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Structure and Function of Ghrelin

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Abstract The endogenous ligand for growth-hormone secretagogue receptor (GHS-R) was purified from the stomach and we named it "ghrelin", after a word root ("ghre") in Proto-Indo-European languages meaning "grow", since ghrelin has potent growth hormone (GH) releasing activity. In addition, ghrelin stimulates appetite by acting on the hypothalamic arcuate nucleus, a region known to control food intake. Ghrelin is orexigenic; it is secreted from the stomach and circulates in the blood stream under fasting conditions, indicating that it transmits a hunger signal from the periphery to the central nervous system. Taking into account all these activities, ghrelin plays important roles for maintaining growth hormone release and energy homeostasis in vertebrates. The diverse functions of ghrelin raise the possibility of its clinical application for GH deficiency, eating disorder, gastrointestinal disease, cardiovascular disease, osteoporosis and aging, etc.

1 Introduction

In recent years, searches for novel ligands using orphan GPCR-expressing cells have resulted in the discovery of several novel bioactive peptides, such as nociceptin/orphanin FQ (Reinscheid et al. 1995), orexin/hypocretin (Sakurai et al. 1998), prolactin-releasing peptide (Hinuma et al. 1998), apelin (Tatemoto et al. 1998), metastin (Ohtaki et al. 2001), neuropeptide B (Fujii et al. 2002; Tanaka et al. 2003), and neuropeptide W (Shimomura et al. 2002; Tanaka et al. 2003). For the orphan-receptor strategy used to identify the endogenous ligands, we first established a cell line that stably expresses an orphan GPCR. Then, a peptide extract is applied to the cell line and a second messenger response is measured. If a target orphan GPCR is functionally expressed on the cell surface and the extract contains the endogenous ligand that can activate the receptor, the second messenger response, as usually monitored by the levels of cAMP or intracellular Ca^{2+} concentration, will increase or decrease. Through monitoring of this assay system, the endogenous ligand can be purified through several chromatographic steps. In this way, orphan receptors represent important tools for the discovery of novel bioactive molecules and for drug development (Civelli et al. 2001).

Among the numerous orphan GPCR receptors, GHS-R (growth hormone secretagogue receptor) attracted the attention of many academic and industrial scientists, since its endogenous ligand could potentially be used directly for treatment of GH deficiency. Many groups tried unsuccessfully to isolate the endogenous GHS-R ligand from extracts of brain, pituitary, or hypothalamus, the known sites of GHS-R expression. Unexpectedly, we succeeded in the purification and identification of the endogenous ligand for the GHS-R from the stomach, and named it "ghrelin" (Kojima et al. 1999). Ghrelin is a growth-hormone-releasing and appetite-stimulating peptide.

In this review, we review the structure, distribution, and physiological functions of ghrelin.

2
Discovery and Structure Determination of Ghrelin

A cultured cell line expressing the GHS-R was established and used to identify tissue extracts that could stimulate the GHS-R, as monitored by increases in intracellular Ca^{2+} levels. After screening several tissues, very strong activity was unexpectedly found in stomach extracts (Kojima et al. 1999). The peptide that stimulated GHS-R was purified from the rat stomach through four steps of chromatography: gel-filtration, two ion-exchange HPLC steps, and a final reverse-phase HPLC (RP-HPLC) procedure. The second ion-exchange HPLC yielded two active peaks (P-I and P-II), from which ghrelin and des-Gln14-ghrelin were purified, respectively (Hosoda et al. 2000b). The active peaks were finally purified by RP-HPLC. The name ghrelin is based on "ghre", a word root in Proto-Indo-European languages for "grow", in reference to its ability to stimulate GH release. Ghrelin is a 28-amino-acid peptide, in which the serine 3 (Ser3) is *n*-octanoylated and this modification is essential for ghrelin's activity (Fig. 1). Ghrelin is the first known and only case of a peptide hormone modified by a fatty acid.

In rat stomach, a second type of ghrelin peptide has been purified and identified as des-Gln14-ghrelin (Hosoda et al. 2000b). Except for the deletion of Gln14, des-Gln14-ghrelin is identical to ghrelin, even retaining the *n*-octanoic acid modification. Des-Gln14-ghrelin has the same potency of activities as that of ghrelin. The deletion of Gln14 in des-Gln14-ghrelin arises due to the usage of a CAG codon to encode Gln, which results in its recognition as a splicing signal. Thus, two types of active ghrelin peptide are produced in rat stomach, ghrelin and des-Gln14-ghrelin. However, des-Gln14-ghrelin is only present in low amounts in the stomach, indicating that ghrelin is the major active form.

