Gastric estrogen directly induces ghrelin expression and production in the rat stomach

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Abstract

Ghrelin, an endogenous ligand for the GH secretagogue receptor, is predominantly produced in the stomach. Little is known about the regulation mechanism of gastric ghrelin. Here, we report that estrogen synthesized in the stomach induces rat gastric ghrelin gene expression and production. We established a gastric ghrelin cell enrichment method using Percoll centrifugation and then studied the effect of estrogen and/or its antagonist on ghrelin expression and production. Treatment with estrogen for 8 h significantly increased the level of ghrelin expression, and ICI-182 780, an estrogen receptor (ER) antagonist, completely reversed this effect. Reverse transcriptase-PCR analysis clearly showed that ERα and aromatase are expressed in the female rat stomach. Moreover, treatment with an aromatase inhibitor, 4-hydroxyandrostenedione (formestane), significantly decreased the level of ghrelin mRNA expression in minced stomach tissue. In vivo studies revealed that the ghrelin mRNA expression and production did not change in gonadectomized rat 3 weeks after surgery. These results strongly suggest that estrogen produced in the stomach directly induces ghrelin expression and production in both female and male rat stomachs.


Introduction

Ghrelin, a gut–brain peptide, was identified as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), and has been shown to have a unique structure in which n-octanoyl modification at the third serine residue is essential for its biological activity (Kojima et al. 1999). It is known that ghrelin stimulates GH release from anterior pituitary cells (Kojima et al. 1999, Yamazaki et al. 2002, Hashizume et al. 2003, Malagon et al. 2003), and several recent studies have indicated that this effect is mediated via not only the direct, but also the indirect pathways, including the vagal afferent nerve (Date et al. 2002, Sakata et al. 2003). In addition to GH release, ghrelin also stimulates food intake and adiposity in humans and rodents (Tsoph et al. 2000, 2001, Wren et al. 2000, 2001, Asakawa et al. 2001, Shintani et al. 2001), indicating that these physiological effects of ghrelin play an important role in growth regulation and energy homeostasis.

A large amount of acylated bio-active ghrelin, the only peripheral orexigenic peptide identified so far, is present in the stomach (Kojima et al. 1999, Ariyasu et al. 2001, Sakata et al. 2002a), and it has been reported that production and expression of ghrelin are regulated by some physiological conditions and factors; for example, it has been shown that gastric ghrelin mRNA and plasma circulating ghrelin levels increased after fasting and are affected by treatment with leptin, insulin, glucagon, and somatostatin (Toshinai et al. 2001, Bagnasco et al. 2002, Shimada et al. 2003, Chanoine & Wong 2004, Kamegai et al. 2004, Lippl et al. 2004, Sanchez et al. 2004). However, the factor that directly regulates ghrelin gene expression is still not clear. In a previous study, we demonstrated that the number of ghrelin cells and level of plasma ghrelin and gastric ghrelin mRNA transiently increased 3 days after ovariectomy in both 4- and 9-week-old female rats (Matsubara et al. 2004). Moreover, we found that ghrelin-immunopositive (ghrelin-ip) cells express estrogen receptor α (ERα; Matsubara et al. 2004), implying that estrogen plays a role in ghrelin expression.

On the other hand, it has been reported that estrogens are produced in extraovarian tissues, such as the testis (Harada & Yamada 1992, Brodie & Inkster 1993), brain (Harada & Yamada 1992, Lephart 1996), and adipose tissue (Ackerman et al. 1981, Nelson & Bulun 2001). Recently, Ueyama et al. (2002) clearly demonstrated that estrogen synthetase (aromatase) is expressed in gastric parietal cells of the rat stomach. Furthermore, Campbell-Thompson et al. (2001) reported that ERα expression was found in the stomach mucosa of rats, and Singh et al. (1997) also revealed the existence of ER mRNA and protein using Northern blot analysis and enzyme immunoassay in the human stomach mucosa. Taken together, these findings suggest that estrogen produced in the stomach regulates ghrelin gene expression. Therefore, in this study, we first established a method to obtain a ghrelin-rich cell fraction from gastric mucosa and then, using these cells or...
minced stomach, we investigated the effect of gastric estrogen on ghrelin gene expression and production.

