Gastric leptin, but not estrogen and somatostatin, contributes to the elevation of ghrelin mRNA expression level in fasted rats

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Abstract

Ghrelin, an endogenous ligand for the GH secretagog receptor, is predominantly produced in the stomach. It has been reported that endogenous ghrelin levels are increased by fasting and decreased after refeeding. It has also been reported that estrogen upregulates ghrelin expression and production and that somatostatin inhibits ghrelin secretion, whereas leptin has a paradoxical effect. Recently, several studies have shown that estrogen, somatostatin, and leptin are produced in the stomach, but the direct effects of these gastric hormones on ghrelin expression in a fasting state remain obscure. In this study, we examined the mRNA expression levels of gastric ghrelin, aromatase (estrogen synthetase), leptin, and somatostatin, and concentrations of stomach leptin and portal vein 17β-estradiol in fasted male rats. After 48 h of fasting, although gastric ghrelin mRNA level was significantly increased, both gastric leptin mRNA level and leptin content were decreased. Further, refeeding of fasted rats resulted in a decrease in ghrelin expression level and an increase in leptin expression level. On the other hand, gastric estrogen and somatostatin levels did not change after fasting. In vitro studies revealed that leptin dose-dependently inhibited ghrelin expression and also inhibited estrogen-stimulated ghrelin expression. Moreover, ghrelin cells were found to be tightly surrounded by leptin cells. RT-PCR analysis clearly showed that long and short forms of the leptin receptor are expressed in the rat stomach. These results strongly suggest that an elevated gastric ghrelin expression level in a fasting state is regulated by attenuated restraint from decreased gastric leptin level.

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Introduction

Ghrelin, a 28-amino acid peptide with an essential n-octanoyl modification on the third amino acid, was identified as the endogenous ligand for the growth hormone (GH) secretagog receptor (Kojima et al. 1999). Initially, ghrelin was known for its potent stimulatory action on GH secretion (Kojima et al. 1999, Yamazaki et al. 2002, Malagon et al. 2003), but later findings showed that it was also involved in the regulation of feeding behavior and energy homeostasis (Tschop et al. 2000, 2001a, Wren et al. 2000, Nakazato et al. 2001, Shintani et al. 2001). Actually, ghrelin is the primary orexigenic signal from the periphery, and it has been reported that peripheral ghrelin exerts its orexigenic effects through circulation (Cowley 2003) or by acting in the periphery to suppress gastric vagal afferents (Date et al. 2002).

Ghrelin is predominantly produced in the stomach (Kojima et al. 1999), and previous studies on the regulation of ghrelin expression and secretion therefore focused on gastric ghrelin. The most important physiological state for the regulation of gastric ghrelin synthesis is feeding. Results of many studies have shown that endogenous ghrelin levels are increased by fasting and decreased after refeeding in several species (Tschop et al. 2000, 2001b, Asakawa et al. 2001, Cummings et al. 2001, Toshinai et al. 2001), and further observations suggest that ghrelin has a role in regulation of energy homeostasis. In addition to feeding, several hormonal states have been shown to be involved in the regulation of ghrelin expression and production.

Recently, Ueyama et al. (2002) clearly demonstrated that gastric parietal cells are capable of producing and secreting a substantial amount of estrogen. Furthermore, in our latest work, we clearly demonstrated that gastric estrogen but not gonadal estrogen directly stimulated ghrelin expression and production in the rat stomach (Sakata et al. 2006). Somatostatin produced in the gastric mucosa is known to suppress secretion of several gastrointestinal hormones in a paracrine fashion, and many studies have shown that somatostatin and its analogs inhibited ghrelin secretion in both humans and rats (Barkan et al. 2003, Shimada et al. 2003, Silva et al. 2005). Moreover, reciprocal circadian rhythms in circulating ghrelin and leptin levels and antagonistic
hypothalamus-mediated control of appetite by ghrelin and leptin have been revealed by several studies (Friedman & Halaas 1998, Tschop et al. 2000, Nakazato et al. 2001, Bagnasco et al. 2002); however, results of studies on the modulation of ghrelin expression and secretion by leptin are inconsistent. One group showed that leptin administration to rats for 5 days stimulated gastric ghrelin mRNA expression (Toshinai et al. 2001), whereas two other groups reported the opposite results (Asakawa et al. 2001, Kamegai et al. 2004). In humans, no effect of leptin administration on circulating ghrelin levels has been found (Chan et al. 2004). Due to these conflicting results, the regulatory role of leptin in ghrelin synthesis remains unclear. On the other hand, leptin was initially thought to be adipocyte derived (Zhang et al. 1994), and it has also been identified in various tissues, including the stomach (Bado et al. 1998). The fact that the release of gastric leptin is rapidly stimulated by food intake or cholecystokinin (CCK) treatment suggests that gastric leptin is involved in the short-term control of energy balance (Bado et al. 1998), although adipocyte leptin is known to be a long-term regulator of energy balance.

