Emotional Stress Enhanced Ghrelin Secretion from the Stomach

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Summary Ghrelin, a novel growth hormone releasing peptide, has been reported to accelerate food intake and gastrointestinal motility. The relationship between ghrelin secretion and emotional stress remains unknown. The present study was designed to investigate ghrelin dynamics in mice after the application of the emotional stress. To apply emotional stress in the mouse model, the communication box paradigm was used. Just after the communication box protocol, the mice were examined. The plasma and gastric ghrelin levels were measured by enzyme immunoassay and radioimmunoassay. Ghrelin immunohistochemistry and quantitative RT-PCR of preproghrelin mRNA in the stomach were performed. The plasma ghrelin levels were significantly increased in the stress-applied mice. Gastric ghrelin levels and the density of ghrelin-immunoreactive cells in the stress-applied mice tended to be decreased as compared with that in the control mice, but the difference was not statistically significant. There was no significant difference in the preproghrelin mRNA expression levels between the stress-applied mice and the control mice. The present study demonstrated for the first time that the plasma level of ghrelin increased in the association emotional stress.

Key Words: ghrelin, emotional stress, communication box, preproghrelin, gastric mucosa

Introduction

Growth hormone (GH) secretion is stimulated not only by GH-releasing hormone (GHRH), but also by GH secretagogues (GHS), acting on the GHS receptor (GHS-R) [1, 2]. In 1999, ghrelin was isolated for the first time from the human and rat stomach as an endogenous ligand of the GHS-R. Ghrelin is a 28-amino-acid peptide and possesses an n-octanoyl modification at the third serine residue, which has been shown to be necessary for its physiological activity [3]. It is produced and secreted from a subset of endocrine cells named A-like cells within the oxyntic glands of the stomach [4]. The physiological roles of ghrelin are to stimulate growth hormone release, gastric motility, and food intake [5].

Considerable evidence has accumulated to demonstrate that feeding behavior is influenced by stress [6–8]. In human beings, many obese patients tend to eat more when they are emotionally tense or depressed or simply bored [9]. In addition, numerous studies have shown that the stomach is influenced by stress [10]. These facts indicate that ghrelin

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may be associated with appetite accompanied by stress.

Although previous studies have shown that ghrelin gene expression in the stomach is increased by both tail pinch stress and starvation stress [11], the relationship between ghrelin secretion and emotional stress remains unknown. The communication box apparatus is sort to be one of the suitable methods to evaluate the social and emotional stress reaction in small animals such as rats or mice [12]. The present study was designed to investigate the ghrelin dynamics in mice after the application of the emotional stress.

Materials and Methods

Animal procedure

The animals were handled according to Declaration of Helsinki and to the guidelines of Keio University Animal Research Committee (No. 045131). Male C57BL/6 mice (n = 16, Japan Clea Co., Tokyo, Japan) weighing about 16–20 g at the beginning of the experiments were used.

Communication box paradigm

Mice were exposed to emotional stress in the compartment of a communication box apparatus [13]. The communication box paradigm originally described by Ogawa et al. [14] was used as previously reported. Briefly, the communication box consists of two types of compartments: compartments A and B (10 x 10 cm each). A total of nine compartments are arranged like a checkerboard and are separated by transparent plastic walls. Parallel stainless steel electric grids, 3 mm in diameter and located every 10 mm, were set on the floors of all compartments. However the floors of the B compartments are covered with insulating plastic panels to shield against electricity. Mice were individually placed in each compartment and intermittent electric shocks (2 mA, 10 s duration, 50 s intershock interval) were delivered through the grid floor by a shock generator (MSG001, Toyo Pharm., Osaka, Japan). The animals in the A compartments (responder) were exposed to emotional stress. During the study, no animal was injured or died from the electrical stimulation.

