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Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus

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Abstract

Ghrelin, an acylated peptide originally identified in rat stomach as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), stimulates both food intake and growth hormone (GH) secretion. Ghrelin is predominantly synthesized by a subset of endocrine cells in the oxyntic gland of human and rat stomach. Previous studies using immunohistochemistry have shown that ghrelin is also present in the hypothalamic arcuate nucleus, a region critical for the control of feeding and GH secretion, but its expression pattern in this region and the details of its molecular form has yet to be clarified. In this report, we examined the presence of ghrelin in the arcuate nucleus using reverse-phase liquid chromatography combined with radioimmunoassay (RIA) and immunohistochemistry. Neurons in the arcuate nucleus were observed to react positively to ghrelin antibodies. In addition, we confirmed the existence of ghrelin mRNA expression using the reverse-transcription polymerase chain reaction (RT–PCR). We also observed the colocalization of GHS-R with neuropeptide Y (NPY) and growth-hormone-releasing hormone (GHRH) in the arcuate nucleus. The present study clearly indicates that ghrelin is synthesized in the arcuate nucleus, which will further our understanding of ghrelin's actions in the central nervous system, including feeding behavior and GH secretion.

Keywords: Ghrelin; GHS-R; Arcuate nucleus; HPLC; RIA; Immunohistochemistry

1. Introduction

Ghrelin, a 28-amino acid peptide with an *n*-octanoyl modification that is indispensable for its activity, was originally discovered in human and rat stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) [1]. Currently, ghrelin homologues have been identified in fish, amphibians, birds, and many mammals. Ghrelin is predominantly produced in the endocrine cells of the stomach and is then released into circulation. Gastrectomy in rats decreases plasma ghrelin concentrations by approximately 80%, indicating that the stomach is the main source of circulating ghrelin [2]. When administered either centrally or peripherally, ghrelin stimulates GH secretion, food intake, and body weight gain [1,3–8]. Several groups have demonstrated that intracerebroventricular administration of ghrelin induces food intake by way of neuropeptide Y (NPY) and agouti-related protein (AGRP) produced in the hypothalamic arcuate nucleus [8–10]. Central effects of ghrelin on feeding are also mediated in part by orexin-A and -B produced in the lateral hypothalamus [11]. These findings suggest that ghrelin is also synthesized in some regions of the brain involved in both feeding and GH secretion. We have already shown using immunohistochemistry that ghrelin-producing neurons are present in the arcuate nucleus, a region critical for feeding and GH secretion [1]. Further verification of the presence of ghrelin and its

Abbreviations: AGRP, agouti-related protein; CH_3CN, acetonitrile; GH, growth hormone; GHS-R, growth hormone secretagogue receptor; NPY, neuropeptide Y; RIA, radioimmunoassay; RP-HPLC, reverse-phase high-performance liquid chromatography; RT–PCR, reverse-transcription polymerase chain reaction.

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receptor in the arcuate nucleus would solidify current models of ghrelin's central activity.

In the present study, we analyzed and characterized ghrelin-immunoreactive molecules in the arcuate nucleus by reverse-phase high-performance liquid chromatography (RP-HPLC) combined with radioimmunoassay (RIA). Using immunohistochemistry, we investigated the ghrelin-immunoreactive neurons in the arcuate nucleus. We also examined the expression of ghrelin mRNA in the arcuate nucleus by reverse-transcription polymerase chain reaction (RT–PCR). We also studied the colocalization in neurons of the ghrelin receptor, GHS-R, with NPY and growth-hormone-releasing hormone (GHRH). Using these methods, we demonstrate that ghrelin is synthesized in the arcuate nucleus.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 300–350 g (Charles River Japan, Shiga, Japan) were used in all experiments. Rats, housed individually in plastic cages at constant room temperature in a 12-h light (07:00–19:00)/12-h dark cycle, were given standard laboratory chow and water ad libitum. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. The protocol was approved by the Miyazaki Medical College Animal Care Research Committee.

2.2. Ghrelin radioimmunoassay (RIA)

Acylated ghrelin content was measured by radioimmunoassay (RIA) recognizing *n*-octanoylated ghrelin [12]. To generate anti-ghrelin antisera, synthetic [Cys^{12}]-ghrelin [1–11] peptide was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH; Pierce, Rockford, IL). This antigenic conjugate solution was administered to three New Zealand white rabbits. The anti-rat ghrelin [1–11] antiserum (#G606) specifically recognized *n*-octanoylated ghrelin and did not recognize des-acyl ghrelin. Synthetic rat [Tyr^{29}]-ghrelin [1–28] was radioiodinated using the lactoperoxidase method. The ^{125}I-labeled peptide was purified on a TSK ODS SIL 120A column by RP-HPLC. Diluted samples or standard peptide solutions (100 μl) were incubated for 24 h with 100-μl diluted antiserum (final dilution of anti-ghrelin [1–11] antiserum, 1:620,000). Following addition of the tracer solution (16,000 cpm in 100 μl), mixtures were incubated for 24 h. Samples were assayed in duplicate; all procedures were done at 4 °C. The limit of detection of rat ghrelin [1–28] on the standard RIA curve was 0.5 fmol per tube. The respective intra- and interassay coefficients of variation at 50% binding for ghrelin RIA were 3.5% and 3.2%. The recoveries of rat ghrelin [1–28] (1 ng) and ^{125}I-rat ghrelin [1–28] (5000 cpm) added to the plasma samples extracted using Sep-Pak C-18 cartridges were 92.2±0.4% (S.E.M.) and 88.9±0.6% (S.E.M.), respectively.

2.3. Quantification of ghrelin in arcuate nucleus

Arcuate nuclei were punched out from the brains of 50 male Wistar rats following anesthesia with pentobarbital (Nembutal, Abbot Laboratories, Chicago, IL) after an overnight 12-h fast. These samples were then boiled at 100 °C for 3 min and applied to a Sep-Pak cartridge. The eluates were subjected to ghrelin RIA, as described above. Portions of the Sep-Pak eluates were applied to RP-HPLC on a TSK ODS SIL 120A column (4.6×150 mm, Tosoh, Tokyo, Japan). RP-HPLC was performed for 40 min at 1.0 ml/min with a linear gradient of acetonitrile (CH_3CN; 10–60%) in 0.1% TFA. All HPLC fractions were quantified by ghrelin RIA.

2.4. Preparation of anti-GHS-R serum

A [Cys0]-rat GHS-R [342–364] peptide was synthesized using the Fmoc solid-phase method on a peptide synthesizer (433A, Applied Biosystems, Foster City, CA) and then purified by RP-HPLC. The synthesized peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce; 6 mg) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and used to immunize New Zealand white rabbits by intra- and subcutaneous injection. Animals were boosted every 2 weeks and bled 7 days after each injection. The specificity of the antiserum was confirmed by its immunoreactivity against GHS-R-expressing (CHO-GHSR62 cells) but not control cells.

2.5. Immunohistochemistry

Three male Wistar rats weighing 250–300 g were used for immunohistochemical study. To enhance the immunostaining of GHS-R-expressing neurons, colchicine (100 μg/rat) was injected into the lateral ventricle 30 h before perfusion. Rats were perfused transcardially with 0.1 M phosphate buffer (pH 7.4) and then with 4% paraformaldehyde in 0.1 M phosphate buffer. The hypothalamus was sectioned into 40-μm thick slices at −20 °C using a cryostat and then treated with 0.3% hydrogen peroxide for 1 h to inactivate endogenous peroxidases. The hypothalamic sections were incubated for 2 days at 4 °C, with anti-ghrelin antiserum diluted 1:1000 or with anti-GHS-R antiserum diluted 1:1000. The pituitary sections were incubated for 2 days at 4 °C with anti-GHS-R antiserum diluted 1:1000. All of the sections were stained using the avidin–biotin complex method, as described previously [13]. We subsequently performed double staining for GHS-

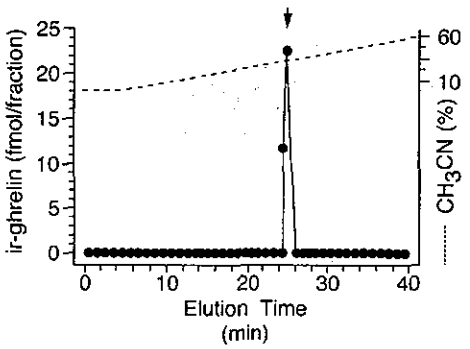

Fig. 1. Representative RP-HPLC profile of ghrelin extracted from rat arcuate nucleus. Wet weight (250 mg) of rat arcuate nucleus was analyzed. Arrow indicates the elution position of *n*-octanoylated ghrelin.

R vs. either NPY or GHRH in some sections of the hypothalamic arcuate nucleus. We also performed double staining for GHS-R vs. GH in some sections of the anterior pituitary. After the sections of arcuate nucleus and anterior pituitary were stained with anti-NPY antiserum (Diasorin, Stillwater, MN; final dilution 1:500) or anti-GHRH antiserum (Chemicon International, Temecula, CA; final dilution 1:1000) and with anti-GH antiserum (NIDDK, National Hormone and Peptide Program, Torrance, CA; final dilution 1:5000), respectively, they were washed with 100 mM glycine–HCl buffer (pH 2.2). Next, the sections of arcuate nucleus and anterior pituitary were stained with anti-GHS-R antiserum using an SG (blue/gray) substrate kit (Vector Laboratories, Burlingame, CA). To test for antiseral specificity, preabsorption tests were done using anti-ghrelin that had been absorbed with 10 μg of ghrelin and GHS-R antiserum that had been absorbed with 10 μg of GHS-R.

2.6. RT–PCR for ghrelin

Total RNA was extracted from the arcuate nuclei of three Wistar rats by the acid guanidinium thiocyanate–phenol–chloroform (AGPC) method [14]. First-strand cDNA was synthesized from 2.5 μg RNA and 7 μM oligo-$(dT)_{18}$ primer with ReverTra Ace-α-™ (Toyobo, Osaka, Japan). The resulting cDNA was subjected to PCR amplification with 2 μM each of the sense and antisense primers and 2.5 units of Pyrobest DNA polymerase (Takara Shuzo, Shiga, Japan). PCR primers for ghrelin were 5′-TTGAGCCCAGAGCACCAGAAA-3′ (sense) and 5′-AGTTGCAGAGGAGGCAGAAGCT-3′ (antisense), corresponding to nucleotide numbers 112–132 and 437–458 (Reference 4, GenBank). PCR was conducted in a reaction volume of 25 μl for 35 cycles comprising denaturation for 5 s at 94°C, annealing for 10 s at 65 °C, and extension for 1 min at 72 °C. The PCR products were electrophoresed on a 2% agarose gel (FMC BioProducts, Rockland, ME).