Materials and Methods

Animals

Intact adult male and female Wistar rats weighing 270–300 and 200–250 g respectively were used in this study. The rats were maintained under 12 h light:12 h darkness cycle (lights on at 0800 h) and room temperature (23±2 °C) with food and water provided ad libitum. All procedures were performed in accordance with the institutional guidelines for animal care at Saitama University.

Dispersion of rat stomach cells

Isolated stomach cells were prepared by the enzymatic dispersion method. Female rats were sacrificed under ether anesthesia, and 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), 0·6 mM NaHCO₃ (pH 7·4)) for 1–1·5 h. Stomach cells were removed from gastric mucosa using a glass pipette with a diameter of approximately 5 mm and passed through a 102 μm filter and then collected by centrifugation at 1500 r.p.m. 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, 0·6 mM NaHCO₃ (pH 7·4)) and was centrifuged again for 5 min. After centrifugation at 1500 r.p.m. for 5 min, the pellet was collected from the bottom of the tube and then stratified on 40% Percoll (Amersham Biosciences Corp.) in medium B. After centrifugation at 1500 r.p.m. for 5 min, the pellet was collected and immersed in ISOGEN for 5 h. Stomach tissue culture

Female and male rats were killed under deep ether anesthesia and stomachs were quickly removed. The mucosa of the stomach body was minced (approximately 1 mm³) with a sharp razor blade in DMEM. These minced stomach tissues were incubated with serum-free DMEM containing 10^{-5} M formestane (Sigma), an aromatase inhibitor, for 8 h at 37 °C in humidified 95% air and 5% CO₂. After incubation, these small tissues were collected, immersed in ISOGEN, and stored at −80 °C until analysis.

Experiment 3: effect of gonadectomy on gastric ghrelin mRNA level and plasma ghrelin concentration

For the gonadectomy study, ovariectomy and castration (CAST) were performed according to the general methods. All surgical operations were performed under sodium pentobarbital anesthesia (50 mg/kg i.p.). The animals were sacrificed by decapitation at 3 weeks after gonadectomy. Stomachs were collected and immersed in ISOGEN for ghrelin mRNA quantification, and trunk blood was also collected for determination of plasma ghrelin concentrations, and stored at −80 °C until analysis.

Immunocytochemical detection of ghrelin in isolated mucosal cells

Immunocytochemical detection of ghrelin cells using rabbit anti-ghrelin serum (# 603, a kind gift from Dr Kangawa, Department of Biochemistry, National Cardiovascular Centre Research Institute, Suita, Japan) was carried out by the avidin–biotin complex (ABC) method. The production and specificity of the anti-rat ghrelin serum used in this study were previously reported (Hosoda et al. 2000), and it is known that this antiserum recognizes the N-terminal region of rat ghrelin. Immunocytochemical staining was performed basically according to the previously reported procedure (Sakata et al. 2002a). Briefly, isolated stomach cells were fixed with 4% paraformaldehyde in 0·067 M phosphate buffer (PB; pH 7·4) for 30 min. After washing with PBS, they were treated with 0·5% sodium metaperiodate to block endogenous peroxidase for 15 min at room temperature and then incubated with TNBS (1% normal horse serum and 0·4% Triton X-100 in PBS) for 1 h. After washing with PBS, the cells were incubated overnight with anti-ghrelin serum diluted 1:100 000 in TNBS in a humidity chamber. An ABC method was used for immunocytochemistry using a staining kit (Vectastain ABC kit, Vector, Burlingame, CA, USA). All incubations were carried out in a humidity chamber at room temperature.

Double staining for aromatase mRNA and ghrelin

Female and male rats were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused first with PBS, and...
then with 4% paraformaldehyde in 50 mM PB (pH 7·4). Stomachs were quickly removed from the rats and postfixed with 4% paraformaldehyde in 50 mM PB overnight. The tissues were immersed in 30% sucrose in PB overnight and embedded in O.C.T. Tissue-Tek Compound (Sakura Fine-technical Co., Ltd, Tokyo, Japan). Serial cryosections (10 μm thick) were mounted on silane-coated slides (ShinEtsu Chemicals, Tokyo, Japan).

