those of MNZ for 7 days. Drug costs of omeprazole, MNZ, and AMX for 7 days are $ 46, and those of lansoprazole, CLR, and AMX for 7 days are $ 76. The 7-day regimen of omeprazole, MNZ, and AMX would save $ 30 per a patient treated. The present study demonstrated that the high eradication rate could be achieved despite of its low cost.

In conclusion, the triple therapy with PPI, MNZ, and AMX appeared to serve as an encouraging first-line strategy in Japan. Given the recent surge of CLR resistance compared to MNX resistance, new strategies for first-line regimens against *H. pylori* infection should be considered.

Conflict of Interest

No potential conflicts of interest were disclosed.

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Efficacy of Sitafloxacin-Based Rescue Therapy for *Helicobacter pylori* after Failures of First- and Second-Line Therapies

Juntaro Matsuzaki,[a] Hidekazu Suzuki,[a] Toshihiro Nishizawa,[a,b] Kenro Hirata,[a] Hitoshi Tsugawa,[a] Yoshimasa Saito,[c] Sawako Okada,[a] Seiichiro Fukuhara,[a] and Toshifumi Hibi[a]

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan[a]; Division of Gastroenterology, National Hospital Organization Tokyo Medical Center, Tokyo, Japan[b]; and Division of Pharmacotherapeutics, Keio University Faculty of Pharmacy, Tokyo, Japan[c]

Sitafloxacin-based triple therapy achieved 83.6% (per-protocol) and 78.2% (intention-to-treat) success in eradicating *Helicobacter pylori* among 78 Japanese patients after clarithromycin-based first-line and metronidazole-based second-line triple therapies failed. Eradication succeeded in 32 out of 43 patients, even with *gyrA* mutation-positive *Helicobacter pylori* (per protocol). The position of the *gyrA* mutation (N87 or D91) was determined to be a better marker than MIC levels for predicting outcomes of sitafloxacin-based treatment.

The emergence of *Helicobacter pylori* strains resistant to both clarithromycin and metronidazole has generated an urgent need for other treatment options for third-line rescue therapy. Possible candidates for such rescue eradication regimens include fluoroquinolones. Quinolone resistance in *H. pylori* is caused by point mutations (N87 and D91) in the quinolone resistance-determining region of the *gyrA* gene of *H. pylori*. The presence of a *gyrA* mutation is predictive of treatment failure with triple therapy, including commonly used quinolones, such as levofloxacin (3, 6). Eradication rates of levofloxacin-based therapies against levofloxacin-resistant strains (MIC, ≥ 1 μg/ml) or *gyrA* mutation-positive strains hover around 33.3% to 41.7% (3, 7). However, a high incidence of *gyrA* mutation was found especially in patients with previous eradication failures (5, 6, 9). Recently, we have shown that a newly developed quinolone, sitafloxacin (STFX), can overcome the resistance of *H. pylori* strains carrying *gyrA* mutations *in vitro* (8). The present study was designed to investigate the efficacy and safety of STFX-based third-line *H. pylori* eradication therapy, especially in *gyrA* mutation-positive strains.

The present study was a prospective trial conducted in Keio University Hospital from April 2009 to October 2011. Eighty-seven patients in whom eradication treatment with clarithromycin-based first-line therapy (triple therapy with clarithromycin [800 mg/day], amoxicillin [1,500 mg/day], and proton pump inhibitors [PPIs] for 7 days) and metronidazole-based second line therapy (triple therapy with metronidazole [500 mg/day], amoxicillin [1,500 mg/day], and PPIs for 7 days) failed were enrolled after obtaining informed consent (UMIN000001558). Before treatment, *H. pylori* isolates were obtained from gastric biopsy specimens. The MICs of STFX against *H. pylori* isolates and the *gyrA* mutation status were determined by the method described previously (5, 6). Seventy-eight patients (37 men and 41 women; mean age, 50.7 ± 13.4 years) were administered STFX-based therapy combined with rabeprazole (10 mg, four times a day [q.i.d.]), amoxicillin (500 mg, q.i.d.), and STFX (100 mg, two times a day [b.i.d.]) for 7 days (intention-to-treat [ITT] population). Three patients with penicillin allergy, 1 patient with loss of follow-up, and 5 patients in whom *H. pylori* could not be detected by culture were excluded from the study. For 73 patients, eradication results were confirmed (per-protocol [PP] population), whereas 5 patients were lost to follow-up. Among 73 patients, 38 had dyspepsia, 22 had peptic ulcer, 2 had early gastric cancer, 1 had mucosa-associated lymphoid tissue (MALT) lymphoma, 1 had idiopathic thrombocytopenic purpura, and 11 received PPIs (rabeprazole, $n = 5$; lansoprazole, $n = 4$; omeprazole, $n = 2$). Successful eradication was confirmed using a [^{13}C]urea breath test (^{13}C-UBT) 12 weeks after the end of therapy. The cutoff value for negative ^{13}C-UBT was less than 2.5%. At least 1 month before performing the ^{13}C-UBT, PPIs and antibiotics were not given. For two patients who showed a borderline value (2.5% to 5.0%) of ^{13}C-UBT, an *H. pylori* stool antigen test was also performed. No severe side effects to this treatment were reported. Mild and transient adverse effects, such as diarrhea (33.3%), soft stool (25.3%), abdominal pain (6.9%), epigastric fullness (6.9%), and dysgeusia (6.9%), were reported. Characteristics of the 73 patients are shown in Table 1.

The eradication rates determined by PP and ITT analyses were 83.6% (61/73 patients) and 78.2% (61/78 patients), respectively. Among 31 patients with *gyrA* mutation-negative *H. pylori*, a PP eradication rate of 96.7% (29/30) and an ITT eradication rate of 93.5% (29/31) were achieved. Moreover, even among 47 patients with *gyrA* mutation-positive *H. pylori*, the PP and ITT eradication rates were 74.4% (32/43) and 68.1% (32/47), respectively. The average MIC of STFX was higher in patients with eradication failure than in patients with eradication success (Table 1). Interestingly, the MICs of STFX in *gyrA* mutation-positive strains differed, depending on the position of the *gyrA* mutation (Fig. 1A). The MICs of STFX were higher in N87-mutated strains (0.21 ± 0.16 μg/ml) than in D91-mutated strains (0.12 ± 0.11 μg/ml) ($P = 0.03$). In fact, eradication rates were lower in patients with N87-mutated strains (61.9% for PP) than in patients with D91-mutated strains (86.4% for PP) ($P = 0.09$). Receiver-operating characteristic (ROC) curves based on the positions of *gyrA* muta-

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Address correspondence to H. Suzuki, hsuzuki@a6.keio.jp.
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