3. Results

3.1. HPLC characterization of ghrelin-immunoreactive molecules and its content in the arcuate nucleus

RP-HPLC coupled with ghrelin RIA was used to analyze the presence of immunoreactive ghrelin molecules in the arcuate nucleus. A large peak corresponding to immunoreactive ghrelin was eluted at the position of *n*-octanoylated ghrelin in arcuate nucleus tissue extract (Fig. 1). The ghrelin content measured by RIA in the arcuate nucleus was 0.56 pg/mg tissue extract.

3.2. Immunohistochemistry

Neuronal cell bodies immunoreactive for ghrelin were found in the arcuate nucleus (Fig. 2A and B). No immunoreactivity for ghrelin was detected in the arcuate nucleus when normal rabbit serum or antisera preabsorbed with an excess of ghrelin was applied (data not shown). Neurons immunostained with GHS-R antisera were also present in the arcuate nucleus (Fig. 3A). GHS-R immunoreactivity colocalized with that of NPY (Fig. 3B) and GHRH (Fig. 3C) in some neurons in the arcuate nucleus. GHS-R was also abundantly expressed in the pituitary,

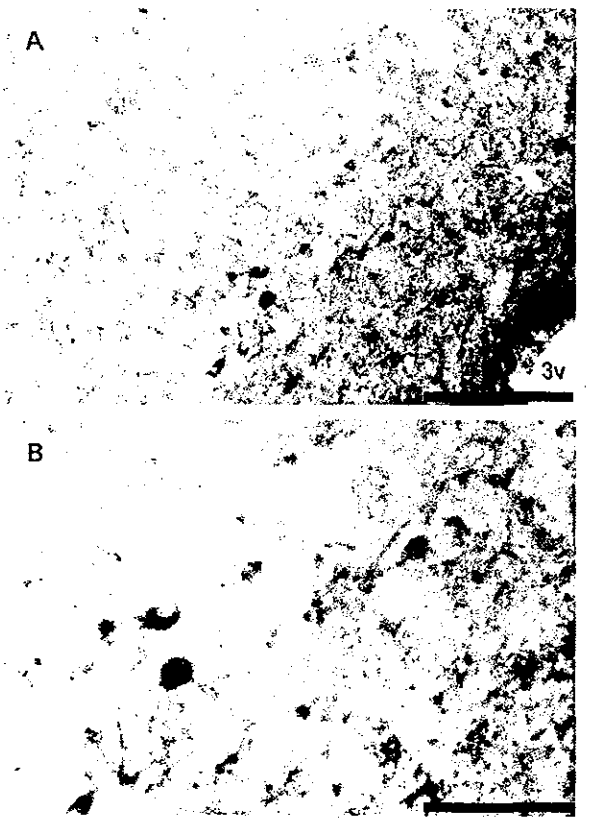

Fig. 2. Immunohistochemical localization of ghrelin in the arcuate nucleus. (A) Ghrelin-immunoreactive neurons in the arcuate nucleus. Bar, 50 μm. (B) High magnification of panel (A). Bar, 25 μm.

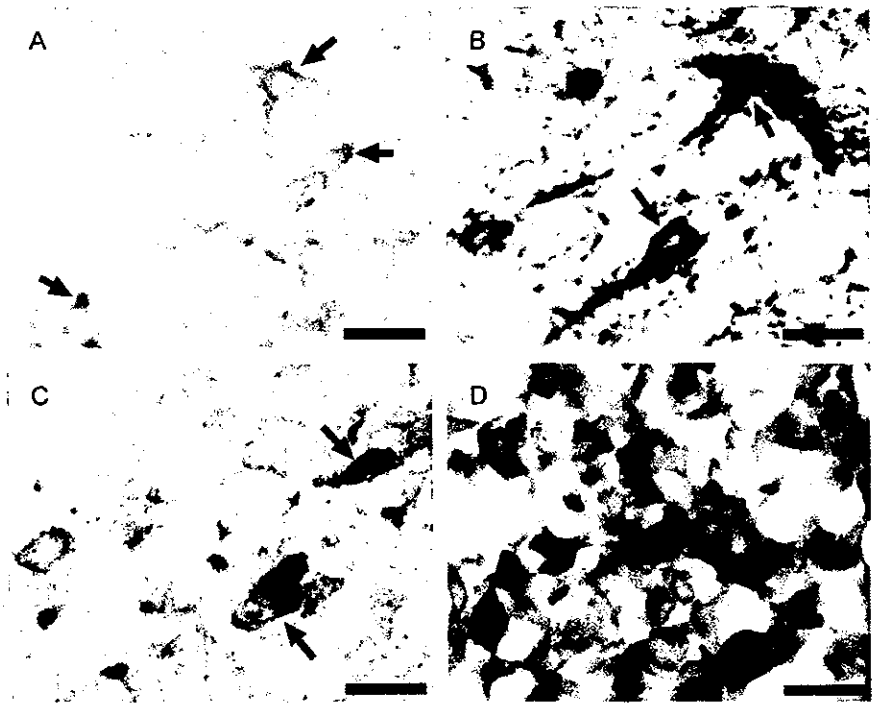

Fig. 3. Immunohistochemical localization of GHS-R in arcuate nucleus and the anterior pituitary. (A) GHS-R-immunoreactive neurons in the arcuate nucleus (arrows). (B) GHS-R-immunoreactive neurons (blue-black) colocalized with NPY neurons (brown; arrows) and (C) GHRH neurons (brown; arrows) in the arcuate nucleus. (D) GHS-R-immunoreactive cells (blue-black) colocalized with GH-producing cells (brown) in the pituitary. Bar, 20 μm in panel (A) to panel (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

where it colocalized with GH-producing cells (Fig. 3D). No GHS-R immunoreactivity was detected in the arcuate nucleus or pituitary gland when normal rabbit serum or antisera preabsorbed with an excess of GHS-R was applied (data not shown).

3.3. RT–PCR amplification of ghrelin transcript

Using ghrelin-specific primers, an RT–PCR product corresponding to the predicted 347-bp size of the ghrelin transcript was present in a rat arcuate nucleus RNA sample (Fig. 4, left panel) but not present when no template was used in the reaction (Fig. 4, right panel).

4. Discussion

Ghrelin is a recently discovered gastrointestinal hormone that appears to play a major role in regulating energy balance [1,3–8]. Ghrelin is predominantly produced in the X/A-like cells of the stomach and may link the gastrointestinal system with hypothalamic control of energy balance, growth, and digestive functions [15]. Central administration of ghrelin has been shown to stimulate GH secretion as well as food intake, fat deposition, and body growth [1,3–8]. Central administration of ghrelin also activates various nuclei in rat hypothalamus, including critical regions for GH and energy homeostasis [8]. These findings strongly suggest the existence of neurons that produce ghrelin and/or its receptor in the brain. In situ hybridization histochemistry has demonstrated expression of the mRNA encoding the ghrelin receptor GHS-R in the pituitary, hypothalamus, pancreas, stomach, and other tissues [16], but immunohistochemical localization of GHS-R has yet to be confirmed.

The present study shows that ghrelin-immunoreactive neurons exist in the ventral portion of the arcuate nucleus, which is consistent with a previous published report [1]. Furthermore, we demonstrated ghrelin immunoreactivity in the arcuate nucleus using RP-HPLC combined with RIA. Ghrelin mRNA expression was also found in the arcuate nucleus. These results imply that the arcuate nucleus may be a major source of ghrelin in the central nervous system of rats. In addition, GHS-R was expressed in NPY- and

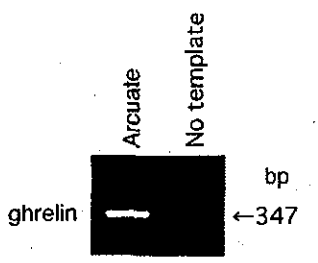

Fig. 4. Representative agarose gel showing the RT–PCR product corresponding to ghrelin mRNA amplified from rat arcuate nucleus.

GHRH-immunoreactive neurons of the rat arcuate nucleus, suggesting that central ghrelin directly affects these neurons which enhance feeding and GH secretion. We also demonstrated that GH-producing cells in the anterior pituitary express GHS-R. This finding indicates that ghrelin also induces GH secretion via an endocrine as well as a neural pathway.

In summary, the present study demonstrated the expression of an active *n*-octanoylated form of ghrelin in the arcuate nucleus. Given the expression of GHS-R in NPY- and GHRH-producing neurons, central ghrelin is expected to play an important role in appetite stimulation, energy homeostasis, and GH secretion. Such identification of ghrelin in the brain will facilitate the investigation of the link between peripheral factors relaying starvation or satiety signals and central ghrelin pathways. Further examination of the distribution of GHS-R-immunoreactive cells throughout the brain and peripheral tissues could lead to discovery of novel functions of ghrelin.

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Review

Ghrelin: a gastric peptide that regulates food intake and energy homeostasis

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Abstract

Ghrelin, an endogenous ligand for the growth-hormone-secretagogue receptor, was isolated from human and rat stomach. It is a 28-amino acid peptide with a posttranslational acyl modification that is indispensable for its activity. In addition to stimulating growth-hormone secretion, food intake, and body weight gain, ghrelin also plays a role in a variety of other systems, including circulation, digestion, and cell proliferation. This review will focus on the discovery, structural characteristics, tissue distribution, and physiological functions of ghrelin, as well as the regulation of its expression and secretion.

Keywords: Ghrelin; GH; GHS; GHS-R

Contents

1. Introduction

Growth hormone (GH), a 191-amino acid polypeptide, is synthesized by somatotroph cells in the anterior pituitary. GH induces protein synthesis and nitrogen retention, and impairs glucose tolerance by antagonizing insulin's action. GH stimulates lipolysis, leading to increased circulating fatty-acid levels, reduced omental fat mass, and enhanced lean body mass. GH promotes sodium, potassium, and water retention, and elevates serum levels of inorganic phosphate. Linear bone growth is mediated by a complex network of hormones and growth factors, including those of insulin-like growth factor-I (IGF-I) [1].

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In the past, GH secretion was thought to be controlled by two peptides: GH-releasing hormone (GHRH) and somatostatin. GHRH is a 44-amino acid hypothalamic peptide that stimulates GH synthesis and release. Somatostatin is synthesized in the medial preoptic area of the hypothalamus and inhibits GH secretion [1]. During the 1970s, when enkephalins were identified as the endogenous ligands for morphine receptors, Bowers [2] found that some opioid-peptide derivatives had weak GH-releasing activity. The discovery that met-enkephalin stimulates GH release from the anterior pituitary in vitro [3] led to the development of small synthetic peptidyl and non-peptidyl molecules called growth-hormone secretagogues (GHSs) [4,5]. In 1996, the GHS receptor (GHS-R), distinct from the GHRH receptor (GHRH-R), was identified. It was then demonstrated that GHSs in fact act through the GHS-R, thus constituting an entirely novel mechanism of regulating GH secretion [6]. The natural endogenous ligand for GHS-R remained unknown until 1999, when ghrelin was identified from rat and human stomach [7].