However, it is not clear whether these hormones produced in the stomach directly contribute to the elevation of ghrelin level in a fasting state. Therefore, in this study, we examined the mRNA expression levels of gastric ghrelin, aromatase (estrogen synthetase), leptin and somatostatin, and concentrations of stomach leptin and portal vein 17β-estradiol in fasted male rats, and we found an inverse relationship between gastric ghrelin and leptin levels. We hypothesized that gastric leptin contributes to the elevation of ghrelin mRNA expression level in a fasting state, and we investigated the effect of gastric leptin on ghrelin mRNA expression using minced stomach and cells isolated from gastric mucosa by a method established in our previous work (Sakata et al. 2006).

Materials and Methods

Animals

Adult (8 weeks old) male Wistar rats weighing 250–300 g were used in this study. The rats were housed in a temperature-controlled room (23 ± 2 °C) with a 12 h light:12 h darkness cycle (lights on 0800 h). Water and food were available ad libitum. All procedures were performed in accordance with the institutional guidelines for animal care at Saitama University.

Experimental design

Experiment 1: effects of fasting and fasting-refeeding

Rats were randomly separated into three groups: free-feeding, fasting, and fasting-refeeding groups. The free-feeding group had free access to food, the fasting group was deprived of food for 48 h, and the fasting-refeeding group was allowed ad libitum refeeding for 5 h after 48 h of fasting. All groups had free access to water. After fasting, half of rats in free-feeding or fasting group were killed under ether anesthesia, and the stomachs were quickly removed. The stomachs were opened and rinsed with 10 mM PBS (pH 7.5) for mRNA quantification, the gastric fundi were collected and stored in ISOGEN (Nippon Gene, Tokyo, Japan) at −80 °C until use; and for stomach leptin measurement, the epithelium of gastric fundus was scraped off, and then the obtained fundic mucosa weighed about 0.2 g was immediately frozen in liquid nitrogen and also stored at −80 °C until leptin assay. The other half of rats were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.), and 1 ml blood from the portal vein close to the liver was collected, centrifuged, and plasma was collected and stored at −80 °C until further measurement for 17β-estradiol concentration. After refeeding, the rats were killed under ether anesthesia, and the stomachs were also removed. Then the stomachs were opened and rinsed with 10 mM PBS, and the gastric fundi were collected and stored in ISOGEN at −80 °C until mRNA quantification.

Experiment 2: effects of somatostatin and leptin on ghrelin mRNA expression in stomach tissue culture

Overnight fasted rats were killed under ether anesthesia, and the stomachs were quickly removed. Stomach tissue culture was performed as previously described (Sakata et al. 2006). Briefly, the mucosa of the gastric fundus was minced (~1 mm³) with a sharp razor blade in phenol red-free Dulbecco’s modified Eagle medium (DMEM; Life Technologies). These minced stomach tissues were then incubated with serum and phenol red-free DMEM containing 10⁻⁷ M somatostatin (Calbiochem, San Diego, CA, USA) or 10⁻⁷ M recombinant rat leptin (Sigma) or a vehicle for 12 h or with serum and phenol red-free DMEM containing increasing doses of recombinant rat leptin (10⁻⁷–10⁻³ M) or a vehicle for 12 h at 37 °C in humidified 95% air and 5% CO₂. After incubation, these stomach tissues were collected and stored in ISOGEN at −80 °C until analysis.

