Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion

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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 gastrotrophic 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...
After 24-h food deprivation (Fig. 1A) was evaluated using powdered food (13) and glass beads (24, 34). Food (Fig. 1A) was intraperitoneally, immediately before the supply of the preweighed powdered food and glass beads (0.2-mm diameter, BZ-02; AS One, Osaka, Japan) or 0.5 ml saline was administered to the rats to eliminate the effect of the difference in the food intake. As shown in Fig. 3C, 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 immediately after the injection to recover the entire stomach, dried, and weighed. The gastric emptying of solid food 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 desacylgghrelin (ghrelin without octanoyl acid modification) level was determined using the Desacyl Ghrelin ELISA Kit (Mitsubishi Chemical Medience, Tokyo, Japan). The mRNA expression levels of preproghrelin mRNA: 5'-GGG ATG ACC ACC TAC ACC GAC CC-3' and 5'-CCC ACT CCT GGA TGG ATG CCA AAT GC-3' were used to assess 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'-GGG ATC CAA GAA GCC ACC AGC' and 5'-GCC CCT AGC AGG TTG ATC TTG CCA AAT GC-3'; GAPDH mRNA: 5'-GCG ACA GTC AAG GCT GAG AAT G -3', 5'-ATG GTG GTG AAG ACC CGA 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%).

**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. 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 immediately after the injection to recover the entire 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 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 stored at –80°C until use. The ghrelin level was determined using the Active Ghrelin ELISA Kit, and the desacylgghrelin (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. 4A. Corrected IR cells = % of number of ghrelin-IR cells × [-(mucosal area without the erosion lesion)/(total area)]. Hematoxylin-eosin (HE) staining was also conducted to evaluate the severity of the injuries induced by I/R.

**GTGT GTG AAG ACG CCA GTA -3** GTGT GTG AAG ACG CCA GTA -3.

**=**

**GAPDH**

**Garstic 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.
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 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 Helicobacter 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 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
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.

**REFERENCES**