Measurement of gastric ghrelin levels

The RIA techniques were used for measuring ghrelin as described previously [16–18]. Briefly, the ghrelin levels were measured using polyclonal rabbit antibodies raised against C-terminal [13–28] (Gln13-Arg28) fragment of rat ghrelin. RIA incubation mixtures, containing 100 µL of standard ghrelin or unknown sample with 200 µL of antisem diluted in RIA buffer containing 0.5% normal rabbit serum, were initially incubated for 12 hours. Then, 100 µL of 125I-labeled tracer (15,000 cpm) was added and the mixture was incubated for 36 hours. Anti-rabbit IgG goat serum (100 µL) was added prior to an additional 24 hour-incubation. Free and bound tracers were then separated by centrifugation at 3,000 rpm for 30 min. Following aspiration of the supernatant, the radioactivity in the pellet was quantitated using a gamma counter (ARC-600, Aloka, Tokyo, Japan). All assays were performed at 4°C. The antisera was equally cross-reactive with human and gerbil ghrelin, and did not recognize the other enteric peptides. The respective intra- and inter-assay coefficients of variation for the C-terminal RIA were 6% and 9%.

Measurement of the density of ghrelin-immunoreactive cells in the stomach

The sections were deparaffinized and then hydrated by transferring the slides through the following solutions: xylene (5 minutes, x2), 96% ethanol (3 minutes, x2), 90% ethanol (3 minutes, x1), and double-distilled water (3 minutes, x1). Endogenous peroxidase was quenched by 0.3% hydrogen peroxide. After washing, non-specific binding was blocked by a blocking reagent (BlockAce, Dainippon Pharm., Osaka, Japan). For ghrelin staining, all the sections were incubated overnight at 4°C with anti-ghrelin [13–28] antisem [19]. After washing with TBS-T, the slides were incubated with biotinylated anti-rabbit IgG (Vectastain, Vector Laborataries, Burlingame, CA, USA) for 30 min at room temperature. The slides were then stained for 10 min at room temperature by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, Vector Laborataries) with

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0.02% 3,3’-deaminobenzidine tetrahydrochloride (Sigma-Aldrich Fine Chem., St Louis, MO) and 0.006% hydrogen peroxide in 50 mM Tris-HCl buffer solution (pH 7.2). After dehydration in a graded alcohol series, the slides were counterstained with hematoxylin. The stained sections were observed under a light microscope equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan) and each image was stored as a PSD file (Adobe Photoshop 7.0). The stained nuclei were counted using a particle analysis program (Ultimage Pro. 2.6.4; Alliance Vision, France). The density of ghrelin-immunoreactive cells (D:ghrelin) was calculated by using the following formula:

\[
D:ghrelin = \frac{Ng}{Nt} \times 100 \%
\]

where Ng and Nt represent the number of ghrelin-immunoreactive cells, and the total cell number, respectively, in the region of interest [20–22].

Measurement of preproghrelin mRNA expression in the stomach

Total mRNA was extracted from the stomach using the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). A TaqMan® quantitative real-time RT-PCR was performed to detect preproghrelin mRNA and GAPDH mRNA with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) [15, 22]. The following primers were used to amplify the preproghrelin mRNA: ghrelin-F (5’-GGA ATC CAA GAA GCC ACC AGC-3’), ghrelin-R (5’-GCT CCT GAC AGC TTG ATG CCA-3’) and ghrelin-Taq (5’-FAM-AAC TGC AGC CAC GTC ATG TAMRA-3’); to amplify GAPDH mRNA as the internal control, the following primers were used: GAPDH-F (5’-TTC AAC GGC ACA GTC AAG GC-3’); GAPDH-R (5’-GCC TTC TCC ATG GTG GTG AAG-3’) and GAPDH-Taq (5’-FAM-CCC ATC ACC ATC TTC CAG GAG CGA GA-TAMRA-3’). The preproghrelin mRNA expression levels were normalized using the GAPDH mRNA expression levels [22].

Statistical analysis

All data were expressed as the mean ± SE; \( P<0.05 \) was considered to denote statistical significance. The data were analyzed using one-way analysis of variance followed by Scheffe’s multiple comparison tests.