The sections were washed with PBS, treated with 2 μg/ml proteinase K for 30 min at 37 °C, and fixed with 4% paraformaldehyde in 0·067 M PB (pH 7·4). After washing with PBS for 1 min, the sections were incubated with 0·2 M HCl in water and then washed with PBS for 1 min. The sections were treated with 0·1 M triethanolamine–HCl (pH 8·0) for 1 min and 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 aromatase cRNA probes (GenBank accession no. M33986, nucleotides 1093–2059) were synthesized using a labeling kit (Roche Diagnostics GmbH) with SP6 or T7 RNA polymerases. The probes were diluted to 1 ng/μl with hybridization buffer (50% formamide, 3× standard saline citrate (SSC), 0·12 M diethyl pyrocarbonate (DEPC)-treated PB (pH 7·4), 1× Denhardt 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 at 28°C for 16 h at 50°C in a humid chamber. The covers were removed by soaking the slides in 5× SSC and immersing in 2× SSC containing 50% formamide for 30 min. The sections were then treated with TNE (10 mM Tris–HCl (pH 7·6), 500 mM NaCl, 1 mM EDTA (pH 8·0)) for 10 min and next with RNase A (5 μg/ml in TNE) for 30 min at 37 °C. The sections were immersed in TNE for 10 min at 37 °C and washed with 2× SSC for 20 min at 50 °C and then with 0·2× SSC for 20 min twice at 50 °C. The sections were then treated with 5 μg/ml RNase A 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 1:2000 in buffer-1. 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, 50 mM MgCl2) for 3 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), 1 mM EDTA (pH 8·0)). In this study, instead of DEPC-treated water, we used Gengard water (Gradient A10, Millipore, Tokyo, Japan) as RNase-free water. After the aromatase mRNA-expressing cells have been detected, immunohistochemistry for ghrelin was performed. After washing with PBS, the sections were incubated with TNBS for 1 h. After the second wash with PBS, the sections were incubated overnight with anti-ghrelin serum diluted 1:100 000 in TNBS in a humidity chamber. After the third wash, the sections were incubated with Alexa594-conjugated goat anti-rabbit IgG (Invitrogen, Corp.) as a second antibody. The sections were washed with PBS, mounted with 90% glycerol in PBS, and then viewed and photographed under a light microscope (BX60, Olympus, Tokyo, Japan).

**Reverse transcriptase (RT)-PCR for ERα and aromatase mRNA**

Total RNA was extracted from the isolated stomach cells or stomach tissues using ISOGEN according to the manufacturer’s instructions. Trace contamination of DNA was removed by DNase digestion (Promega). cDNA was synthesized from 1 μg total RNA using Superscript III reverse transcriptase (Invitrogen). The following primers were designed to amplify a rat ERα fragment (370 bp; accession no. Y00102): sense primer, ACCCATGGGAACAT-TTCTGGG; antisense primer, CCCTAAGTGATGTCGCT. The following primers were designed to amplify a rat aromatase fragment (493 bp; accession no. M33986): sense primer, GGAATCCATCAAG-CAGCAT; antisense primer, TTCCACCTCGGA-TACTCTG. PCR was performed using HotStar Taq DNA Polymerase (Qiagen GmbH) according to the manufacturer's instructions. Initial template denaturation was programmed for 15 min at 95°C. The cycle profiles were programed as follows: 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 1 min at 72°C (extension). Forty cycles of the profile were run, and PCR products were visualized by 2% agarose gel electrophoresis.

**Quantitative RT-PCR for ghrelin mRNA**

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, CAGGTTCGAGCTTCTTCTGA; antisense primer, GACAGCTGTATGCCCAACA. Real-time quantitative PCR was performed using SYBR Premix Ex Taq (Takara BIO, Shiga, Japan) according to the 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: 5 s at 95°C (denaturation) and 15 s at 60°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 amplon sequence. Amplicon size and specificity were confirmed by melting curve analysis and 2% agarose gel electrophoresis.
Ghrelin C-RIA

