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 Hibi1

1Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; 2Tsumura Research Laboratories, Tsumura & Co., Ibaraki, Japan; 3National 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. Various investigations have demonstrated that ischemia and reperfusion (I/R) contribute significantly to postischemic mucosal injury, but also transient delay in gastric emptying in a rat model of gastric I/R (28). These changes appear 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.

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, 10–136). 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. 1A). 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. 1B). 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 Shinnanomachi, Shinjuku-ku, Tokyo 160-8582, JAPAN (e-mail: hszuiki@a6.keio.jp).
was evaluated using powdered food (13) and glass beads (24, 34).

A food (Fig. 1) intraperitoneally, immediately before the supply of the preweighed Peptide Institute, Osaka, Japan) or 0.5 ml saline was administered before and after the feeding period. In the experiment to determine the hanging-wire cages. After reperfusion for 12 h or 48 h, the rats were

B: measurement of gastric emptying rates or plasma ghrelin levels at 12 h or 48 h after I/R in fed 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. 1C).

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 intraarterially, immediately before the supply of the preweighed food (Fig. 1A).

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. 1B), 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. 1B) or after 48-h pair feeding (Fig. 1C), 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 at 20 min at 97°C in Dako REAL Target Retrieval Solution (DAKO Japan, Tokyo, Japan). Non-specific 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. 4A. 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 ATGCCA-3′; GAPDH mRNA: 5′-GCC ACA GTC AAG GCT GAG AAT G-3′, 5′-ATG GTG GTG AAG AGC 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. 2A). 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. 2B). 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 2C 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. 3C) conditions to eliminate the effect of food intake. As shown in Fig. 3A, fasting
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. 3B. 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. 3C).

Immunohistochemical staining for ghrelin-producing cells. Ghrelin-immunoreactive (IR) cells were counted in the mucosal layer of the fundic gland region (Fig. 4A). 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. 4B). However, because erosive lesion areas were observed at 12 h and 48 h after I/R (Fig. 4A, right), we corrected the numbers of ghrelin-IR cells by the percentages of the remaining mucosal areas not showing erosive lesions (Fig. 4C). 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. 5A). 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. 5B).

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. 6A). However, administration of ghrelin significantly restored the decreased cumulative food intake (2 and 3 h) in I/R rats (Fig. 6B). 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. 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.

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.
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 h after I/R)
The ghrelin-IR cells were significantly decreased in number attributed 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 lesion 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

![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 sham-operated rats 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.]
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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