Enhanced ghrelin secretion in rats with cysteamine-induced duodenal ulcers

Seiichiro Fukuhara,1,2 Hidekazu Suzuki,1,2 Tatsushi Masaoka,1,2 Mamoru Arakawa,1,2 Hiroshi Hosoda,3 Yuriko Minegishi,2 Kenji Kangawa,3 Hiromasa Ishii,1 Masaki Kitajima,4 and Toshifumi Hibi1

Departments of 1Internal Medicine and 4Surgery, and 2Center for Integrated Medical Research, Keio University School of Medicine, Tokyo; and 3Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan

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Fukuhara, Seiichiro, Hidekazu Suzuki, Tatsushi Masaoka, Mamoru Arakawa, Hiroshi Hosoda, Yuriko Minegishi, Kenji Kangawa, Hiromasa Ishii, Masaki Kitajima, and Toshifumi Hibi. Enhanced ghrelin secretion in rats with cysteamine-induced duodenal ulcers. Am J Physiol Gastrointest Liver Physiol 289: G138–G145, 2005. First published March 18, 2005; doi:10.1152/ajpgi.00298.2004.—Ghrelin possesses an octanoyl modification at the third serine residue; this modification has been shown to be necessary for the protein’s physiological activity (13). Ghrelin is produced and secreted by a subset of endocrine cells, known as A-like cells, that are found within the oxyntic glands of the stomach (4). Rindi et al. (21) investigated the characteristics of the gastric ghrelin-secreting cells in the rat by electron microscopy and suggested that ghrelin secretion is probably accomplished by exocytosis, because small electron-dense granules were found to be localized near the basal cytoplasmic membrane. The physiological roles of ghrelin include the stimulation of GH release, gastric motility, and food intake (18).

Cysteamine (mercaptoethylamine, HS-CH₂-CH₂-NH₂) has been reported to induce duodenal ulcers in rats (23). The mechanisms underlying the development of cysteamine-induced duodenal ulcers include a reduction in duodenal mucosal blood flow (8) and a decrease in Brunner gland secretion (11), both of which depress local defense mechanisms. Recent reports also demonstrate the alteration of redox state and reduced mucosal oxygenation in the early peptic ulcerogenic duodenal mucosa after cysteamine treatment (10). Other mechanisms include an increase in gastric acid secretion as a result of increased serum gastrin levels (9, 12, 14). Because treatment with somatostatin prevents cysteamine-induced duodenal ulcers (22) and cysteamine depletes tissue somatostatin levels (30), the main factor underlying the development of cysteamine-induced duodenal ulcers may be the depletion of somatostatin.

In the present study, we investigated the plasma and gastric levels of ghrelin, the number of ghrelin-immunoreactive cells in the stomach, and the relationship among ghrelin dynamics, inflammation, and the severity of ulcer lesions in rats treated with cysteamine.

MATERIALS AND METHODS

Animal procedures. The present study was approved by the Keio University Animal Research Committee (No. 023009). Seven-week-old male Wistar rats were used for the study. Rats (n = 15) were orally administered either cysteamine (400 mg/kg) in 1 ml of saline or saline alone three times at 4-h intervals and then euthanized 10 or 50 h after the first dose of cysteamine or saline. Furthermore, to evaluate the effect of proton-pump-inhibitor against cysteamine administration, rats (n = 21) were treated with lansoprazole (LPZ). Namely, LPZ was dissolved in 0.5% carboxymethylcellulose (CMC) and 1% NaHCO₃, and the rats were given subcutaneous injections of LPZ (30 mg/kg) or CMC alone at 24 h, 0.5 h before, and 24 h after the first dose of cysteamine. The body weight of the rats was measured just before the first dose of cysteamine and just before the euthanasia. The pH of the gastric mucosal surface was measured by a pH meter with a flat probe (model D-51; Horiba, Kyoto, Japan).

In a separate experiment, rats (n = 25) were administered cysteamine (400 mg/kg) in 1 ml of saline or saline alone orally and then euthanized 0.5 or 2 h after the cysteamine administration.

Evaluation of duodenal ulcers. Ulcer lesions were evaluated macroscopically using a dissecting microscope and photographed using a digital camera (model COOLPIX990; Nikon, Tokyo, Japan). The ulcer area size was measured using an area analysis program (Ultimage Pro. 2.6.4; Alliance Vision, Montélimar, France).