In this review, the discovery, structural characteristics, tissue distribution, and physiological functions of ghrelin, as well as the regulation of its expression and secretion and future directions of research, are discussed.

2. Structural characteristics and tissue distribution of GHS-R

GHS-R is a G-protein coupled receptor (GPCR); the gene encoding it is located at 3q26.2. The GHS-R exists in two isoforms, 1a and 1b [6,8], the former of which is the functional receptor for ghrelin. GHS-R is distributed among the hypothalamus, pituitary, brain, stomach, intestine, kidney, pancreas, heart, and aorta of rodents and humans [6,9]. The wide distribution of GHS-R may explain the multifaceted roles of ghrelin and GHSs. GHRH was found to stimulate GH release through its binding to the GHRH-R, itself a GPCR, which leads to increased intracellular cAMP. On the other hand, GHSs and ghrelin stimulate GH release by increasing intracellular Ca^{2+} levels through the activation of the phospholipase-IP3 pathway (Fig. 1).

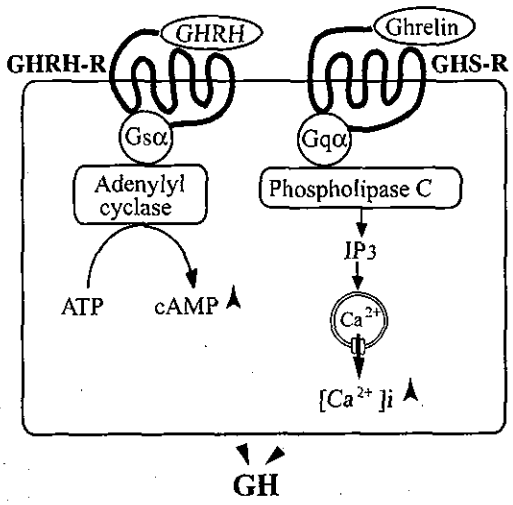

Fig. 1. Pathways of growth-hormone (GH) release from a somatotroph. GH-releasing hormone (GHRH) binds to its receptor (GHRH-R) and increases the intracellular cAMP level. Ghrelin binds to the ghrelin receptor (formerly called the growth-hormone secretagogue receptor, GHS-R) and increases the intracellular Ca^{2+} level ($[Ca^{2+}]i$) through activation of the phospholipase-IP3 pathway.

3. Discovery of ghrelin

After the discovery of GHS-R, a search for its natural endogenous ligand was undertaken using the orphan-receptor strategy. A cultured cell line transfected with the GHS-R was used to identify a natural endogenous ligand from tissue extracts that stimulate GHS-R, as monitored by increases in intracellular Ca^{2+} influx. As it was known that the GHS-R was expressed in the pituitary, hypothalamus, and hippocampus, its ligand was thought to exist also in the brain [10]. However, the weak activation of GHS-R by brain extracts indicated that the endogenous ligand might only be present at low levels in the brain. Several other tissue extracts were screened for activation of GHS-R artificially expressed in CHO cells; unexpectedly, a very strong response was found in stomach-tissue extract. Active peptide was purified from the extract by gel filtration, ion exchange, and reverse-phase chromatography. The purified peptide was named "ghrelin", from the word root "ghre" in Proto-Indo-European languages, which means "grow". Ghrelin also means that this peptide stimulates GH release.

4. Structural characteristics and tissue distribution of ghrelin

Ghrelin is a 28-amino acid peptide modified at its third residue, a serine (Ser3), by a middle-chain fatty acid, *n*-octanoic acid (Fig. 2). The Ser3-acylation is essential for its biological activity, especially the binding and activation of the ghrelin receptor (formerly known as GHS-R) [7]. A complete knockout of the GHS-R gene in mice resulted in animals that do not respond to ghrelin injection with GH release or appetite induction [11]. These results suggest that ghrelin in fact acts through GHS-R, which can thus be called the ghrelin receptor. Acyl modifications have been reported in integral membrane proteins, but not in bioactive secreted peptides. At present, ghrelin has been found in fishes, amphibians, birds, and many mammals. There is no structural homology between ghrelin and peptidyl GHSs. The seven-amino-acid sequence at the N-terminal and the acyl modification of the third residue are well conserved in multiple species [12], suggesting that

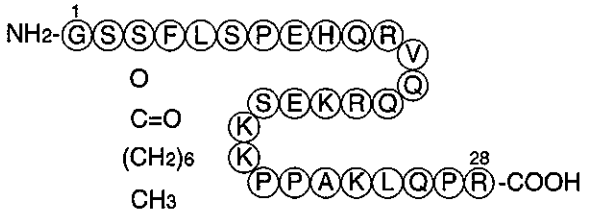

Fig. 2. Amino acid sequence of human ghrelin. The hydroxyl group of the Ser3 residue is esterified by *n*-octanoic acid.

these features are important for ghrelin's biological activities.

The human ghrelin gene is located on chromosome 3p25-26 and consists of four exons and three introns. The structure of the rat ghrelin gene is identical to that of the human gene, but the mouse ghrelin gene comprises five exons and four introns [13]. Analysis of ghrelin's genomic structure has shown that the mature peptide is encoded in exons 1 and 2. The rat and human ghrelin precursors both comprise 117 amino acids [7]. In this 117-residue preproghrelin, the mature ghrelin sequence in the rat follows directly after a 23-residue signal peptide. Two isoforms of ghrelin precursor mRNA are produced in rat stomach cells from the same gene by an alternative splicing mechanism [14]. One encodes the ghrelin precursor, whereas the other codes for a precursor for des-Gln14-ghrelin, which lacks a glutamine (Gln) at position 14. Des-Gln14-ghrelin is another endogenous ligand for the ghrelin receptor; it is a 27-amino acid peptide with an *n*-octanoyl modification at Ser3 [14]. It accounts for around 10% of all immunoreactive ghrelin in the rat stomach and stimulates increases in intracellular Ca^{2+} and GH secretion to the same degree as the 28-amino acid ghrelin [14]. However, the greater abundance of the 28-amino acid isoform makes it the major bioactive form. A structure–function analysis showed that the des-*n*-octanoyl form of ghrelin does not induce changes in intracellular Ca^{2+} of target cells [15]. Identification of the acyl-modifying enzyme and factors involved in its regulation will be invaluable in furthering our understanding of ghrelin's biosynthetic processing in vivo.

Ghrelin mRNA is predominantly expressed in the stomach [7,16,17], but low levels have been reported in the bowel, pituitary gland, kidney, lung, placenta, testis, pancreas, leukocyte, hypothalamus, and trace amounts have been detected by real-time PCR in the adrenal gland, adipocytes, gall bladder, skeletal muscle, myocardium, skin, spleen, liver, ovary, and prostate [9].

In the rat stomach, ghrelin is abundant in the oxyntic mucosa of gastric fundus, and a double-staining study showed that ghrelin-positive cells account for 20% of chromogranin A-immunoreactive endocrine cells [17], indicating that ghrelin is produced in these endocrine cells of the oxyntic gland. Four types of endocrine cells, enterochromaffin-like (ECL) cells, Delta (D) cells, enterochromaffin (EC) cells, and X/A-like cells, have been identified in the oxyntic mucosa by light- and electron-microscopic immunohistochemistry [18]. These four cells are present in the rat oxyntic gland at the following relative percentages: 60–70% ECL cells, 20% X/A-like cells, 2–5%

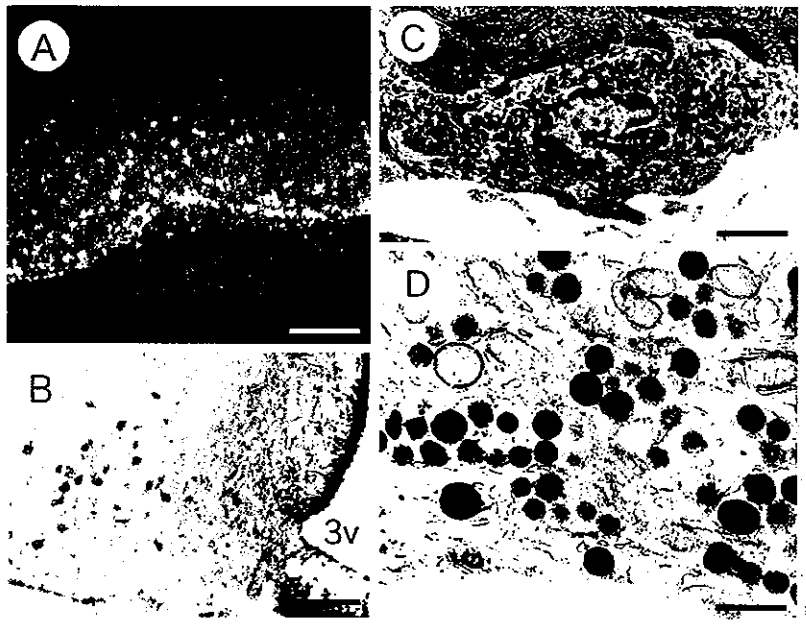

Fig. 3. Ghrelin-expressing cells in rat stomach and brain. (A) Hybridization signals are abundant from the neck to the base of the oxyntic gland by in situ hybridization for ghrelin mRNA. (B) Ghrelin-positive neurons in the ventral–lateral border of the hypothalamic arcuate nucleus. 3v; third ventricle. (C) The ovoid cell has many round, compact, electron-dense granules in its cytoplasm. (D) Higher magnification of C. Granules in the cytoplasm are labeled with immunogold staining for ghrelin. Scale bars: 500 μm in A–B; 2 μm in C; 500 nm in D.

D cells, and 0–2% EC cells. In humans, the oxyntic gland is composed of 30% ECL cells, 20% X/A-like cells, 22% D cells, and 7% EC cells [19]. X/A-like cells are round to ovoid, with compact, electron-dense granules. Although they represent a major endocrine cell population in the oxyntic mucosa of both rats and humans, their products and functions have not previously been characterized. Morphological analysis shows that ghrelin-immunoreactive cells share many characteristics with X/A-like cells, including their localization, abundance, and ultrastructure (Fig. 3A,C,D) [17]. These data indicate that the ghrelin cells are in fact the X/A-like cells. Ghrelin cells in the stomach are the second-most abundant endocrine cells, after ECL cells [17], and are "closed-type" cells that have no physical connection to the stomach's lumen. Electron-microscopic analysis has shown that they are closely associated with the capillary network running through the lamina propria [17]. In the rat gastrointestinal tract, "open-type" ghrelin cells increase in abundance along a gradient from the stomach to the lower gastrointestinal tract. Ghrelin circulates in the plasma, suggesting that ghrelin cells may function in an endocrine fashion [17].