In mammals, ghrelin homologues have been identified in human, rhesus monkey (Angeloni et al. 2004), rat, mouse, mongolian gerbil (GenBank Accession number: AF442491), cow (GenBank Accession number: AB035702), pig (GenBank Accession number: AB035703), sheep (GenBank Accession num-

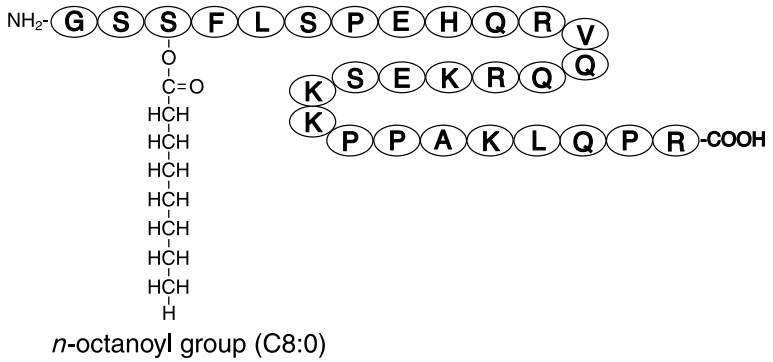

Fig. 1 Structures of human ghrelins. Human ghrelin is a 28-amino-acid peptide, in which Ser3 is modified by a fatty acid, primarily *n*-octanoic acid. This modification is essential for ghrelin's activity

ber: AB060699), dog (Tomasetto et al. 2001), and cat (Ida et al. 2007), etc. The amino-acid sequences of mammalian ghrelins are well conserved; in particular, the ten amino acids are with respect to their N-termini identical (Fig. 2). This structural conservation and the universal requirement for acyl-modification of the third residue indicate that this N-terminal region is of central importance to the activity of the peptide.

Ghrelin has also been identified and the structures determined in birds, fishes, amphibians and reptiles (Kojima and Kangawa 2005) (Fig. 2). All vertebrate ghrelins are mainly produced in stomach, or stomach-like organs, and modified by medium-chain fatty acid. The fatty acids used for acyl-modification are *n*-octanoic, *n*-decanoic acid or other minor medium-chain fatty acids. The characteristic features of non-mammalian ghrelins are their multiple forms in tissues: ghrelins could be classified by the type of acyl-modification and amino acid length.

3 Des-Acyl Ghrelin

A non-acylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in both stomach and blood (Hosoda et al. 2000a). In blood, des-acyl ghrelin circulates in amounts far greater than acylated ghrelin. Des-acyl ghrelin does not replace radiolabelled ghrelin at the binding sites of acylated ghrelin in hypothalamus and pituitary and shows no GH-releasing and other endocrine activities in rats. Moreover, des-acyl ghrelin does not possess endocrine activities in humans.

One question is whether there is a specific receptor for des-acyl ghrelin. Baldanzi and coworkers have suggested the existence of another ghrelin re-