Experiment 3: effect of leptin on estrogen-stimulated ghrelin mRNA expression in isolated stomach cells

Stomach cells were isolated by the enzymatic dispersion method established in our previous study (Sakata et al. 2006) with little modification. Briefly, male rats were killed under ether anesthesia, and the stomachs were quickly removed and then turned inside out. The stomachs were inflated and incubated in dispase I solution (1000 PU/ml dispase I (Godo Shusei, Tokyo, Japan), 135 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, 10 mM glucose, 10 mM HEPES (DOJINDO, Kumamoto, Japan), and 0.6 mM NaHCO₃ (pH 7.4)) for 1.5 h. Stomach cells were removed from gastric mucosa using a glass pipette with a diameter of ~5 mm and passed through a 102 μm filter and then collected by centrifugation.
at 400 g for 5 min. The pellet was suspended in medium B (135 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 0.6 mM NaHCO₃ (pH 7.4)) and was centrifuged at 400 g for 5 min, the pellet was collected from the bottom of the tube and then centrifuged on the 40% layer on 50% Percoll medium and centrifuged again for 5 min. After centrifugation, cell solution fractioned on 50% Percoll medium was collected. These ghrelin-rich cells were resuspended in phenol red-free DMEM (Life Technologies Inc.) with 10% charcoal–dextran-treated fetal bovine serum. The cells were plated on a poly-l-lysine–coated culture dish (2 × 10⁵ cells/ml, 5 ml/dish) and incubated in humidified 95% air and 5% CO₂ for 1 h. After preincubation for 12–16 h, the cells were washed twice with phenol red-free DMEM. Then the cells were incubated in phenol red-free DMEM with 1% charcoal–dextran–treated fetal bovine serum containing 10⁻⁷ M recombinant rat leptin and 10⁻⁵ M water-soluble 17β–estradiol (E₂; Sigma) or 10⁻⁵ M water-soluble 17β–estradiol alone for 8 h in humidified 95% air and 5% CO₂ at 37 °C. After 8 h of incubation, the cells were collected and stored in ISOGEN at −80 °C until use for ghrelin mRNA quantification.

**Double staining**

**Tissue preparation for double staining** After killed under ether anesthesia, rat stomachs were quickly removed and opened along their longitudinal axes. The stomachs were fixed with 4% paraformaldehyde (PFA) in 50 mM phosphate buffer (PB), pH 7.4, for 24 h, and then embedded in paraplast (Oxford, Labware, MO, USA). Serial sections (5 μm in thickness) were cut and mounted on slides coated with silane (Shin-Etsu Chemicals, Tokyo, Japan).

**Double staining for leptin mRNA and ghrelin** Double staining was performed by a previous reported procedure (Sakata et al. 2006) with some modification. Briefly, the sections were deparaffinized with xylene for 20 min and immersed in descending ethanol series (100, 90, 80, and 70%) for 15 s each. Then the sections were washed with PBS, treated with 32 μg/ml proteinase K for 30 min at 37 °C, and fixed with 4% PFA in 0.067 M PB (pH 7.4) for 10 min. After washing with PBS for 1 min, the sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed with PBS for 1 min, immersed in a graded ethanol series (70, 80, and 90%) for 15 s each and then immersed twice in 100% ethanol for 15 s, and dried for 20 min. Digoxigenin (DIG)-labeled antisense and sense rat leptin cRNA probes (GenBank accession no. D45862, nucleotides 22–560) were synthesized using a labeling kit (Roche Diagnostics GmbH) with T7 or SP6 RNA polymerases. The probes were diluted to 1 ng/μl with hybridization buffer (50% formamide, 3 × SSC, 0.12 M diethyl pyrocarbonate–treated PB (pH 7.4), 1 × Denhardt’s solution, 125 μg/ml tRNA, 0.1 mg/ml sonicated salmon sperm DNA, and 10% dextran sulfate) and dropped on the tissue sections. A sense RNA probe was used as a negative control. The sections were covered with Parafilm (American National Can, Chicago, IL, USA) and incubated for 16 h at 42 °C in a humid chamber. After incubation, the covers were removed, and the sections were immered in 2 × SSC, containing 50% formamide at 42 °C for 30 min. The sections were then treated with TNE buffer (10 mM Tris–HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA) for 10 min and next with RNase A (1 μg/ml in TNE) for 30 min at 37 °C. The sections were immersed in TNE for 10 min at 37 °C, washed with 2 × SSC for 20 min at 42 °C, and then with 0.2 × SSC for 20 min twice at 42 °C. The sections were incubated for 5 min in buffer-1 (100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.01% Tween 20), immersed in 1.5% blocking reagent (Roche Diagnostics GmbH) in buffer-1 for 1 h at 37 °C, and then washed in buffer-1 for 5 min. After washing, the sections were incubated with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics Corporation) diluted 1:1000 in buffer-1 for 1 h at 37 °C. The sections were then washed in buffer-1 for 15 min twice and in buffer-2 (100 mM Tris–HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂) for 5 min. A chromagen solution (337 μg/ml 4-nitroblue tetrazolium chloride and 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate in buffer-2) was added, and the sections were incubated until a visible signal was detected. The reaction was stopped by adding a reaction stop solution (10 mM Tris–HCl (pH 7.6) and 1 mM EDTA). In this study, we used Gengard water (Gradient A10, Millipore, Tokyo, Japan) as RNase-free water. After the leptin mRNA-expressing cells have been detected, immunohistochemistry for ghrelin was performed using a rabbit anti-ghrelin serum (no. 603, Department of Biochemistry, National Cardiovascular Centre Research Institute, Suita, Japan). The production and the specificity of this anti-rat ghrelin serum were previously reported, and it is known that this antisem recognizes the N-terminal region of rat ghrelin (Hosoda et al. 2000). After washing thrice with PBS, the sections were incubated with TNBS buffer (1% normal horse serum and 0.4% Triton X-100 in PBS) for 30 min. After the second wash with PBS, the sections were incubated with anti-ghrelin serum diluted 1:10 000 in TNBS in a humid chamber for 2 h. After the third wash with PBS, the sections were incubated with Alexa594–conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) as a second antibody for 2 h. The sections were washed with PBS thrice, mounted with 5% DABCO containing 90% glycerol in PBS, and then viewed and photographed under a light microscope (BX60, Olympus, Tokyo, Japan).