In the central nervous system, ghrelin mRNA and immunoreactive peptide levels are very low. Immunohistochemical analysis showed that ghrelin-positive neuronal cells are localized to a limited region of the hypothalamic arcuate nucleus (ARC) (Fig. 3B) [7], which is known to be rich in GHRH neurons and involved in the regulation of food intake. Ghrelin is synthesized in the hypothalamic ARC [7], but the number of ghrelin-positive neurons is small. Hypothalamic ghrelin might act on the anterior pituitary via the portal vein to induce GH release, or it may also be involved in regulating feeding behavior and energy homeostasis via neural pathways.

5. Physiological functions of ghrelin

5.1. GH secretion

Ghrelin stimulates GH secretion from rat anterior pituitary cells in vitro in a dose-dependent manner [7]. The GH-releasing potency of ghrelin is comparable to that of GHRH in vitro, and is higher than that of GHRH in freely moving animals [7]. Intravenous (IV) administration of ghrelin specifically stimulates GH secretion in rats [7]. Ghrelin also stimulates GH release in vivo upon intracerebroventricular (ICV) administration [20]. IV and ICV injection of ghrelin stimulates GH release in rats in vivo with minimum doses of 1.5 nmol and 10 pmol [21], respectively, demonstrating the higher potency of ICV administration. IV injection of ghrelin also induces GH secretion in healthy humans in a dose-dependent manner, with a minimum dose of 0.2 mg/kg [21,22].

Co-administration of ghrelin and GHRH elicits a significantly synergistic effect on GH secretion [23]. Furthermore, infusion of GHRH in freely moving rats results in a significant increase in the expression of genes encoding ghrelin and its receptor in the pituitary gland [24]. Finally, GHRH antisera and GHRH antagonists attenuate the GH-secreting activity of ghrelin. These results indicate that GHRH may modulate ghrelin's role in GH secretion [24].

The vagus nerve, a cranial nerve that contains both efferent and afferent fibers, is involved in GH secretion from the pituitary gland. The afferent fibers of the vagus nerve form a neuroanatomical link between the alimentary tract and the nucleus of the solitary tract (NTS) in the hindbrain [25]. Approximately 90% of vagus nerve fibers in the subdiaphragma are afferent and are unmyelinated, thin, and capsaicin-sensitive. Some of these afferent endings present within the gastrointestinal mucosa and submucosa are optimally positioned to detect substances in the lumen. Selective chemical and surgical deafferentation of the gastric vagal nerve can attenuate the GH secretion induced by peripheral administration of ghrelin, suggesting that stomach-derived ghrelin physiologically stimulates GH release in vivo via the vagus nerve (Fig. 4) [21].

5.2. Regulation of food intake and energy homeostasis

Ghrelin is a strong orexigenic and adipogenic molecule in mammals [26–28]. The hypothalamus is a center for the control of energy homeostasis [29], and the gastrointestinal tract and brain are closely linked in the regulation of food intake. After ICV administration of ghrelin, Fos protein, a marker of neuronal activation, can be detected in areas where the ghrelin receptor is distributed [26]. Fos is especially highly expressed in the dentate gyrus and hippocampus, where the ghrelin receptor is abundant [26]. Inhibiting ghrelin-receptor expression in the hypothalamus of transgenic rats by expressing anti-sense ghrelin receptor mRNA decreases GH secretion, food intake, and body fat mass [30], suggesting that in the hypothalamus, the ghrelin receptor is important in regulating GH secretion and energy homeostasis.

Ghrelin induces weight gain and adiposity [26–28,31–33]. ICV administration of ghrelin to free-feeding rats during both light and dark phases increases food intake in a dose-dependent manner [26]. Neutralization of ghrelin with anti-ghrelin immunoglobulin G also dose-dependently suppresses starvation-induced feeding [26], suggesting that endogenous ghrelin is a strongly orexigenic. Ghrelin has no effect on locomotor activity or stress-related behavior. ICV administration of ghrelin to genetically GH-deficient rats stimulates food intake, indicating that ghrelin's orexigenic activity is independent of the GH signaling pathway [26]. Continuous ICV administration of ghrelin induces food intake and an increase in fat mass by selectively utilizing carbohydrates, leading to weight gain [27]. IV administration of ghrelin to healthy humans increases energy intake from a buffet lunch by 28±3.9% and also increases the

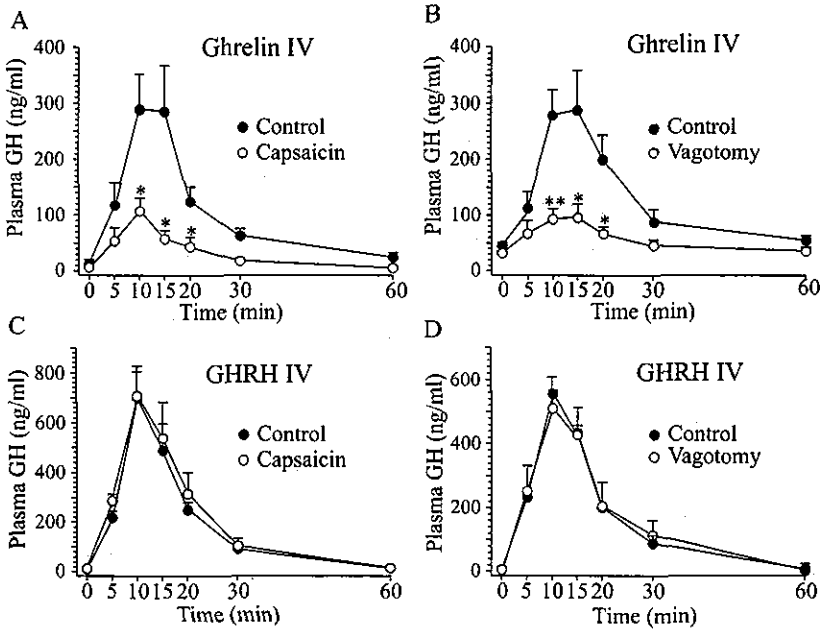

Fig. 4. Effects of gastric afferent nerve activity on ghrelin-induced growth-hormone (GH) secretion. (A) and (B) Plasma GH response (mean±S.E.M.) after intravenous (IV) administration of ghrelin to rats that had undergone perivagal capsaicin application (A) or vagotomy (B). (C) and (D) Plasma GH response after IV administration of GHRH to rats that had undergone perivagal capsaicin application (C) or vagotomy (D). *P<0.01; **P<0.001 vs. control. Redrawn from Ref. [21].

visual analogue score for appetite [32]. These data indicate that ghrelin is a peripheral orexigenic and adipogenic peptide.

Ghrelin-producing neurons are present in the ARC of the hypothalamus, which is also a target for leptin. Leptin is a strong peripheral anorectic protein secreted from adipose tissues [29,34] that reduces food intake and fat mass without altering lean body mass [35]. Microinjection of ghrelin into the ARC in rats induces food intake [36]. Neuropeptide Y (NPY) and agouti-related protein (AgRP), two leptin-responsive orexigenic peptides, are produced together with the leptin receptor in the same neurons of the ARC. ICV administration of ghrelin was shown to induce Fos expression in 39% of NPY/AgRP-expressing neurons [26] and increase both NPY and AgRP mRNA levels in the ARC [24,33,37,38]. Ghrelin-induced feeding is suppressed by receptor antagonists and specific antisera against NPY and AgRP [26,33]. Ghrelin-immunoreactive axonal terminals make direct synaptic contacts with NPY/AgRP-producing neurons in the ARC and orexin-producing neurons in the lateral hypothalamus [39]. Moreover, plasma ghrelin concentration and hypothalamic NPY mRNA level in streptozotocin (STZ)-induced diabetic rats are significantly higher than those in control rats and can be normalized by insulin treatment [40]. Furthermore, hyperphagia in STZ-induced rats is partially reversed by the administration of a ghrelin-receptor antagonist. These results indicate that ghrelin is an upstream regulator of the orexigenic peptides NPY and AgRP, and that it antagonizes leptin's effects on NPY/AgRP-expressing neurons, resulting in an increase in feeding and body weight. By activating the NPY/Y1 receptor-signaling pathway, ghrelin acts as a natural antagonist to leptin [26,29,31,33].

Meal-related metabolites such as monoamines and peptides, as well as mechanical and chemical stimuli, transmit satiety signals to the NTS via vagal afferent fibers or to the hypothalamus via the bloodstream. Ghrelin had been thought to enter the brain across the blood–brain barrier, but intraperitoneal injection of ghrelin to totally vagotomized mice does not stimulate food intake [41]. Blockade of the gastric vagal afferent fibers by vagotomy or perivagal application of caspsaicin abolishes ghrelin-induced feeding and activation of NPY- and GHRH-producing neurons (Fig. 5) [21]. The ghrelin receptor is synthesized in vagal afferent neurons in the nodosa ganglion and transported to their afferent terminals in the stomach. IV administration of ghrelin suppresses firing of the vagal afferents [21]. These findings together suggest that the gastric vagal afferents comprise the major pathway conveying ghrelin's starvation signals to the brain. Conversely, the electrical activity of efferent fibers of the vagus nerve is stimulated by ghrelin administration. These data indicate that the vagus nerve is important for conveying ghrelin's signals not only from the stomach to the central nervous system but also vice versa (Fig. 6).

5.3. Cardiovascular system

Ghrelin plays a number of roles in the cardiovascular system, including protecting against cardiac dysfunction. IV

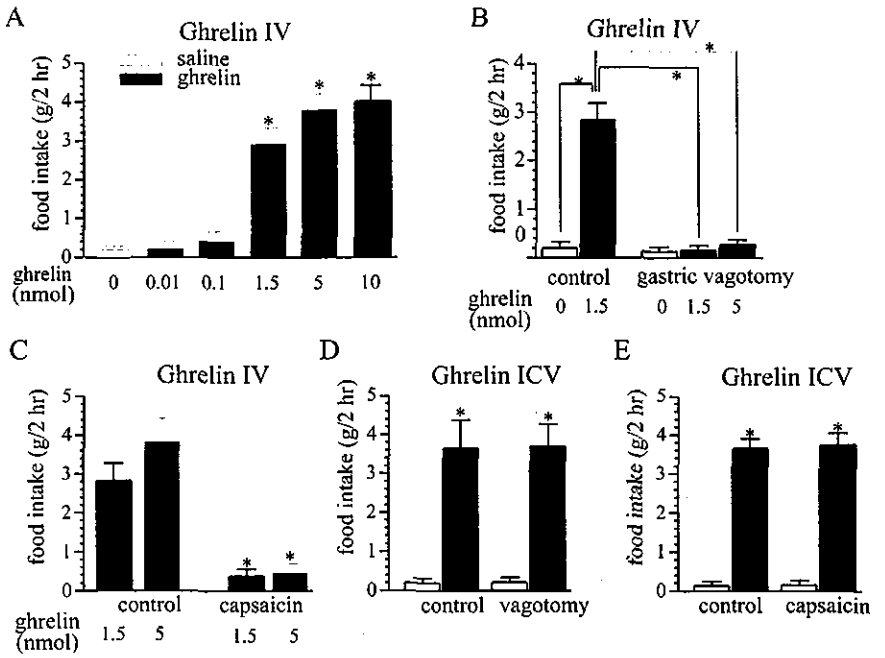

Fig. 5. Effects of gastric afferent nerve activity on ghrelin-induced feeding. (A) Two-hour food intake (mean±S.E.M.) of rats after a single intravenous (IV) administration of ghrelin. (B) and (C) Food intake after IV ghrelin administration in rats treated with vagotomy (B) and perivagal capsaicin (C). (D) and (E) Food intake after intracerebroventricular (ICV) administration of ghrelin in rats treated with gastric branch (D) and perivagal capsaicin (E). *$P<0.01$ vs. control. Redrawn from Ref. [21].