Ghrelin concentrations were determined by a double-antibody RIA using rabbit anti-ghrelin serum (# 107, a kind gift from Dr Kangawa). The production and specificity of the anti-rat ghrelin serum used in this study were previously reported (Hosoda et al. 2000), and it is known that this antiserum recognizes the C-terminal region of rat ghrelin. Ghrelin C-RIA was performed basically according to the previously reported procedure (Hosoda et al. 2000). Two hundred microliters RIA-buffer (0·05 M Na2HPO4-12H2O, 0·08 M NaCl, 0·025 M EDTA-2Na, 0·05% NaN3, 0·5% skim milk) and 100 μl rabbit anti-ghrelin serum (1:3000 dilution in RIA buffer) was added to each tube. Then 100 μl 125I-ratGhrelin was added to each assay tube. After incubation for 24 h at 4 °C, 1000 μl goat anti-rabbit IgG serum (1:50 dilution in RIA buffer containing 10% PEG2000) was added to each tube. Then the precipitates were separated by centrifugation (3000 r.p.m., 15 min, 4 °C) and the radioactivities were counted using a gamma-counter (Auto-Gamma Counting Systems; PACKARD Instrument Co., Meriden, CT, USA). Each sample was assayed, and a standard curve was obtained from measurements in duplicate.

Statistical analysis

Statistical analysis was performed using Fisher’s protected least significant difference test with Stat View statistics software (SAS Institute, Cary, NC, USA). P<0·05 was considered statistically significant. Values were given as mean ± S.E.M.

Results

Preparation of ghrelin-rich cells and analysis of ERα and aromatase mRNA expression in isolated stomach cells

To study the direct effect of estrogen on ghrelin cells, we first established a procedure for obtaining a ghrelin cell-rich fraction from dispersed gastric mucosal cells. Immunocytochemical analysis showed that ghrelin-ip cells, which were detected using an antibody recognizing acylated-type ghrelin, were found in the ghrelin cell-rich fraction (Fig. 1). The percentage of ghrelin-ip cells in the primary digested cell fraction (approximately 3–5%) was increased by the Percoll procedure (10–15%). RT-PCR analysis clearly demonstrated that these isolated cell populations as well as whole stomach tissue express ERα and aromatase mRNA (Fig. 2).

Effect of estrogen on isolated female rat stomach cells

Using immunohistochemistry, we previously demonstrated that ERα immunoreactivity colocalized in ghrelin cells (Matsubara et al. 2004). To elucidate the direct effect of estrogen on ghrelin cells, we investigated whether estrogen increases ghrelin mRNA expression and production in isolated stomach cells.

Quantitative real-time RT-PCR showed that ghrelin mRNA levels were significantly increased by estrogen treatment in a dose-dependent manner ($10^{-5}$ and $10^{-7}$ M; Fig. 3A). Moreover, ghrelin concentration in the medium was also elevated by estrogen treatment (Fig. 3B).

Treatment with ER antagonist and an aromatase inhibitor

Pretreatment with ICI-182 780, an ER antagonist, significantly inhibited the effect of ghrelin mRNA production by estrogen in the minced stomach tissue (Fig. 4A). It has been reported that estrogen is produced in gastric parietal cells as well as in the ovary (Ueyama et al. 2002). Moreover, the results of a previous study (Ueyama et al. 2002) and our results demonstrated that estrogen synthetase, aromatase, is present in rat gastric mucosal cells. Therefore, we analyzed ghrelin gene expression in stomach tissue culture after treatment with formestane, an aromatase inhibitor, for 8 h and found that ghrelin mRNA expression level was significantly decreased compared to that in the control group (Fig. 4B). We also found that formestane treatment of minced gastric tissue from male rats significantly suppressed ghrelin mRNA expression (Fig. 4C). No difference was found between β-actin mRNA levels in the aromatase inhibitor and vehicle groups (data not shown).

Stomach ghrelin mRNA expression and plasma ghrelin concentration in gonadectomized rats

Ovariectomized (OVX) female rats were used to study the involvement of ovarian estrogen in gastric ghrelin expression. Stomachs and blood were collected 3 weeks after OVX surgery. Ghrelin mRNA expression and plasma ghrelin concentration in the OVX rats were not different from those in the sham-operated rats (Fig. 5A and B). As this was
found in female rats, ghrelin mRNA expression levels in male rats were not different in sham and CAST groups, and plasma concentration of ghrelin also did not change in the CAST group (Fig. 5C and D).

Localization of ghrelin-immunopositive cells and aromatase mRNA-expressing cells

Many aromatase mRNA-expressing cells detected by in situ hybridization were found in the glandular body of the fundic gland (Fig. 6A). As previously reported, ghrelin-ip cells were scattered throughout the gastric mucosa (Fig. 6B). Aromatase mRNA-expressing cells in both male and female rats were found to be located close to ghrelin cells, and sometimes these cells were found to be in contact with each other (Fig. 6C and D).