**Double-label fluorescent immunostaining for leptin and ghrelin** The sections were deparaffinized with xylene and rehydrated through descending concentrations of
ethanol. Next, the sections were treated with 0.5% sodium metaperiodate to block endogenous peroxidase for 15 min at room temperature. After washing with distilled water, the sections were incubated with TNBS for 1 h and then washed thrice with PBS, incubated overnight with rabbit polyclonal antibody against leptin (Ob A20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100 (2 μg/ml) in TNBS in a humid chamber. After washing thrice with PBS, a second incubation with Alexa594-conjugated donkey anti-rabbit IgG (Molecular Probes) was carried out for 2 h, and this was followed by further washing with PBS. For ghrelin double staining, the sections were incubated with a monoclonal antibody against octanoylated (i.e., acylated) ghrelin (11F9) for 3 h, washed with PBS. After final incubation with Alexa488-conjugated donkey anti-mouse IgG (Molecular Probes) as a secondary antibody for 2 h, the sections were washed with PBS, mounted with 5% DABCO containing 90% glycerol in PBS, and then viewed and photographed under a light microscope (BX60, Olympus).

Reverse transcriptase (RT)-PCR for short and long forms of the leptin receptor (OB-R) mRNA

Total RNA was extracted from the isolated stomach cells or stomach tissues using ISOGEN according to manufacturer’s instructions. Trace DNA contamination was removed by DNase digestion (Promega). cDNA was synthesized from 1 μg total RNA using Superscript III reverse transcriptase (Invitrogen) for OB-Ra and from 2 μg total RNA using Primerscript reverse transcriptase (Takara, Tokyo, Japan) for OB-Rb. The following primers were designed to amplify a rat long form (OB-Rb) OB-R fragment (1131 bp; accession no. D85558): sense primer, TGGCCCATGAGTAAGGTCTGA and antisense primer, CAGACAGTGAAGCTGGGATA. The following primers were designed to amplify a rat short form (OB-Ra) OB-R fragment (399 bp; accession no. AF304191): sense primer, GATGATATCGCCAAACAGCA and antisense primer, CCCAAGTCGAACATACAAACC. PCR amplification was carried out with Ex Taq polymerase (Takara) according to manufacturer’s instructions. Initial template denaturation was programmed for 5 min at 94 °C. For OB-Rb, the cycle profiles were programed as follows: 1 min at 94 °C (denaturation), 1 min at 64 °C (annealing and extension), and 1 min at 74 °C (extension). Forty-five cycles of the profile were run. For OB-Ra, the cycle profiles were programed as follows: 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing and extension), and 1 min at 74 °C (extension). Forty cycles of the profile were run. Then PCR products were visualized by 1% agarose gel electrophoresis for OB-Rb and 2% agarose gel electrophoresis for OB-Ra.