infusion of ghrelin significantly decreases mean arterial pressure without changing heart rate and also increases cardiac output in healthy volunteers [42] and patients with chronic heart failure [43]. Infusion of ghrelin into the forearm artery of normal subjects increases blood flow in a dose-dependent manner. Plasma levels of ghrelin are

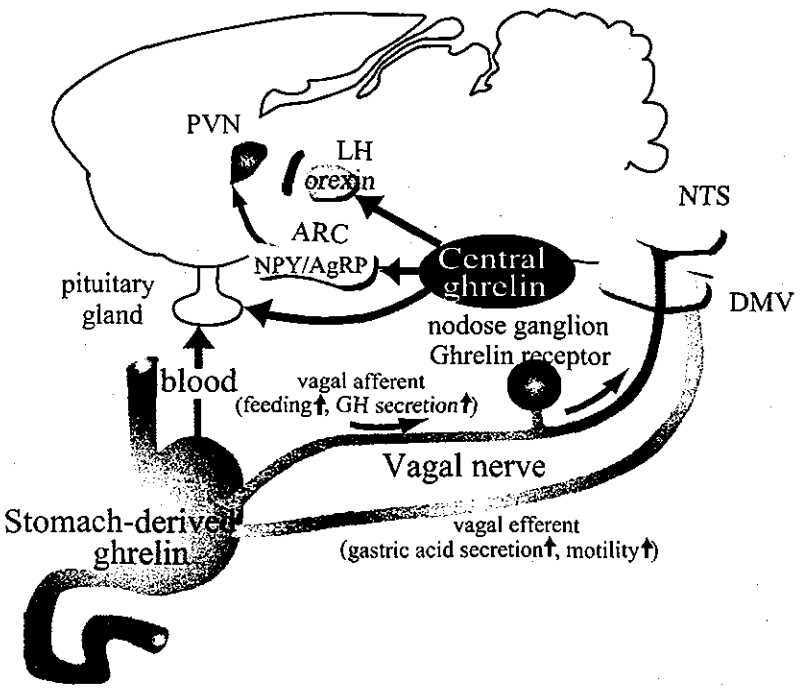

Fig. 6. Pathways of ghrelin's action in the regulation of feeding and growth-hormone (GH) release. Ghrelin's signal for starvation and GH secretion to the brain is conveyed via gastric vagal afferent nerves. AgRP; agouti-related peptide protein, ARC; arcuate nucleus, DMV; dorsomotor nucleus of the vagus, LH; lateral hypothalamus, NPY; neuropeptide Y, NTS; nucleus of solitary tract, PVN; paraventricular nucleus.

significantly higher in the cachectic chronic heart failure patients than in those without cachexia [44]. Chronic administration of ghrelin for 3 weeks was shown to improve left-ventricular dysfunction concomitant with elevations in plasma GH and IGF-I; it also attenuated the development of left ventricular remodeling and cardiac cachexia in rats with chronic heart failure [45].

5.4. Gastrointestinal effects

IV administration of ghrelin to rats stimulates gastric-acid secretion and gastric motility in a dose-dependent manner [24,26]. This effect is cancelled by both vagotomy and administration of atropine, but not by administration of histamine H2-receptor antagonist, suggesting that ghrelin affects gastric function via the vagus nerve [46]. ICV administration of ghrelin in anesthetized rats also stimulates gastric-acid secretion, an effect that is abolished by both vagotomy and pre-treatment with atropine [47]. However, centrally administered GHS and ghrelin inhibit gastric-acid secretion in conscious rats [48]. These conflicting effects might be due to the state of anesthesia of the rodents; however, they both suggest that ghrelin participates in the central regulation of gastric-acid secretion via the vagus nerve and functions as a metabolic signaling molecule.

5.5. Carbohydrate metabolism

Ghrelin modulates circulating glucose levels via GH release, increasing insulin resistance, and stimulating gluconeogenesis [49]. Because some conflicting results have been reported, the effect of ghrelin on insulin secretion remains controversial; IV administration of ghrelin inhibits insulin secretion even on a background of increased glucose levels in human [50]. Similar results were observed in some animal studies; however, some studies have found that ghrelin in fact stimulates insulin secretion.

IV administration of ghrelin in humans induces hyperglycemia by reducing insulin secretion without affecting GH secretion [50]. When coadministrated with a GH receptor antagonist, ghrelin increased insulin resistance significantly in humans [49].

Ghrelin has been shown to co-localize with glucagon in rat islet α cells. It also increases the cytosolic free Ca^{2+} concentration in α cells and stimulates insulin secretion [51]. A recent paper reported that animals lacking transcription factors important for pancreatic β cell development, Nkx2.2 or Pax4, replaced these cells in the islet with ghrelin-producing ones. Normal murine pancreas also contains a small population of ghrelin-producing cells, which have been designated as novel ε cells in the islets. These results indicate that pancreatic β and ε cells may have a common precursor and that a genetic mechanism may underlie the relative influence of insulin and ghrelin in regulating glucose metabolism [52].

6. Regulation of ghrelin expression and secretion

In humans, the fasting plasma level of ghrelin is around 140±14 fmol/ml, as determined by a ghrelin-specific radioimmunoassay that recognizes the C-terminal region of ghrelin [44]. Immunoreactive acylated ghrelin exists in human plasma at a concentration of 5.4±1.4 fmol/ml.

Plasma ghrelin concentration peaks in humans at 2 AM [53,54]. It increases about two-fold before each meal and decreases back to baseline within 1 h after eating, a pattern opposite to that of insulin [54–56]. Plasma ghrelin concentration is not changed by water intake [41,53,55,56]. Administration of ghrelin to healthy subjects induces hunger sensations [49,57]. These results suggest that preprandial elevation of ghrelin is a signal to initiate food intake. Plasma ghrelin level is increased by a low-protein meal and decreased by a high-fat diet [58]. It is profoundly decreased after gastric bypass surgery [59], suggesting that ingested nutrients might be important in inducing ghrelin secretion from the stomach. In general, however, the mechanisms underlying the regulation of ghrelin secretion remain poorly understood.

Plasma ghrelin concentration is lower in obese humans [53,59,60] and in Pima Indians who have obesity and type-2 diabetes mellitus [60]. On the contrary, plasma ghrelin is higher under fasting conditions [27,53,54] and in individuals with anorexia, bulimia nervosa, cachexia [44,61–63], and Prader-Willi syndrome (PWS) [64]. These results, except for the correlation with PWS, suggest that plasma ghrelin level correlates negatively with body mass index [44,53,60,61]. Increased plasma ghrelin levels in PWS patients might be related to their insatiable appetite and obesity. Plasma ghrelin levels are increased in patients with anorexia nervosa but normalize after weight gain [61].

Ghrelin mRNA expression in the gastric fundus is increased after 48 h of fasting and by administration of insulin and leptin [65]. Hyperinsulinemic euglycemic clamp studies in humans have shown that insulin regulates plasma ghrelin levels [66]. Ghrelin mRNA is increased in ob/ob mice, which are deficient in leptin [41], and in untreated STZ-induced diabetic rats [40]. It is decreased in db/db mice, which are deficit in the leptin receptor [41]. The increased ghrelin expression in ob/ob mice is downregulated by leptin administration. These results indicate that leptin is an upstream regulator of gastric ghrelin.

7. Conclusions and perspectives

Ghrelin is mainly produced in the stomach and plays a role in the regulation of energy balance (Fig. 6). Ghrelin has also been reported to have many other effects, including stimulation of adrenocorticotropic hormone, cortisol, prolactin, and aldosterone secretion, cell proliferation, sleep, and thermoregulation [67]. The *n*-octanoyl acid modifica-

tion in ghrelin is unique in the fields of bioactive peptides and endocrinology.

Feeding is regulated not only by the central nervous system but also by peripheral tissues [68]. Ghrelin is the first orexigenic signal to be derived from the stomach and is thought to counter the satiety signal of leptin secreted from adipose tissue. The discovery of ghrelin has raised the novel idea that the stomach plays an important role in GH secretion and feeding regulation, providing a multitude of directions for clinical and basic research on GH and feeding.

Clinical applications of ghrelin in humans are already been tested. Ghrelin might be a useful tool for the diagnosis of GH deficiency and treatment of chronic heart failure, cachexia, feeding disorders, or somatopause. A ghrelin antagonist for the treatment of obesity may be developed in the future. Conversely, ghrelin-resistant conditions (hyperghrelinemia) may be amenable to treatment with ghrelin or GHS, as insulin treatment is used for insulin resistance syndromes. Further research on ghrelin will contribute to our understanding of physiological and pathophysiological feeding mechanisms and provide a novel therapeutic tool for patients with altered nutritional homeostasis.