Discussion

In this study, using Percoll gradient centrifugation, we successfully established a method for obtaining ghrelin cell-rich fraction (10–15% ghrelin-ip cells). This fraction is useful for evaluation of the direct effects of various stimulatory or inhibitory factors. We found that estrogen treatment significantly stimulated ghrelin mRNA expression and ghrelin production in a dose-dependent manner and that ICI-182 780, a pure ER antagonist, abolished the stimulatory effect of estrogen, indicating that estrogen works on ghrelin gene expression through the ER. In a previous study, we demonstrated that ghrelin cells have an ERα (Matsubara et al. 2004). In addition, Kishimoto et al. (2003) cloned and analyzed the 5′-flanking promoter region of the human ghrelin gene and reported the existence of two half-site estrogen response elements (half ERE). Taken together, our results suggest that estrogen directly acts on and induces ghrelin mRNA expression via the ER.

Ueyama et al. (2002) demonstrated that parietal cells in the gastric mucosa produce and secrete a substantial amount of estrogen and that inhibition of aromatase activity by treatment with formestane resulted in a decrease in the production of gastric estrogen. We hypothesized that estrogen produced in the stomach directly regulates ghrelin gene transcription in a

![Figure 2](https://example.com/figure2.png)

**Figure 2** Reverse transcriptase-PCR analysis of estrogen receptor α (ERα) and aromatase mRNA expression. Both ERα and aromatase mRNA were expressed in the stomach and isolated cells. (1, 4) Whole stomach tissue; (2, 5) ghrelin-rich isolated cell fraction; (3, 6) water.

![Figure 3](https://example.com/figure3.png)

**Figure 3** Effect of 17β-estradiol (E₂) treatment on ghrelin mRNA expression and peptide production in isolated stomach cells. (A) Changes in ghrelin mRNA expression after E₂ treatment. (B) Changes in ghrelin concentration in the media. Both ghrelin mRNA expression and peptide production were significantly increased by E₂ administration in a dose-dependent manner. Values are mean±S.E.M. of four individual dishes. Significant differences compared with control values (P<0.05) are expressed by an asterisk (*).
physiological state, and we demonstrated that aromatase mRNA is expressed in the stomach and that formestane treatment significantly decreased ghrelin mRNA level in vitro. These results indicate that gastric estrogen is the key regulator of ghrelin mRNA expression. On the other hand, ghrelin mRNA expression and production did not change in 3 weeks in gonadectomized rats. The fact that plasma estrogen concentrations are very low in this condition suggests that ghrelin mRNA transcription is regulated by stomach estrogen but not by gonadal estrogen, which is thought to be a source of circulating estrogen. Ueyama et al. (2004) studied the main steroidogenic pathway in rat stomach and showed that the rat stomach is incapable of producing pregnenolone and progesterone, suggesting that stomach estrogen is synthesized from circulating progesterone or testosterone. To our knowledge, the source of supply of estrogen precursor is considered to be the gonads or adrenal gland. Indeed, a previous study showed that plasma progesterone levels in OVX rats were lower than the levels in sham-operated rats (Lu & Judd 1982). However, the present study showed that gastric ghrelin expression did not change in OVX rats, indicating that even a decreased level of plasma estrogen precursor in OVX rats is sufficient for producing a physiologically effective amount of estrogen in the stomach. In addition, it has been shown that the amount of circulating estrogen precursors was much greater than that of estrogen concentrations (Lu & Judd 1982), and a decreased serum concentration of estrogen precursor after gonadectomy (approximately 7 ng/ml) may produce a sufficient amount of estrogen in the stomach (Lu & Judd 1982) because progesterone concentration in a physiological state was shown a small step down (approximately 2 ng/ml) in the portal vein close to the liver compared to that of artery (Ueyama et al. 2004). Taken together, these results suggest that the amount of estrogen precursor in blood as the source of gastric estrogen under physiological conditions is very large. Ueyama et al. (2004) also showed a significant increase in estrogen concentration in the portal vein compared with that in the artery, suggesting that the amount of estrogen produced in the stomach is much greater than that of plasma estrogen concentration. In addition, as mentioned above, two half ERE exist in the human ghrelin gene, and it has been reported that a half-site of the ERE palindrome exhibited lower ER-binding affinity and transcriptional activity (Klinge et al. 2001, Martini & Katzenellenbogen 2001), suggesting that a relatively high concentration of estrogen may be needed to induce gastric ghrelin mRNA expression. Moreover, we revealed that ghrelin cells and aromatase-expressing cells in the gastric mucosa were localized close together, suggesting that ghrelin cells are exposed to estrogen. Therefore, gastric ghrelin cells may be exposed to a higher concentration of gastric estrogen than that of plasma estrogen. Kellokoski et al. (2005) reported that peroral estrogen treatment, but not transdermal estrogen treatment, increased plasma ghrelin levels, suggesting that a direct effect and high levels of estrogen treatment is essential for an increase in stomach ghrelin expression. This may be the reason why gastric ghrelin mRNA expression and plasma