Quantitative RT-PCR for mRNA of ghrelin, aromatase, leptin, and somatostatin

RNA extraction and cDNA synthesis were performed as described above. The following primers were designed to amplify a rat ghrelin fragment (191 bp; accession no. AB029433): sense primer, CAGGTTCCAGGTCTCTTGGA and antisense primer, GACAGCTTGATGCCAACCA. The following primers were designed to amplify a rat aromatase fragment (194 bp; accession no. M33986): sense primer, GAATCCATCAAGCAGCT and antisense primer, TGATAAGGAGTGCTTGGCAG. The following primers were designed to amplify a rat leptin fragment (187 bp; accession no. D45862): sense primer, GAGACCTCTCCCATCTGCTG and antisense primer, CATTAGGCGCATAATGCA. Real-time quantitative PCR was performed using SYBR Premix Ex Taq (Takara BIO, Shiga, Japan) according to manufacturer’s instructions. Amplification reactions were performed using a LightCycler (Roche Diagnostics). Initial template denaturation was performed for 30 s at 95 °C. The cycle profiles were programed as follows: for ghrelin, β-actin, and somatostatin, 5 s at 95 °C (denaturation) and 15 s at 60 °C (annealing and extension); for aromatase, 5 s at 95 °C (denaturation) and 20 s at 60 °C (annealing and extension); and for leptin, 5 s at 95 °C (denaturation) and 18 s at 65 °C (annealing and extension). Forty-five cycles of the profile were run, and the final cooling step was continued for 30 s at 40 °C. Quantitative measurement of each mRNA was achieved by establishing a linear amplification curve from serial dilutions of each plasmid containing the amplicon sequence. The relative amount of each mRNA was normalized by the amount of β-actin mRNA. Amplicon size and specificity were confirmed by melting curve analysis and 2% agarose gel electrophoresis.

ELISA for estrogen and leptin

Estrogen ELISA Portal vein 17β-estradiol concentration was measured using an estradiol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) without extraction according to manufacturer’s instructions. This assay is based on the competition between free estradiol and an estradiol tracer (estradiol linked to an acetylcholinesterase (AchE) molecule) for a limited number of estradiol-specific rabbit antiserum-binding sites. The 96-well plate which was pre-coated with mouse monoclonal anti-β estradiol IgG was supplied by this kit. The plate was treated with estradiol AchE tracer, rabbit antiserum-estradiol, and either a series of estradiol standard diluted by EIA buffer using a concentration range of 7.8–1000 pg/ml or unknown plasma samples. The plate was then covered with plastic film and incubated for 1 h at room temperature. After incubation, the plate was washed six times with wash buffer to remove any unbound reagents and
incubated with Ellman’s reagent (which contains the substrate to AChE) to develop the color using an microtube mixer (orbital shaker) in the dark for 1.5 h. Then the plate was placed in a microplate reader (Bio-Rad), and the optical density was obtained at 405 nm. The detection limit is 8 pg/ml. Cross-reactivities of various steroids with the antiserum were as follows: 100% estradiol, 17% estradiol-3-glucuronide, and 4% estrone. All samples and standards were prepared in duplicate.

**Leptin ELISA**

Fundic mucosa was homogenized at 4°C in 1:5 (w/v) of a Krebs–Ringer bicarbonate–HEPES solution (129 mM NaCl, 5 mM NaHCO3, 4-8 mM KCl, 1-2 mM KH2PO4, 1-0 mM CaCl2, 1-2 mM MgSO4, 0-1% BSA, 10 mM HEPES, and 2-8 mM glucose (pH 7-4)) using a Teflon/glass homogenizer. The homogenate was centrifuged at 10 000 g for 10 min at 4°C, and the resulting supernatant was used for leptin measurement. Leptin concentration in fundic homogenate was measured with a Quantikine mouse leptin ELISA kit (R&D Systems, Minneapolis, MN, USA). This kit also recognizes rat leptin well and has been validated to determine the leptin concentration in the rat stomach (Pico et al. 2002). Both intra- and inter-assay precision were <8%, with a detection limit of 22 pg/ml. Some cross-reactivities were as follows: human leptin 0-24% and 79% rat leptin. All samples and standards were prepared in duplicate.

**Data analysis**

All of the data are expressed as means ± S.E.M. The results were statistically evaluated by Student’s t-test and Fisher’s protected least significant difference test with Stat View statistics software (SAS Institute, Cary, NC, USA). P<0-05 was considered statistically significant.