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Samples of the gastric and duodenal wall were fixed in 10%-neutralized formalin and prepared for histological examination using routine methods. The tissue sections (4–6 μm thick) were stained with hematoxylin-eosin (H-E), and the depth of the duodenal ulcers and the presence of neutrophil infiltration were evaluated histologically. Ulcer depth was scored according to the Murakami classification using a scale of 0–4 (0 = no ulcer, 1 = superficial mucosal lesion, 2 = penetration of the muscularis mucosae, 3 = penetration to the level of the muscularis externa, and 4 = penetration of the muscularis externa). Neutrophil accumulation was histologically evaluated by determining the ratio of the number of neutrophils observed to the size of the area examined.

**Measurement of somatostatin- and ghrelin-immunoreactive cell density in the stomach.** Tissue sections were deparaffinized and hydrated. Endogenous peroxidase was quenched by treatment with 0.3% hydrogen peroxide for 20 min. Nonspecific binding was blocked using a blocking reagent (BlockAce; Dainippon Pharmaceuticals, Osaka, Japan). For double staining, the samples were first stained for somatostatin. Sections were incubated for 1.5 h at room temperature with antisomatostatin antiserum (Nichirei, Tokyo, Japan). After being washed with TBS-T, the slides were incubated with EnVision+ Peroxidase rabbit (DAKO Japan, Kyoto, Japan) for 30 min at room temperature, then visualized after color development in 3,3'-diaminobenzidine tetrahydrochloride solution for 3 min. The samples were then stained for ghrelin. All slices were incubated overnight at 4°C with antighrelin-(13–28) antiserum (final dilution, 1:10,000) (3). After being washed with TBS-T, the slides were incubated with EnVision+ alkaline phosphatase (ALP; DAKO) for 30 min at room temperature and then visualized after color development in ALP solution for 3 min. The sections were then counterstained with hematoxylin. Stained sections were evaluated under a light microscope equipped with a 3CCD digital camera (model C7780; Hamamatsu Photonics, Hamamatsu, Japan). All nuclei were counted using a particle analysis equation $D:ghrelin = (Ng/Nt) \times 100\%$, where $Ng$ and $Nt$ represent

<table>
<thead>
<tr>
<th>Body Weight, g</th>
<th>MPO Activity in the Stomach, mU/mg protein</th>
<th>Neutrophil Accumulation, per mm²</th>
<th>Somatostatin-immunoreactive cells/total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>50 h after the first cysteamine treatment</td>
<td>Stomach</td>
<td>Duodenum</td>
</tr>
<tr>
<td>Control rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>224.6±2.5</td>
<td>219.0±4.7</td>
<td>0.46±0.19</td>
<td>59.6±8.7</td>
</tr>
<tr>
<td>Cysteamine-treated rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>226.0±2.1</td>
<td>190.5±2.0*</td>
<td>0.95±0.10†</td>
<td>179.5±16.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE. The body weight of the animals was measured before the first injection of cysteamine and before euthanasia. The mean body weight of the cysteamine-treated rats was significantly lower than that of the control rats. MPO activity in the stomach of the cysteamine-treated rats was significantly higher than that in the control rats. Neutrophil accumulation in the stomach and duodenum of the cysteamine-treated rats was significantly higher than that of the control rats. The plasma somatostatin level in the cysteamine-treated rats was significantly lower than that in the control rats. The density of somatostatin-immunoreactive cells in the gastric corpus and antrum (%) of the cysteamine-treated rats was also significantly lower than that of the control rats. *$P < 0.001$, compared with control; †$P < 0.05$, compared with control.

Fig. 1. The macroscopic and microscopic status of the stomach and duodenum. A: whole status of the entire stomach and duodenum in control rats. B: hematoxylin-eosin (H-E) staining of the duodenum in control rats. The duodenal ulcer was not observed. C: whole status of the entire stomach and duodenum in cysteamine-treated rats. Cysteamine-induced duodenal ulcers were observed near the gastric antrum. D: H-E staining of the duodenum in cysteamine-treated rats. The duodenal ulcer penetrated the muscularis propria. *, Oral side.
the number of ghrelin-immunoreactive cells and the total cell number, respectively, in the region of interest (28). The density of somatostatin-immunoreactive cells was also computed using a similar method.

**Measurement of plasma somatostatin level.** The plasma somatostatin levels of samples were measured using an RIA according to a previous method (1).

**Measurement of plasma and gastric ghrelin levels.** Two polyclonal rabbit antibodies were raised against the NH2-terminal (1–11) (Gly1-Lys11) and COOH-terminal (13–28) (Gln13-Arg28) fragments of rat ghrelin (6). (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 (Pierce, Rockford, IL) (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 from immunization of New Zealand white rabbits by subcutaneous injection. Using these antibodies, two kinds of RIAs to measure the gastric and plasma ghrelin levels were performed as described previously (2, 27, 28).