Results

There was no significant difference in body weight between the stress-applied mice and the control mice. No significant difference in macroscopic findings of the gastric mucosa was shown in each group.

The plasma levels of total ghrelin were significantly increased in the stress-applied mice as compared with those in the control mice (stress-applied mice: 392.5 ± 102.1 fmol/ml, control mice: 261.1 ± 46.9 fmol/ml, \( P<0.01 \)) (Fig. 1).

Gastric total ghrelin in the stress-applied mice tended to be lower than those in control mice, but the difference was not statistically significant (\( P=0.07 \)). There was no significant difference in the preproghrelin mRNA expression levels between the stress-applied mice and the control mice. The density of ghrelin-immunoreactive cells in the gastric corpus of the stress-applied mice was decreased as compared with that in the control mice, but the difference was not statistically significant (\( P=0.12 \)) (Table 1).

Discussion

The present study showed for the first time that plasma

![Fig. 1. Plasma total ghrelin levels measured by ELISA in control mice (n = 8) and stress-applied mice (responder mice, n = 8). The plasma total ghrelin levels increased significantly in the responder mice. *P<0.01 compared with the control mice.](attachment:image)

Table 1. The date are expressed as the mean values ± SD. Gastric ghrelin levels, Preproghrelin mRNA expression levels and density of ghrelin-immunoreactive cells in control mice and stress-applied mice (responder mice). Preproghrelin mRNA expression levels is normalized to the GAPDH mRNA expression levels measured using real-time RT-PCR.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Responder</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric ghrelin levels (fmol/mg)</td>
<td>4122.2 ± 558.9</td>
<td>3369.5 ± 870.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Preproghrelin mRNA expression levels</td>
<td>0.92 ± 0.30</td>
<td>1.07 ± 0.21</td>
<td>0.34</td>
</tr>
<tr>
<td>Density of ghrelin-immunoreactive cells (%)</td>
<td>3.1 ± 0.7</td>
<td>2.5 ± 0.7</td>
<td>0.12</td>
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levels of ghrelin were enhanced after emotional stress was applied by the communication box apparatus.

Although there are many methods to apply stress to animals, such as electrical stimuli loading of the sender animals in the communication box, water-immersion restraint, and cold exposure, most methods expose animals to direct stimuli. In the communication box, although the responder was exposed only to affective stimuli, which were conveyed from the sender by visual, auditory, and olfactory sensation, the responder was never exposed to actual physical stimuli produced by electric shocks to the feet. The communication box apparatus might be one of best choices to simulate psychosocial and emotional stress in a usual human life.

The results of the present study demonstrated that the plasma ghrelin levels were significantly increased in the stress-applied mice as compared with those in the control mice, and gastric ghrelin levels in the stress-applied mice tended to be lower than those in control mice. The increased plasma ghrelin levels and the decreased gastric ghrelin levels in the stress-applied mice may be due to an increase in ghrelin release by the stomach into the blood stream.

A functional relationship between the cholinergic system and ghrelin secretion has been demonstrated. The food deprivation-induced elevation in plasma ghrelin levels in rats has been found abolished by subdiaphragmatic vagotomy and substantially reduced by atropine [23]. Fabio et al. [24] demonstrated that circulating levels of ghrelin were increased by pirenzepine, a muscarinic antagonist, and reduced by pyridostigmine, an indirect cholinergic agonist. Thus, ghrelin secretion is under the stimulatory control of the cholinergic, namely muscarinic, receptor. On the other hand, Cho et al. [25] reported that cold restraint stress provoked prolonged vagal hyperactivity. As a possible explanation for the enhanced plasma ghrelin by stress, it has been suggested that the vagal hyperactivity by stress may stimulate the secretion of ghrelin.

In conclusion, the present study demonstrated for the first time that the plasma level of ghrelin increased in association with the emotional stress, suggesting that ghrelin may be involved in the pathogenesis under the condition with emotional stress.

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