**Results**

**Effects of fasting and fasting-refeeding**

To determine whether gastric estrogen, leptin, and somatostatin contribute to the elevation of ghrelin expression level in a fasting state, we fasted male rats for 48 h and examined the mRNA expression levels of gastric ghrelin, aromatase, leptin, and somatostatin. We also determined the stomach leptin content and portal vein 17β-estradiol concentration. As previously shown, gastric ghrelin mRNA expression level in rats that had been fasted for 48 h was about 2-5 times higher than that in fed rats (Fig. 1A). In a previous study, we found that gastric estrogen directly stimulated ghrelin expression and production (Sakata et al. 2006). In this study, however, both aromatase mRNA expression level and portal vein 17β-estradiol concentration in the fasted rats were not different from those in the fed rats (Fig. 1B and C). Somatostatin mRNA expression levels were also similar in the fasted and fed rats (Fig. 1D). In contrast, gastric leptin mRNA expression level and leptin content significantly decreased after fasting to about 47 and 36% respectively, of those in fed rats (Fig. 1E and F). Further, refeeding of fasted rats for 5 h also induced inverse changes in gastric ghrelin and leptin expression levels, a decrease in ghrelin expression level (Fig. 1A) and an increase in leptin expression level (Fig. 1E), and the expression levels of both ghrelin and leptin recovered to the levels in fed rats (Fig. 1A and E).

In vitro effects of somatostatin and leptin on ghrelin mRNA expression

To determine the direct effect of somatostatin or leptin on ghrelin mRNA expression, we investigated whether treatment of somatostatin or leptin affects ghrelin mRNA expression in minced stomach tissue or isolated stomach cells. In the minced stomach tissue, somatostatin at a dose of 10-8 M suppressed ghrelin mRNA expression, although this effect was not statistically significant (Fig. 2A); on the other hand, leptin significantly inhibited ghrelin mRNA expression level in a dose-dependent manner (10-9–10-7 M; Fig. 2B). Using a previously established method (Sakata et al. 2006), we obtained a ghrelin cell-rich fraction from gastric mucosa. We treated these cells with estrogen alone or estrogen combined with leptin and found that ghrelin mRNA expression level was significantly increased by estrogen treatment as in our previous study, whereas this effect was significantly reversed by leptin pre-treatment (Fig. 2C).

**Distribution of leptin and ghrelin cells in the stomach**

Both leptin mRNA-expressing cells detected by in situ hybridization (Fig. 3A) and leptin-immunopositive cells (leptin-ip cells; Fig. 3D) were mainly located in the lower half of the fundic gland. No signals were detected by in situ hybridization in the negative control section (sense probe; Fig. 3A, inset). Ghrelin-ip cells were observed throughout the gastric mucosa (Fig. 3B and E), and leptin mRNA-expressing cells or leptin-ip cells were adjacent to ghrelin-ip cells, some ghrelin-ip cells being closely surrounded by leptin-expressing or leptin-ip cells or in direct contact with them (Fig. 3C and F).

**RT-PCR analysis of OB-Ra and OB-Rb mRNA expression**

RT-PCR analysis clearly demonstrated that mRNAs of both OB-Ra (Fig. 4A) and OB-Rb (Fig. 4B) were expressed in whole stomach tissue and isolated stomach cells.

**Discussion**

The results of our previous study showing a stimulatory effect of gastric estrogen on both ghrelin expression and production
(Sakata et al. 2006) prompted us to further investigate the regulation of ghrelin synthesis by gastric estrogen under various physiological conditions including fasting, a negative energy balance state. In the present study, however, neither the expression level nor the secretion level of gastric estrogen was altered by fasting, although the expression level of ghrelin mRNA was significantly increased as predicted. The lack of response of gastric estrogen to fasting may be due to the minor role of estrogen in short-term regulation of feeding and does not support the idea that gastric estrogen is a major contributor to fasting-induced increase in gastric ghrelin expression. Meanwhile, although our in vitro study revealed an inhibitory effect of somatostatin on ghrelin expression, the expression level of somatostatin mRNA in the gastric fundus was also not changed by fasting. Interestingly, this result is similar to that of a previous study showing that fasting reduced antral but not fundic somatostatin mRNA level (Yamada et al. 1997). These findings clearly support the hypothesis that in a fasting state somatostatin is involved in the regulation of antral ghrelin rather than fundic ghrelin, which is the major source of ghrelin.