**Measurement of MPO activity.** Tissue samples of gastric mucosa were collected in tubes containing PBS and protease inhibitors (100 μM PMSF, 10 μg/ml aprotinin) and sonicated over ice in 30 consecutive 0.5-s bursts at 0.5-s intervals at a power setting of 150 W (VCX 750; Sonics & Materials, Newton, CT). The total protein level in the homogenates was measured using a modified Lowry method (15), as described by Smith et al. (25).

MPO activity, an index of polymorphonuclear cell accumulation, was determined using a previously described method (5, 29).

**Measurement of preproghrelin mRNA expression in the stomach.** Total mRNA was extracted from the stomach tissue using a RNeasy Mini Kit (Qiagen). A TaqMan quantitative real-time RT-PCR was then performed to detect preproghrelin mRNA and GAPDH mRNA using an ABI PRISM 7700 sequence detection system (PE Applied Biosystems) (16, 28).

The following primers were used to amplify the preproghrelin mRNA: ghrelin-F (5’-GGAGAACGAACCAGC-3’), ghrelin-R (5’-GCTCGACGTCCTGGAGAGC-3’), and ghrelin-Taq (5’-FAM-ACACGCTCTGGAGAGC-GC-TAMRA-3’). To amplify GAPDH mRNA as an internal control, the following primers were used: GAPDH-F (5’-TTCACGGACAACTGGAG-3’), GAPDH-R (5’-GCCCTCTCCATGTGTTG-3’), and GAPDH-Taq (5’-FAM-CCACCACCTGTTCCAGGAGGA-GA-TAMRA-3’).

The preproghrelin mRNA expression levels were normalized using the GAPDH mRNA expression levels.

**Fig. 2.** The dynamics of ghrelin 50 h after the first dose of cysteamine. The plasma ghrelin levels (A: total ghrelin; B: active ghrelin) in the cysteamine-treated rats (filled bars: n = 9) were significantly increased compared with those in the control rats (open bars: n = 6). The gastric ghrelin levels (C: total ghrelin; D: active ghrelin) in the cysteamine-treated rats were significantly decreased compared with those in the control rats. E: preproghrelin mRNA expression in the cysteamine-treated rats was significantly decreased compared with that in the control. F: density of the ghrelin-immunoreactive cells in the gastric corpus of the cysteamine-treated rats was significantly decreased compared with that in the control rats. ***P < 0.001, **P < 0.01, * P < 0.05, compared with the control.

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The plasma total and active ghrelin levels significantly correlated with plasma somatostatin level.

The gastric total and active ghrelin levels significantly correlated with MPO activity.

The gastric total and active ghrelin levels significantly correlated with the ulcer depth.

The gastric total and active ghrelin levels significantly correlated with the ulcer area.

Fig. 3. A and B: relationship between plasma ghrelin levels (A: total ghrelin; B: active ghrelin) and plasma somatostatin. The plasma total and active ghrelin levels significantly correlated with plasma somatostatin level. C and D: relationship between gastric ghrelin levels (C: total ghrelin; D: active ghrelin) and MPO activity. The gastric total and active ghrelin levels significantly correlated with MPO activity. E and F: relationship between gastric ghrelin levels (E: total ghrelin; F: active ghrelin) and duodenal ulcer depth. The gastric total and active ghrelin levels significantly correlated with the ulcer depth. G and H: relationship between gastric ghrelin levels (G: total ghrelin; H: active ghrelin) and duodenal ulcer area. The gastric total and active ghrelin levels significantly correlated with the ulcer area.
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Table 2. Body weight, MPO activity in the stomach, neutrophil accumulation in the stomach and duodenum, dynamics of somatostatin 10 h after the first cysteamine treatment

<table>
<thead>
<tr>
<th></th>
<th>Body Weight, g</th>
<th>MPO Activity in the Stomach, mU/mg protein</th>
<th>Neutrophil Accumulation, per mm²</th>
<th>Plasma Somatostatin, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>10 h after the first cysteamine treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control rats</td>
<td>251.6±3.3</td>
<td>240.0±3.1</td>
<td>0.73±0.10</td>
<td>72.3±10.2</td>
</tr>
<tr>
<td>Cysteamine-treated rats</td>
<td>252.6±1.8</td>
<td>242.8±1.5</td>
<td>0.96±0.05†</td>
<td>120.3±7.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. The body weight of the animals was determined before the first injection of cysteamine and before euthanasia. The mean body weight of the cysteamine-treated rats was significantly lower than that of the control rats. MPO activity in the stomach of the cysteamine-treated rats was significantly higher than that in the control rats. Neutrophil accumulation in the stomach and duodenum of the cysteamine-treated rats was significantly higher than that of the control rats. The plasma somatostatin level in the cysteamine-treated rats was significantly lower than that in the control rats. *P < 0.001, compared with control; †P < 0.05, compared with control.