Figure 4 Effects of ER antagonist and aromatase inhibitor on ghrelin mRNA expression. (A) Effect of an ER antagonist, ICI-182 780, on ghrelin mRNA expression in stomach cells isolated from female rats. ICI-182 780 completely abolished the effect of E2 treatment. (B) Effect of formestane, an aromatase inhibitor, on ghrelin mRNA expression in minced stomach tissue from female rats. Treatment with formestane significantly decreased ghrelin mRNA expression in the stomach. (C) Effect of an aromatase inhibitor, formestane, on ghrelin mRNA expression in the minced stomach tissue from male rats. Treatment with formestane significantly decreased ghrelin mRNA expression as was found in tissue from female rats. Values are mean ± S.E.M. of four individual dishes. Significant differences compared with control values (P<0.05) are expressed by an asterisk (*).
Gastric estrogen and ghrelin expression

It has been reported that many physiological states or factors regulate gastric ghrelin expression. Moreover, significant sexual dimorphic differences were found in plasma ghrelin levels in humans and mice (Akamizu et al. 2005, Shaw et al. 2005) and in the numbers of ghrelin-ip and -ex cells in the neonatal rat stomach (Sakata et al. 2002b). Although there is no information on the relationship between ghrelin concentration and gonadectomy, it has been reported that many physiological states or factors regulate gastric ghrelin expression. Moreover, significant sexual dimorphic differences were found in plasma ghrelin levels in humans and mice (Akamizu et al. 2005, Shaw et al. 2005) and in the numbers of ghrelin-ip and -ex cells in the neonatal rat stomach (Sakata et al. 2002b). Although there is no information on the relationship between ghrelin concentration and gonadectomy, it has been reported that many physiological states or factors regulate gastric ghrelin expression.
between gastric estrogen level and physiological conditions, studies on the regulatory mechanisms of estrogen produced in the stomach under various physiological conditions may be essential for understanding ghrelin regulation. In addition to aromatase, it has been reported that other steroidogenic enzymes, 17\(\alpha\)-hydroxylase/17,20-lyase (P450 17\(\alpha\)) and 17\(\beta\)-hydroxysteroid dehydrogenase type III, were detected in the gastric mucosa (Ueyama et al. 2004). Therefore, it would also be interesting to investigate the rate-limiting process of gastric estrogen by these enzymes in various physiological states.

In conclusion, we demonstrated for the first time that estrogen in the stomach upregulates ghrelin mRNA expression in the rat stomach. The results of this study provide a new insight into ghrelin gene regulation and may contribute to the establishment of strategies for controlling plasma or gastric ghrelin levels in some disease cases.

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Figure 6 Double staining of ghrelin-ip and aromatase mRNA-expressing cells. Most aromatase mRNA-expressing cells were found in the glandular body of the fundic gland. (A, inset) No signals were found in the negative control section (sense probe). (B) Ghrelin-ip cells were found sporadically throughout the gastric mucosa. (C) and (D) Localization of aromatase cells (bright field) and ghrelin cells (dark field). Aromatase mRNA-expressing cells (arrows) and ghrelin-ip cells (arrowheads) were localized close together. Several cells were found to be in contact with each other. (C) Female stomach. (D) Male stomach. Scale bar = (A, A inset, B) 100 \(\mu\)m and (C) and (D) 50 \(\mu\)m. MU, mucosa; SL, smooth muscle layer.
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