On the other hand, results of several studies have shown that leptin mRNA levels in the stomach and adipocytes and levels of gastric and circulating leptin are decreased by fasting in rats (Frederich et al. 1995, Pico et al. 2002). In accordance with these findings, both gastric leptin mRNA and leptin levels were found to be significantly decreased by fasting in the present study. However, another study revealed that fasting only produced a slight but not significant decrease in gastric leptin (Bado et al. 1998). This discrepancy can be attributed to the different fasting durations used in two studies, 18 h in the earlier study and 48 h in our study. Actually, gastric ghrelin mRNA level also did not change in rats that had been fasted for 24 h (data not shown). Indeed, an inverse pattern was also observed between circulating ghrelin and leptin levels in response to fasting (Sanchez et al. 2004). Since opposing effects of circulating ghrelin and leptin on appetite control via NPYergic signaling in the arcuate nucleus–paraventricular nucleus (ARC–PVN) axis of the hypothalamus have been revealed by many studies (Friedman & Halaas 1998, Tschop et al. 2000, Nakazato et al. 2001), under a fasting condition, enhanced stimulation by ghrelin

Figure 1 mRNA expression levels of gastric ghrelin, aromatase, leptin, and somatostatin measured by quantitative RT-PCR and concentrations of stomach leptin and portal vein 17β-estradiol determined by ELISA under different feeding conditions. The mRNA expression level of gastric ghrelin was significantly increased by 48 h of fasting, whereas refeeding of fasted rats for 5 h restored gastric ghrelin mRNA level to the control expression level (A). Both the mRNA expression level of gastric aromatase (B) and 17β-estradiol concentration in the portal vein (C) did not change after fasting. mRNA expression levels of somatostatin were similar in fasted and fed rats (D). The mRNA expression level of gastric leptin (E) and stomach leptin concentration (F) were significantly decreased by fasting, and 5 h of refeeding resulted in recovery of gastric leptin mRNA level to the control expression level (E). Ghrelin, aromatase, leptin, and somatostatin mRNA levels are expressed relative to β-actin mRNA levels. Data are presented as means ± S.E.M. n=3–4/group. *P<0.05; **P<0.01; NS, no significant difference.


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concomitant with attenuated restraint from leptin on NPYergic signaling must directly contribute to the robust feeding and energy saving. It has been reported that both decreases in body fat content and circulating insulin level contribute to the suppression of circulating leptin level in a fasting state (Weigle et al. 1997). Although the mechanism of reduction in gastric leptin level under a fasting condition remains unknown, one could assume that this fasting-induced decrease in gastric leptin level would enable gastric leptin to exert regulatory effects on energy saving and food intake by indirectly activating nerve endings on gastric and intestine mucosa or by directly modulating gastrointestinal hormone secretion in a paracrine or endocrine manner. Therefore, the inverse relationship between gastric ghrelin and leptin levels led us to hypothesize that the elevation of gastric ghrelin expression level in a fasting state is mediated by decreased gastric leptin level. Moreover, in the present study, the expression of gastric ghrelin and that of leptin also showed opposite responses to refeeding, i.e., reduced ghrelin mRNA level and increased leptin mRNA level, indicating a similar role of gastric leptin in regulating ghrelin expression under the refeeding condition. To test this hypothesis, we studied whether leptin directly regulates ghrelin mRNA expression in vitro and found that leptin treatment inhibited ghrelin mRNA expression in minced stomach tissue in a dose-dependent manner and also inhibited estrogen-stimulated ghrelin mRNA expression in isolated stomach cells. Consistent with results of several studies (Wang et al. 1996, Mix et al. 2000, Sobhani et al. 2000), our RT-PCR analysis clearly showed that mRNA of both OB-Ra and OB-Rb was expressed in the gastric fundus, indicating a role of the leptin receptor in leptin-induced suppression of ghrelin expression. Further detection of the expression of leptin receptors on ghrelin-producing cells will probably help to confirm this. Moreover, we revealed that leptin-producing cells were mainly located in the lower half of the gastric mucosa, where most of the ghrelin cells were tightly surrounded by leptin-producing cells, suggesting that gastric leptin has a paracrine role in regulation of ghrelin cells and that ghrelin cells may be exposed to a higher concentration of gastric leptin than that of plasma leptin since leptin infusion at 0.1 nM, which can mimic the plasma leptin concentration under basal conditions in rats, has been shown to be incapable of suppressing ghrelin release from the isolated rat stomach (Kamegai et al. 2004).