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Impaired Production of Gastric Ghrelin in Chronic Gastritis Associated with *Helicobacter pylori*

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Ghrelin is primarily secreted from the stomach and has been implicated in the coordination of eating behavior and weight regulation. The effects of *Helicobacter pylori* infection on plasma ghrelin concentration and gastric ghrelin production still have not been well known. We determined plasma ghrelin concentration in a total of 160 consecutive individuals with normal body mass index including 110 *H. pylori*-infected and 50 *H. pylori*-negative subjects. The expression levels of ghrelin mRNA and ghrelin-producing cells in the gastric mucosa were quantified with real-time quantitative RT-PCR and immunohistochemistry, respectively. The severity of gastric atrophy was evaluated by serum pepsinogen concentrations. Plasma ghrelin concentration, gastric ghrelin mRNA, and ghrelin-positive cell numbers in gastric mucosa were significantly lower in *H. pylori*-infected subjects. The decrease in plasma ghrelin concentration in *H. pylori*-positive subjects was accompanied by an attenuation of ghrelin mRNA expression and a reduction of ghrelin-positive cell numbers in the gastric mucosa. Moreover, lower serum pepsinogen I concentrations and I/II ratio were significantly associated with lower plasma ghrelin concentrations in *H. pylori*-positive subjects. These findings suggest that impaired gastric ghrelin production in association with atrophic gastritis induced by *H. pylori* infection accounts for the decrease in plasma ghrelin concentration. (*J Clin Endocrinol Metab* 90: 10–16, 2005)

GHRELIN, A 28-AMINO acid peptide isolated from rat and human stomach possesses strong GH-releasing activity and plays central as well as peripheral roles in food intake, gastric motility, and acid secretion (1, 2). Ghrelin has been shown to evoke weight gain by actions in the hypothalamus (3). Plasma ghrelin concentrations rise before meals and fall after meals. This peptide also contributes to the regulation of both somatic growth and adipose tissue mass and is therefore a short-term, meal-related orexigen as well as a long-term regulator of body weight (4–6). Circulating ghrelin concentrations in newborns are not associated with gender, body weight, or hormonal parameter (7). In children and adults, however, plasma ghrelin concentrations are lower in obese subjects, compared with those with normal body weight and lean subjects (8, 9). The decrease of plasma ghrelin concentrations appears to compensate for the positive energy balance in obese individuals (9). The majority of circulating ghrelin is produced in the mammalian gastric mucosa by enteroendocrine cells/oxyntic glands, probably the X/A-like cells (10). Thus, there exists the possibility that chronic persistent damage of the gastric mucosa, such as chronic gastritis, might affect ghrelin production, leading to changes in food intake and body weight. *Helicobacter pylori* is a Gram-negative bacterium that colonizes the stomach. *H. pylori* infection is involved in the pathogenesis of gastritis, gastric and duodenal ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma (11–13). More than 50% of the adult population are infected with *H. pylori* worldwide (14, 15). *H. pylori* infection first leads to atrophic gastritis and intestinal metaplasia, which may further lead to dysplasia and gastric carcinoma (16). Thus, it is an intriguing question whether *H. pylori* infection affects gastric ghrelin production and consequently alters plasma ghrelin concentration.

In this respect, Nwokolo *et al.* (17) reported that plasma ghrelin concentrations increased after the eradication of *H. pylori*. On the contrary, Gokcel *et al.* (18) reported that *H. pylori* infection has no effect on plasma ghrelin levels. Thus, the relationship between *H. pylori* infection and plasma ghrelin concentrations is still controversial, prompting us to further assess the effects of *H. pylori* infection on plasma ghrelin concentrations. Because previous studies examined serum ghrelin concentrations without investigating gastric ghrelin production (17, 18), the direct relationship between *H. pylori* infection and gastric ghrelin production, which could influence plasma ghrelin concentrations, is still to be demonstrated. We thus conducted this study to investigate the association of *H. pylori* infection with both ghrelin mRNA and protein production in the stomach, concomitantly examining plasma ghrelin concentrations. To this end, we applied real-time quantitative RT-PCR and immunohistochemistry of endoscopic biopsy specimens. Moreover, because body weight is an important factor that determines plasma ghrelin concentrations, only individuals with normal body mass index (BMI) were enrolled in this study. We report here that *H. pylori* infection is associated with lower gastric ghrelin mRNA and protein as well as serum ghrelin concentrations.

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Abbreviations: BMI, Body mass index; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Subjects and Methods

Participants

We enrolled 160 consecutive asymptomatic men with normal BMI undergoing gastric cancer surveillance in Tochigi, Japan. They were divided into two groups (110 *H. pylori*-positive subjects and 50 *H. pylori*-negative controls) according to the presence or absence of *H. pylori* in the gastric mucosa evaluated by the bacterial culture and histological examination. The percentage of the *H. pylori*-positive subjects was similar to that of the same generation in Japan. *H. pylori*-positive subjects included 23 patients with chronic gastritis alone, 50 patients with chronic gastritis and gastric ulcer, 27 patients with chronic gastritis and duodenal ulcer, eight patients with chronic gastritis and gastric polyp, and two patients with chronic gastritis and gastric adenoma. Neither atrophic changes nor any other abnormal findings were observed in the 50 controls without *H. pylori* infection by endoscopic or histological examination. Their characteristics were similar to those of the *H. pylori*-positive subjects in age, gender, BMI, serum cholesterol, and fasting blood sugar as shown in Table 1. No subjects had evidence of a cachectic state such as advanced cancer, thyroid disease, liver disease, or infection. Subjects with diabetes mellitus or renal dysfunction (serum creatinine ≥1.5 mg/dl) were excluded. None of the 160 individuals recruited had a history of eradication therapy for *H. pylori* infection or received any antibiotic treatment during the study. Written informed consents were obtained from all participants in accordance with the Declaration of Helsinki and its later revision. This study was approved by the Ethics Committee of the Jichi Medical School. Subjects with *H. pylori* infection were classified into three groups according to the fasting levels of plasma ghrelin as shown in Table 2: low ghrelin group (<70 fmol/ml; n = 34), middle ghrelin group (70–150 fmol/ml; n = 36), and high ghrelin group (>150 fmol/ml; n = 40). Age, BMI, serum lipid data, and fasting blood sugar were similar in those three groups.

Specimens

Five adjacent biopsy specimens from the greater curvatures at the midcorpus of the stomach as well as five from the antrum were obtained endoscopically from all subjects. One biopsy specimen from the corpus of the stomach and one from the antrum were cultured individually to evaluate for the presence of *H. pylori* infection. Three biopsy specimens from the corpus and three from the antrum were immediately snap frozen and stored in liquid nitrogen for later use. The remaining corpus and antral specimens were fixed and stained with hematoxylin and eosin, Giemsa, and antighrelin antibody. Histological assessments were performed by a single observer (H.Os.). *H. pylori* infection was evaluated by the bacterial culture and histological examination.

Hormone assays

Blood was drawn into chilled tubes containing EDTA-2Na (1 mg/ml) and aprotinin (500 U/ml), and plasma was harvested after immediate centrifugation and stored at −30 C until assay. Plasma ghrelin levels were measured by a RIA developed in our laboratory. In brief, antiserum against the C-terminal region of human ghrelin was raised in New Zealand white rabbits that were immunized against synthetic human ghrelin (13–28). Human Tyr^0-ghrelin (position13–28) was radioiodinated by the lactoperoxidase method for use in the assay. Inter- and intraassay variation was less than 8 and 6%, respectively. The limit of detection of this assay is 12 fmol per tuve of human ghrelin. We described previously the properties of the antiserum for ghrelin used in this study (9, 10).

TABLE 1. Characteristics of *H. pylori*-negative and -positive subjects

	H. pylori-negative (n = 50)	*H. pylori*-positive (n = 110)	*P*
Age (yr)	47.4 ± 1.1	48.8 ± 0.6	0.58
BMI (kg/m^2)	22.5 ± 0.4	21.9 ± 0.2	0.21
T-Chol (mg/dl)	196 ± 4	194 ± 3	0.71
HDL-C (mg/dl)	61.4 ± 2.7	60.7 ± 1.2	0.79
Triglyceride (mg/dl)	99 ± 6	93 ± 4	0.34
FBS (mg/dl)	96 ± 2	94 ± 1	0.18

Data are the mean (± SE). T-Chol, Total cholesterol; HDL-C, high-density lipoprotein cholesterol; FBS, fasting blood sugar.

TABLE 2. Characteristics of low, middle, and high ghrelin groups in *H. pylori*-positive subjects

	Low (n = 34)	Middle (n = 36)	High (n = 40)
Age (yr)	50.1 ± 1.1	48.3 ± 0.9	48.0 ± 1.1
BMI (kg/m^2)	21.9 ± 0.3	21.9 ± 0.3	21.8 ± 0.2
T-Chol (mg/dl)	196 ± 6	186 ± 5	198 ± 5
HDL-C (mg/dl)	59.8 ± 2.6	61.1 ± 2.1	61.1 ± 1.8
Triglyceride (mg/dl)	96 ± 7	88 ± 7	94 ± 7
FBS (mg/dl)	95 ± 2	93 ± 1	93 ± 2

Data are the mean (± SE). T-Chol, Total cholesterol; HDL-C, high-density lipoprotein cholesterol; FBS, fasting blood sugar.

Immunohistochemistry

We generated antighrelin antiserum as described previously (10). Briefly, synthetic [Cys^{12}]rat-ghrelin (position 13–28) (4 mg) was conjugated with maleimide-activated mariculture keyhole limpet hemocyanin (6 mg; Pierce Chemical Co., Rockford, IL). The antigenic conjugate solution was administered to a New Zealand White rabbit. The antirat ghrelin antiserum (G107) specifically recognizes ghrelin and has 100% cross-reactivity with human ghrelin in immunohistochemistry (10).

Paraffin-embedded sections of the biopsy samples taken from the greater curvature of the stomach were deparaffinized in xylene, immersed in citrate buffer [10 mM (pH 6.0)], heated at 120 C for 20 min in an autoclave, and left at room temperature for 60 min. After incubation with blocking reagent (Dako Japan, Kyoto, Japan) for 10 min, individual sections were incubated with antiserum for ghrelin (diluted to 1:500) in a moist chamber at 4 C overnight. Normal mouse IgG_1 was used for control studies. The slides were then washed five times with PBS and incubated with dextran polymer system/peroxidase (EnVision+; Dako Japan) at 37 C for 60 min. The chromogen was developed by incubating the slides with diaminobenzidine solution for 3 min. The slides were counterstained with hematoxylin.

RNA extraction

Total RNA was isolated from the biopsy specimen with ISOGEN (Nippon Gene, Tokyo, Japan). Two microgram of total RNA from each sample was reverse transcribed by using random nanomers and reverse transcriptase (TOYOBO, Osaka, Japan) according to the manufacturer's protocol.

Real-time quantitative RT-PCR

The expression level of ghrelin mRNA was evaluated by using a real-time quantitative RT-PCR method with an ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, CA). The sense primer for ghrelin was 5′-GGCAGGCTCCAGCTTCCT-3′ and the antisense primer was 5′-TGGCTTCTTCGACTCCTTTCTC-3′. The reaction mixture was prepared according to the manufacturer's protocol using TaqMan PCR kits (PE Applied Biosystems). The reactions also contained target hybridization ghrelin probe labeled with a reporter fluorescent dye, 6-carboxyfluorescein, at the 5′ end (5′-AGCCCTGAACACCA-GAGA-3′). The thermal cycling conditions included 50 C for 2 min and 95 C for 10 min, followed by 15 sec of denature at 95 C and 1 min of annealing/extension at 60 C for 40 cycles.

The quantitative amplification and expected sigmoid curve of PCR were obtained. The PCR products were also examined by 2% agarose gel electrophoresis to confirm successful amplification of the expected size of the gene. As a control, the mRNA was also subjected to real-time quantitative RT-PCR for measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using TaqMan GAPDH control reagents (PE Applied Biosystems). For relative quantification of the ghrelin expression, calibration curves were constructed using the mRNA obtained from normal gastric mucosa without *H. pylori* infection. mRNA for GAPDH was used as an endogenous control. The levels of ghrelin mRNA were calculated from the ratio of ghrelin mRNA level to GAPDH

mRNA level and shown as the 1000 times data of the mean ratio of three corpus samples or three antral samples. Ghrelin mRNA expression levels in the gastric mucosa were compared between *H. pylori*-positive and -negative subjects or among groups with different levels of plasma ghrelin.