Statistical analysis. All data were expressed as the means ± SE. The data were analyzed using a one-way ANOVA, followed by Scheffé’s multiple comparison tests. A value of P < 0.05 was considered statistically significant.

RESULTS

Experiment 1 (50 h after the first dose of cysteamine). Seven of nine cysteamine-treated rats developed ulcers in the proximal duodenum (Fig. 1C). Some of the duodenal ulcers penetrated the muscular layer (Fig. 1D). The mean body weight of the cysteamine-treated rats was significantly lower (P < 0.001) than that of the control rats (190.5 ± 2.0 and 219.0 ± 4.7 g, respectively; Table 1). The MPO activity in the stomachs of the cysteamine-treated rats was significantly higher (P < 0.05) than that of the control rats (Table 1). Neutrophil accumulation in the stomach and duodenum was prominent in cysteamine-treated rats (Table 1). The plasma somatostatin level in the cysteamine-treated rats was significantly lower (P < 0.001) than that in the controls. The density of somatostatin-immunoreactive cells in the gastric corpus and antrum of the cysteamine-treated rats was also significantly lower (P < 0.001) than that of the control rats (Table 1).

The plasma levels of total and active ghrelin in the cysteamine-treated rats were significantly higher (total P < 0.01; active P < 0.05) than those in the control rats (Fig. 2, A and B), whereas the gastric levels of total and active ghrelin were significantly lower (total P < 0.001; active P < 0.01; Fig. 2, C and D). Preproghrelin mRNA expression in the cysteamine-treated rats was significantly lower (P < 0.05) than in the control rats (Fig. 2E). The density of ghrelin-immunoreactive cells in the gastric corpus of the cysteamine-treated rats was also significantly lower (P < 0.05) than that of the control rats (Fig. 2F).

A significant correlation between the plasma somatostatin and plasma ghrelin levels was observed (Fig. 3, A and B). The
gastric ghrelin levels were also correlated with the MPO activity (Fig. 3, C and D) and the duodenal ulcer depth and area (Fig. E-H).

Experiment 2 (10 h after the first dose of cysteamine). Six of ten cysteamine-treated rats developed ulcers in the proximal duodenum. The body weight of the cysteamine-treated rats was not significantly lower than that of the control rats (Table 2). The MPO activity in the stomach of the cysteamine-treated rats was significantly higher \((P < 0.05)\) than that of the control rats (Table 2). Neutrophil accumulation in the stomach and duodenum in cysteamine-treated rats was marked (Table 2). The plasma somatostatin level in the cysteamine-treated rats was significantly lower \((P < 0.05)\) than that in the control rats (Table 2).

The plasma levels of total and active ghrelin in the cysteamine-treated rats were significantly higher (total \(P < 0.01\); active \(P < 0.05\)) than those in the controls (Fig. 4, A and B). The density of ghrelin-immunoreactive cells in the gastric corpus of the cysteamine-treated rats was significantly lower \((P < 0.05)\) than that of the control rats (Fig. 4C). The plasma ghrelin levels in rats with cysteamine-induced duodenal ulcers were significantly higher than those in cysteamine-treated rats with no ulcers (Fig. 5).

Experiment 3 (0.5 or 2 h after cysteamine treatment). All of the cysteamine-treated rats did not develop duodenal ulcers. The body weight of the cysteamine-treated rats was not significantly lower than that of the control rats (Table 3). Furthermore, neutrophil accumulation in the stomach and the duodenum in cysteamine-treated rats was not significantly elevated at 0.5 or 2 h after the first cysteamine dose (Table 3). The plasma level of total ghrelin in the cysteamine-treated rats was significantly higher than those in the controls 2 h after the cysteamine treatment \((P < 0.01; \text{Fig. 6B})\). But this phenomenon was not observed 0.5 h after the cysteamine treatment (Fig. 6A).

Experiment 4 (effect of LPZ). In the cysteamine + LPZ-treated rats, although no cysteamine-induced duodenal ulceration was detected, the plasma total ghrelin level was nonetheless significantly elevated to the level comparable with those observed in rats treated with cysteamine alone \((P < 0.05, \text{Fig. 7})\). In addition, the pH of the cysteamine + LPZ-treated rats \((1.64 \pm 0.48)\) was significantly elevated compared with that of the control rats \((4.84 \pm 0.47; P < 0.01)\).