Our present findings support the idea that gastric leptin directly suppresses ghrelin expression in the rat stomach and that elevation of gastric ghrelin expression level in a fasting state is mediated by decreased gastric leptin level. Several lines of evidence support this hypothesis. In zucker fatty (fa/fa) rats, an animal model that is characterized by a lack of leptin signaling due to a default in the leptin receptor, both mRNA level of ghrelin in the stomach and circulating ghrelin level have been shown to be augmented (Beck et al. 2003, 2004). Moreover, fasting of young zucker fatty rats for 48 h induced only a slight increase in circulating ghrelin level, which was significantly lower than that in lean control rats, and even no change in circulating ghrelin levels has been shown in older fatty rats after fasting (Ariyasu et al. 2002). It should be noted that the synthesis of ghrelin in the stomach is also positively
regulated by gastric estrogen and negatively regulated by gastric somatostatin. Therefore, the following regulatory model of the elevated expression level of ghrelin in a fasting state can be proposed. Under a basal (fed) condition, the expression of ghrelin is maintained at a certain level due to a balance between positive regulation from gastric estrogen and negative regulation from gastric leptin and somatostatin. Under a fasting condition, when fasting suppresses gastric leptin level with no change in gastric estrogen and somatostatin levels, this balance is broken by attenuated negative regulation due to decreased gastric leptin level and finally results in increased ghrelin expression. This model does not, however, exclude the involvement of other factors such as neural control through the vagal nerve system or hormones outside the stomach such as insulin, but an elevated gastric ghrelin expression level in a fasting state is due at least in part to attenuated restraint by decreased gastric leptin level. Further studies are needed to determine the regulatory mechanisms of leptin produced in the stomach in a fasting state. Insulin has been shown to stimulate the secretion of gastric leptin, but this effect is dependent on the integrity of the vagal nerve system (Sobhani et al. 2002). It has been reported that decreased plasma insulin level is involved in suppression of circulating leptin level in a fasting state (Weigle et al. 1997). Another study further revealed that withdrawal of insulin from adipose cells results in a dramatic decrease in leptin mRNA content (Leroy et al. 1996). These findings strongly support the possible contribution of insulin to fasting-induced reduction in gastric leptin level. It would also be interesting to investigate the changes in estrogen, leptin, and somatostatin in relation to ghrelin in the stomach in other physiological states.

In summary, the present study is the first to demonstrate that leptin produced in the stomach directly suppresses ghrelin

Figure 3 Double staining of leptin mRNA-expressing cells and ghrelin-immunopositive (ip) cells (A–C) and leptin- and ghrelin-ip cells (D–F). Both leptin mRNA-expressing cells (A) and leptin-ip cells (D) were found in the lower half of the fundic gland, and no signals were detected by in situ hybridization in the negative control section (sense probe; A, inset). Ghrelin-ip cells were found to be randomly scattered in the gastric mucosa (B and E). Leptin mRNA-expressing cells (C, blue, arrows) and leptin-ip cells (F, red, arrows) were adjacent to ghrelin-ip cells (C, pink, arrowheads and F, green, arrowheads), and some ghrelin-ip cells were closely surrounded by leptin-expressing or leptin-ip cells or in direct contact with them (C and F). Scale bar = 200 μm (A, A inset, B, D and E) and 20 μm (C and F) respectively. MU, mucosa and SL, smooth muscle layer.

Figure 4 RT-PCR of OB-Rα and OB-Rβ mRNA. Both (A) OB-Rα and (B) OB-Rβ mRNAs were expressed in the stomach and isolated cells. Lane 1 (A and B), wide-range DNA ladder; lane 2 (A and B), stomach tissue (gastric fundus); lane 3 (A and B), ghrelin-rich isolated stomach cell fraction; and lane 4 (A and B), negative control (distilled water). Expected sizes for PCR products: 399 bp for OB-Rα and 113 bp for OB-Rβ.
expression in the rat stomach and that elevation of gastric ghrelin expression level in a fasting state is mediated at least in part by decreased gastric leptin level. The findings of the present study provide new evidence on how ghrelin expression is regulated in a fasting state and shed new light on the physiological interaction of ghrelin and leptin in the periphery. These results may have implications in the control of high ghrelin levels in some negative energy balance states, which may directly contribute to increased food intake and adiposity.

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