Counting the ghrelin-producing cells

The slides after the immunostaining were viewed at ×100 magnification and digitized with a digital HD microscope (VH-7000, Keyence, Tokyo, Japan). The immunoreactive cells in the gastric mucosa were counted and presented as the number of positive cells per branch of oxyntic gland because they were localized in the lower half of fundic epithelial glands. The numbers of ghrelin-expressing cells in the gastric mucosa were also compared between *H. pylori*-positive and -negative subjects or among groups with different levels of plasma ghrelin.

Statistical analyses

The level of ghrelin mRNA expression was expressed as the median (first quartile to third quartile). The number of the immunoreactive cells and clinical data were presented as mean (± SE). The Mann-Whitney *U* test was used to compare ghrelin mRNA levels in gastric mucosa from *H. pylori*-positive and -negative subjects. Two-tailed unpaired *t* test was used to compare the numbers of the immunoreactive cells in the gastric mucosa between *H. pylori*-positive and -negative subjects as well as clinical data between two groups. An ANOVA based on Fisher's protected least significant difference test was used for the assessment of the relationship between the plasma ghrelin levels and either the serum pepsinogen levels or the number of the immunoreactive cells in gastric mucosa among *H. pylori*-positive subjects. Differences at $P < 0.05$ were considered significant.

Results

Plasma ghrelin concentrations are lower in H. pylori-positive subjects

Because the relationship between *H. pylori* infection and plasma ghrelin concentration remains controversial, we first attempted to assess the effect of *H. pylori* infection on the plasma ghrelin concentrations. To this end, we compared plasma ghrelin concentrations between *H. pylori*-positive and -negative subjects. The effect of body weight level was minimized because BMIs were similar in both groups (Table 1). As shown in Fig. 1, plasma ghrelin concentrations were significantly lower in *H. pylori*-positive subjects than in *H. pylori*-negative controls. A similar difference was also significantly observed between patients with chronic gastritis alone and *H. pylori*-negative controls (data not shown). Because the majority of ghrelin is known to be produced in the stomach, we hypothesized that *H. pylori* infection may at-

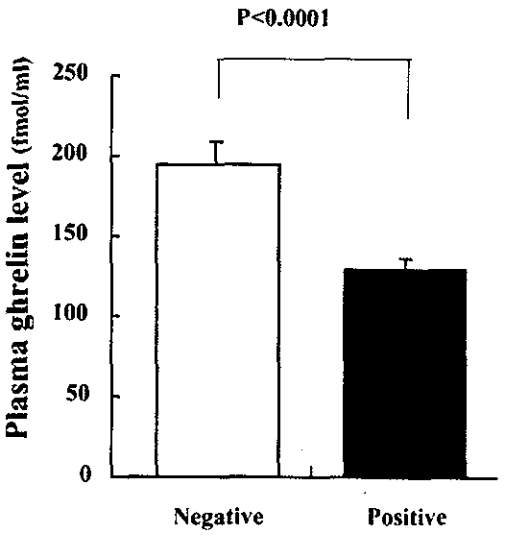

FIG. 1. Comparison of plasma ghrelin levels between *H. pylori*-positive and -negative subjects. Plasma ghrelin levels in *H. pylori*-positive subjects were significantly lower than *H. pylori*-negative controls (mean ± SE, 128 ± 8 *vs.* 194 ± 15 fmol/ml, $P < 0.0001$ by unpaired two-tailed *t* test).

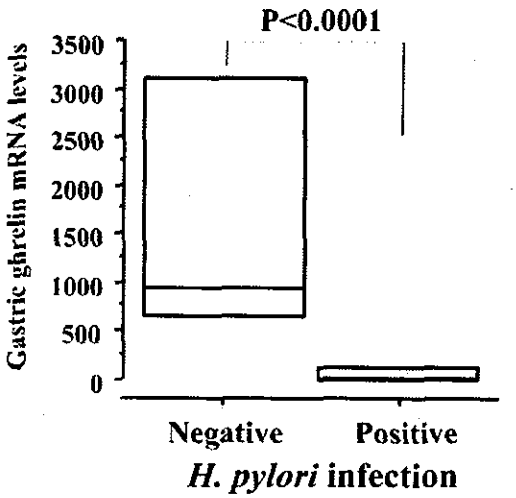

FIG. 2. Comparison of gastric ghrelin mRNA expression levels between *H. pylori*-positive and -negative subjects. There was a remarkable decrease in the expression levels of ghrelin mRNA in the gastric mucosa from *H. pylori*-positive subjects than *H. pylori*-negative subjects (median [first to third quartile]; 22 (8–91): n = 110; and 1011 (716–3200): n = 50; $P < 0.0001$ by Mann-Whitney *U* test).

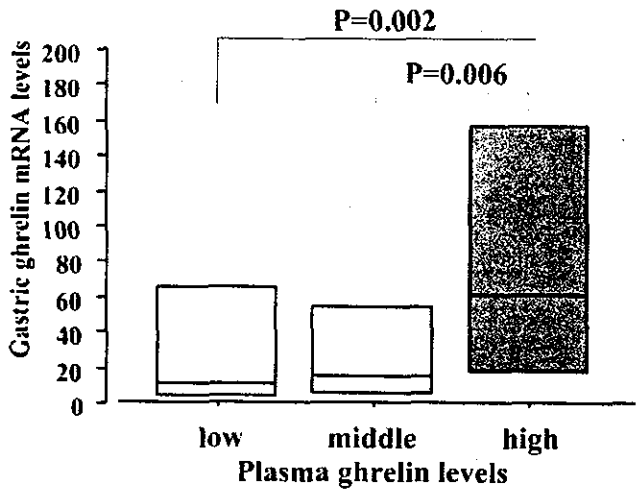

FIG. 3. Comparison of plasma ghrelin levels and gastric ghrelin mRNA levels in *H. pylori*-infected subjects. Values are expressed as the median (first to third quartile). There was a correlation between plasma ghrelin concentrations and gastric ghrelin mRNA levels with a correlation coefficient value of 0.36 ($P = 0.0001$). Gastric ghrelin mRNA levels in low 12 (4–66) and middle 16 (6–55) group were significantly lower than high ghrelin group 61 (17–157) ($P = 0.002$ and $P = 0.006$ by Mann-Whitney *U* test, respectively).

tenuate ghrelin production in the stomach and may consequently reduce plasma ghrelin concentrations in *H. pylori*-positive subjects. To examine this hypothesis, we next examined the specific changes of gastric ghrelin production in association with *H. pylori* infection.

Ghrelin mRNA in gastric mucosa is lower in H. pylori-positive subjects

In an effort to examine the effects of *H. pylori* infection on ghrelin production in the gastric mucosa, we compared gastric ghrelin mRNA expression levels between *H. pylori*-positive and -negative subjects using real-time quantitative RT-PCR using corpus mucosa because gastric ghrelin is predominantly produced in the corpus rather than in the antrum (10). As shown in Fig. 2, gastric ghrelin mRNA levels of corpus mucosa were significantly lower in *H. pylori*-positive patients than *H. pylori*-negative controls. A similar difference was also significantly observed between patients with chronic gastritis alone and *H. pylori*-negative controls (data not shown). These results suggest that the expression of ghrelin mRNA in the gastric mucosa is markedly decreased in association with *H. pylori* infection. It is important to note that the average of gastric ghrelin mRNA expression levels in *H. pylori*-positive subjects was less than one 45th of that in *H. pylori*-negative controls. Moreover, as shown in Fig. 3, plasma ghrelin concentrations were in parallel with the ghrelin mRNA expression levels in *H. pylori*-positive subjects. Taken together, these results suggest that the attenuation of the ghrelin production in the gastric mucosa accounts for the decrease in the plasma ghrelin concentrations in *H. pylori*-positive individuals.

Ghrelin-producing cells in the gastric mucosa are lower in H. pylori-positive subjects

As an independent test to examine the effect of *H. pylori* infection on gastric ghrelin production and its relation with plasma ghrelin concentrations, we next investigated the numbers of ghrelin-producing cells of the corpus mucosa in *H. pylori*-positive and -negative subjects. For this purpose, biopsy samples taken from gastric mucosa were immunostained using an antighrelin polyclonal antibody. Immunoreactive cells were seen in the lower half of fundic epithelial glands as described previously (10). No immunoreactivity was detected in the tissue when control serum was used for staining (data not shown). Immunoreactivity was concentrated in the basal cytoplasm of the positive cells as shown in Fig. 4, A and B. As shown in Fig. 4C, the number of ghrelin-positive cells in the gastric mucosa of *H. pylori*-positive individuals was significantly lower than those of *H. pylori*-negative individuals. Furthermore, the numbers of ghrelin-positive cells in the gastric mucosa fell significantly in accompaniment to the decrease in plasma ghrelin concentrations in *H. pylori*-positive subjects (Fig. 5). These results reinforce that the attenuation of the gastric ghrelin production caused by *H. pylori* infection accounts for the decrease in the plasma ghrelin concentrations in *H. pylori*-positive individuals.