DISCUSSION

The present study demonstrated, for the first time, that plasma ghrelin levels are higher and gastric ghrelin levels are lower in rats with cysteamine-induced duodenal ulcers, compared with the levels in control rats. In the cysteamine-treated rats, both the number of somatostatin-immunoreactive cells in the stomach and the plasma somatostatin levels were significantly lower than in the control rats. These data are consistent with the results of previous reports (23, 30).

In terms of the relationship of ghrelin levels with the levels of somatostatin, the plasma levels of ghrelin inversely correlated with the plasma levels of somatostatin. Shimada et al.
(24) previously reported that intravenous administration of somatostatin caused a decrease of the plasma levels of ghrelin. Furthermore, it has been reported that cysteamine inactivates somatostatin directly through the hydrolysis of the disulphide bond (19). In the present study, we also found that the plasma ghrelin level in the cysteamine-treated rats was not changed 0.5 h after the cysteamine administration, although the somatostatin level in the cysteamine-treated rats was significantly decreased at this time point (data not shown). Thus cysteamine treatment might stimulate the secretion of ghrelin from the A-like cells of the gastric fundus by annulling the inhibitory effect of somatostatin on ghrelin secretion.

Tschope et al. (31) previously reported that intracerebroventricular administration of ghrelin was associated with a dose-dependent increase of food intake and body weight. Also, fasting was associated with an increase in the rat plasma ghrelin concentrations, which was reversed by refeeding or oral glucose administration. Thus food intake and body weight are influenced by the ghrelin dynamics. Because the mean body weight of the cysteamine-treated rats was significantly reduced at 50 h but not at 0.5, 2, and 10 h after the first cysteamine dose in the present study, the change in the body weight of rats may not be attributable simply to alteration of the ghrelin dynamics induced by cysteamine. Moreover, because the plasma ghrelin levels were also significantly increased in cysteamine-treated rats without ulcers, it appears that the alteration of the ghrelin dynamics observed following cysteamine administration is not a direct consequence of the ulcer formation. These results lend further support to contention that direct suppression of somatostatin released by cysteamine is responsible for the increase in the plasma levels of ghrelin observed in cysteamine-treated rats.

The lower gastric preproghrelin mRNA expression level and the smaller number of ghrelin-immunoreactive cells in the stomach of cysteamine-treated rats, compared with the findings in the control rats, suggest that cysteamine administration may attenuate the synthesis of ghrelin in A-like cells.

Gastric ghrelin levels were inversely correlated with the severity of the duodenal ulcers, as evaluated by the macroscopic area of the ulcer, the histological depth of the ulcer lesion, neutrophil accumulation, and the activity of gastric MPO. As previously reported, the formation of cysteamine-induced ulcers was followed by duodenal inflammation (20). These results suggest that gastric antral and duodenal mucosal inflammation might extend to localized areas containing A-like cells.

Recently, Khomenko et al. (10) reported that cysteamine altered redox state, HIF1-α transcriptional interactions, and reduced duodenal mucosal oxygenation in the early preulcerogenic phase after cysteamine treatment and emphasized the importance of preulcerogenic signal after cysteamine administration. In the present study, it was also found in the preulcerogenic phase that the plasma ghrelin levels were significantly increased even at 2 h after the first cysteamine dose, at which time no mucosal neutrophil accumulation was observed, indicating that the increase in the plasma ghrelin level preceded the gastric neutrophil accumulation; these findings also suggest that the increase in plasma ghrelin levels observed following cysteamine administration might be related to the inhibition of somatostatin release by the drug. Thus an increase in the plasma ghrelin levels is thought to be an upstream event to duodenal ulceration in rats treated with cysteamine.

In the present study, although LPZ treatment significantly attenuated duodenal ulceration induced by cysteamine, the elevation of plasma ghrelin level by cysteamine was not attenuated by LPZ (Fig. 7). Because ghrelin has been reported to stimulate both gastric acid secretion and gastric motility (17), the increased gastric acid secretion and its facilitated transportation to the duodenum in the presence of ghrelin might play important roles in the duodenal ulcer formation. Taken together, it is proposed that inhibition of endogenous somatostatin release induced directly by cysteamine treatment promotes ghrelin secretion from the A-like cells of the gastric fundus in the preulcerogenic phase; then, the enhanced levels of ghrelin, in turn, stimulate gastric acid secretion and possibly facilitate the transport of this acid to the duodenum by enhancing the gastric motility to induce duodenal ulceration.

GRANTS

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