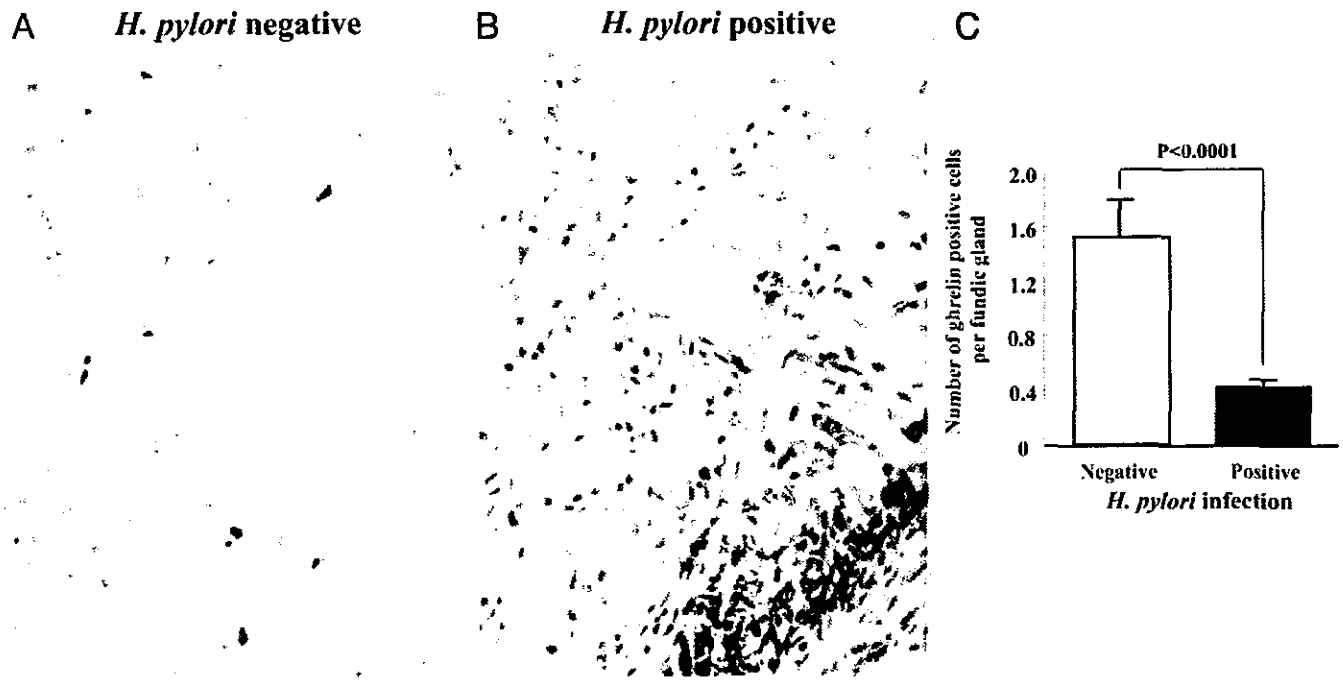

FIG. 4. Immunostaining of ghrelin in the fundic glands. A, Normal epithelium without *H. pylori* infection. B, Inflammatory gastric mucosa with *H. pylori* infection. Ghrelin-producing cells were abundant in the mucosa from *H. pylori*-negative subject, whereas they were seldom observed the mucosa from *H. pylori*-positive subject (magnification, ×200). C, Numbers of the immunoreactive cells in *H. pylori*-positive and -negative subjects. Immunoreactive cells in the gastric mucosa were counted and presented as the number of positive cells per branch of oxyntic glands. Values are expressed as the mean ± SE. The numbers of the immunoreactive cells in the gastric mucosa were significantly lower in the *H. pylori*-positive subjects than the *H. pylori*-negative controls ($P < 0.0001$ by unpaired two-tailed t test).

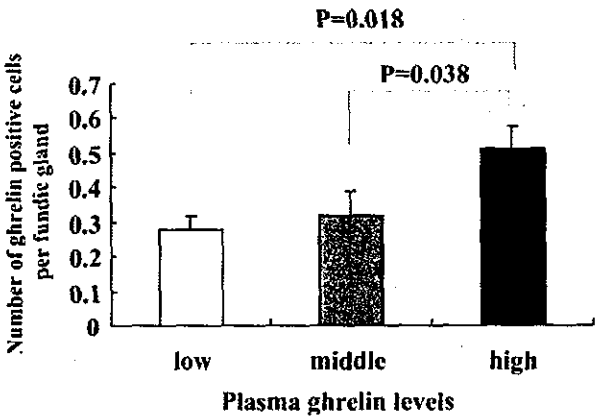

FIG. 5. Comparison of the frequencies of the immunoreactive cells in *H. pylori*-positive subjects among different levels of plasma ghrelin groups. Values are expressed as the mean ± SE. There was a correlation between plasma ghrelin concentrations and the frequencies of the immunoreactive cells with a correlation coefficient value of 0.41 ($P < 0.0001$). The numbers of the positive cells were significantly lower in the low and middle ghrelin groups than those in the high ghrelin group (low *vs.* high: $P = 0.018$; middle *vs.* high: $P = 0.038$ by variance (ANOVA) based on Fisher's protected least significant difference test).

Plasma ghrelin concentrations are associated with serum pepsinogen concentrations in H. pylori-positive subjects

In the last sets of examinations, we further attempted to demonstrate the association between *H. pylori* infection and plasma ghrelin concentrations. Because *H. pylori* infection first induces gastric atrophy in its pathological course, we compared plasma ghrelin concentration with serum pepsinogen concentrations in *H. pylori*-positive patients. Pepsinogen I and pepsinogen II differ in their location in the stomach. Both are located in the chief and mucous neck cells of the oxyntic gland mucosa in the gastric corpus, but only pepsinogen II is present in the gastric antrum. A pepsinogen I to II ratio less than 3 is considered to be a reliable marker for severe atrophic gastritis (19, 20). Therefore, the plasma ghrelin levels in *H. pylori*-positive patients were compared with serum pepsinogen concentrations and serum pepsinogen I to II ratios. As shown in Fig. 6, serum levels of pepsinogen I and the ratio of pepsinogen I to II fell significantly as plasma ghrelin concentrations decreased, indicating the positive association between plasma ghrelin and pepsinogen I concentrations as well as pepsinogen I to II ratios in *H. pylori*-positive patients. Collectively, these results reveal that plasma ghrelin concentrations are associated with the progression of gastric atrophy.

Discussion

Plasma ghrelin levels have been associated with several clinical factors including BMI, food intake, and serum insulin levels (9, 21, 22). Although ghrelin-producing endocrine cells have been found mainly in the oxyntic mucosa of the stomach, ghrelin is also released from other tissues including small and large intestines, lung, kidney, the nucleus of the hypothalamus, and A cells of the pancreatic islet (23, 24). In fact, plasma ghrelin concentrations in gastrectomized patients still remain about one third of those in normal subjects (6). Thus, it is important to clarify which organ primarily influences changes in plasma ghrelin concentrations in each disease. In this study, we have demonstrated that plasma ghrelin concentrations are influenced by *H. pylori* infection. In particular, we focused on the gastric mucosa to better understand the effects of *H. pylori* infection on the alteration of ghrelin expression. The expression levels of ghrelin mRNA and the numbers of ghrelin-producing cells in the gastric mucosa were much lower in patients with *H. pylori* infection. Plasma ghrelin concentrations correlated with the gastric ghrelin mRNA as well as the frequency of ghrelin-immunoreactive cells in the gastric mucosa. Finally, we compared plasma ghrelin concentrations with serum pepsinogen levels, a marker for gastric atrophy. Plasma ghrelin concentrations in *H. pylori*-positive patients correlated with serum pepsinogen I concentration as well as pepsinogen I to II ratio. In addition, we demonstrated that groups with histologically higher degrees of gastric atrophy in the *H. pylori*-positive subjects tend to have lower plasma ghrelin concentrations (data not shown). These findings strongly suggest that the reduction of ghrelin-producing cells in the gastric mucosa by *H. pylori* infection results in the lower plasma ghrelin concentration in *H. pylori*-positive patients.

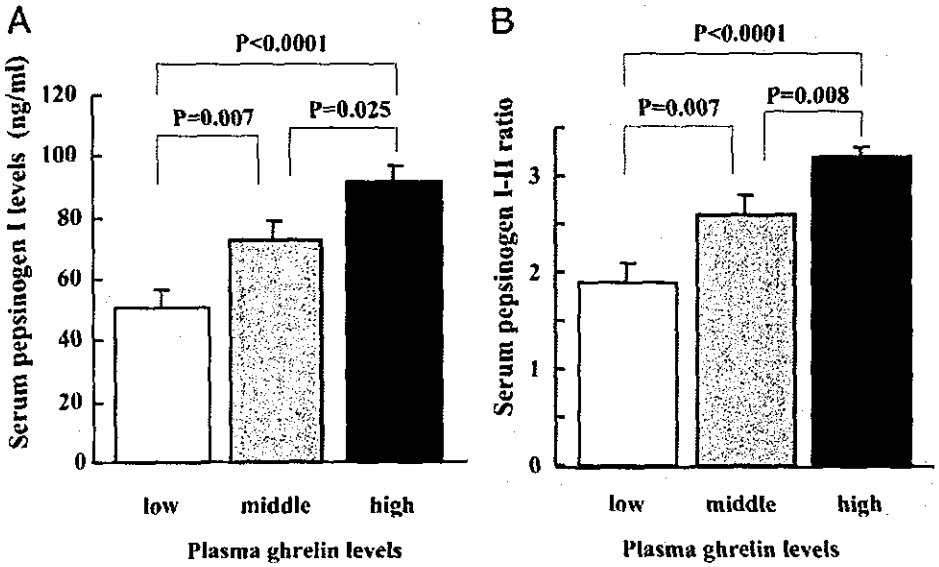

FIG. 6. Comparison of plasma ghrelin levels and serum pepsinogen levels in *H. pylori*-infected subjects. Values are expressed as the mean ± SE. Both serum pepsinogen I levels and pepsinogen I to II ratio significantly fell with the decrease of plasma ghrelin levels (A and B). An ANOVA based on Fisher's protected least significant difference test was used.

Our current data are consistent with the report of Nwokolo's group (17) that plasma ghrelin concentrations increased after *H. pylori* eradication. In their study, however, gastric ghrelin before and after *H. pylori* eradication was not measured. Moreover, effects of a change in BMI before and after *H. pylori* eradication on plasma ghrelin concentrations could not be excluded. In addition, the number of the enrolled subjects in their study was relatively small (10 subjects). Therefore, our present study expanded their observations by enrolling many more subjects with comparable BMI and showing a direct association between *H. pylori* infection and lower gastric ghrelin production.

On the other hand, Gokcel's group compared plasma ghrelin concentrations between *H. pylori*-positive and -negative subjects, and, opposed to our results, they found no differences. Although their study design was similar to ours, they did not provide any data on the gastric atrophy or gastric ghrelin production in their subjects. It is, therefore, only a speculation, but the discrepancy of the results may be due to the different features of gastric atrophy between Western and Japanese populations including disease frequency and severity. The earlier age of acquiring *H. pylori* infection in Japan, compared with Western countries, may also explain the high incidence of atrophic gastritis in Japanese adults and lower concentrations of plasma ghrelin concentrations as well.

It would be intriguing to clarify how a persistent decrease in plasma ghrelin concentration influences human growth and body weight. Recently several reports demonstrated that *H. pylori*-positive children have a high incidence of growth retardation (25, 26). *H. pylori* is acquired early in life in most of the developing world. Together with our results, the decrease of plasma ghrelin levels accompanied by *H. pylori* gastritis may have considerable influences on growth retardation in childhood. In our study, the plasma ghrelin levels in *H. pylori*-positive subjects were lower than *H. pylori*-negative subjects, even in patients with mild atrophic changes, implying that even mild gastric inflammation by *H. pylori* infection in children may reduce the production of gastric ghrelin. Further study in children including plasma ghrelin levels, degree of atrophy in the stomach, and presence of *H. pylori* infection may clarify these relationships.

In conclusion, our study indicates that plasma ghrelin concentrations are influenced by the presence of chronic gastritis in association with *H. pylori* infection. Decreases in gastric ghrelin production may account for lower concentrations of plasma ghrelin in *H. pylori*-positive individuals. These observations provide novel insights for understanding the physiological function of ghrelin and its relation to various diseases.

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