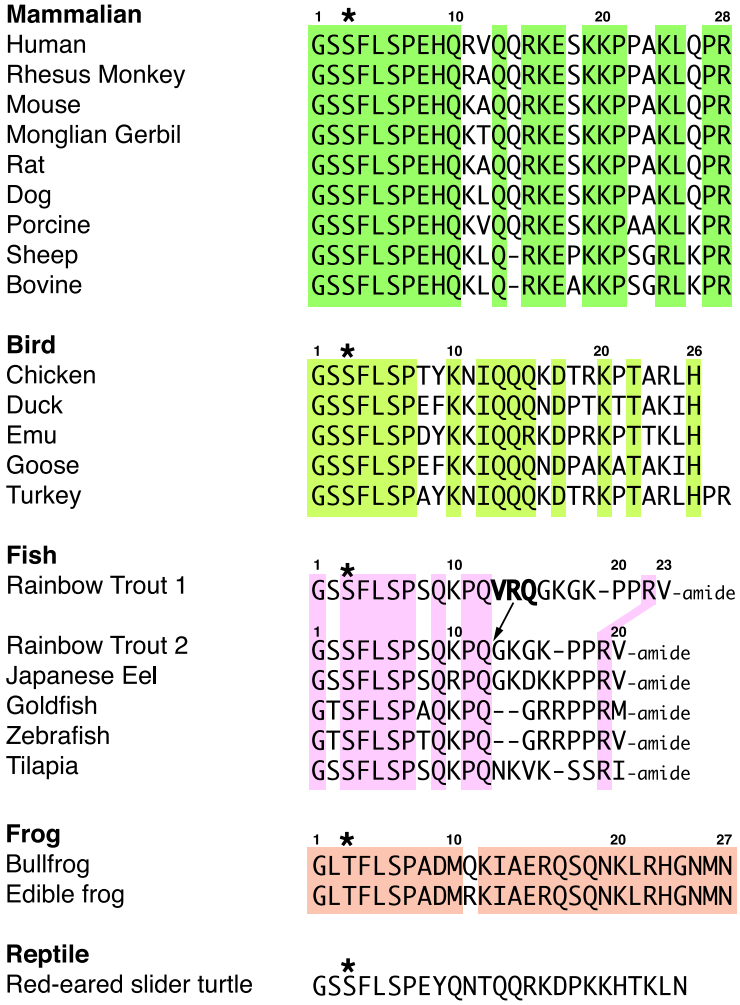

Fig. 2 Sequence comparison of vertebrate ghrelins. Identical amino acids in each species of mammal, bird, fish and frog are *colored*. The *asterisks* indicate acyl-modified third amino acids. N-terminal cores with acyl-modification sites are well conserved among all vertebrate ghrelins. A unique third residue (Thr3) in two frog ghrelins differs from the Ser3 in ghrelins of other species. Because serine and threonine both possess hydroxyl groups on their side chains, they can both be modified by fatty acids. Indeed, the frog ghrelins are modified by either *n*-octanoic or *n*-decanoic acids. Fish ghrelins were found to contain an amide structure at their COOH-terminal ends, though these amide structures are not necessary for activity

ceptor in the cardiovascular system (Baldanzi et al. 2002). They showed that ghrelin and des-acyl ghrelin both recognize common high-affinity binding sites on H9c2 cardiomyocytes, which do not express the ghrelin receptor,

GHS-R. However, BLAST searches of the human genome using ghrelin receptor (GHS-R) cDNA as a search sequence have not revealed any ghrelin receptor homologues. Further study is required to determine whether desacyl ghrelin is biologically active and binds to an as-yet-unidentified receptor.

4
Ghrelin Gene and the Structure of the Ghrelin Precursor

The human ghrelin gene is localized on the chromosome 3p25–26. The human ghrelin receptor gene has also been identified on chromosome 3, at position q26–27 (Smith et al. 1997).

The human ghrelin gene, like the mouse gene, comprises five exons (Kanamoto et al. 2004; Tanaka et al. 2001). The short first exon contains only 20 bp, which encode part of the 5′-untranslated region. There are two different transcriptional initiation sites in the ghrelin gene; one occurs at –80 and the other at –555 relative to the ATG initiation codon, resulting in two distinct mRNA transcripts (transcript-A and transcript-B). Transcript-A is the main form of gastric ghrelin mRNA.

The 28 amino acids of the functional ghrelin peptide are encoded in exons 2 and 3. In the rat and mouse ghrelin genes, the codon for Gln14 (CAG) is used as an alternative splicing signal to generate two different ghrelin mRNAs (Hosoda et al. 2000b). One mRNA encodes the ghrelin precursor, and another encodes a des-Gln14-ghrelin precursor. Des-Gln14-ghrelin is identical to ghrelin, except for the deletion of Gln14.

The amino-acid sequences of mammalian ghrelin precursors are well conserved. In these precursors, the 28-amino-acid active ghrelin sequence immediately follows the signal peptide. The cleavage site for the signal peptide is the same in all mammalian ghrelins. Although propeptides are usually processed at dibasic amino acid sites by prohormone convertases, the C-terminus of the ghrelin peptide sequence is processed at an uncommon Pro-Arg recognition site. The Pro-Arg sequence is also used for the C-terminal processing of atrial natriuretic peptide (ANP) (Seidah and Chretien, 1999; Steiner, 1998). Zhu et al. reported that the protease that acts at the Pro-Arg site is prohormone convertase 1/3 (PC1/3) (Zhu et al. 2006).

5
Enzyme for Acyl-Modification of Ghrelin

An enzyme that catalyzes the acyl-modification of ghrelin has not yet been identified. The universal incorporation of *n*-octanoic acid in mammals, fish, birds, and amphibians suggests that this putative enzyme is rather specific in its choice of medium-chain fatty acid substrates.

Ingestion of either medium-chain fatty acids (MCFAs) or medium-chain triacylglycerols (MCTs) specifically increases production of acyl-modified ghrelin without changing the total (acyl- and des-acyl-) ghrelin level (Nishi et al. 2005). When mice ingested either MCFAs or MCTs, the acyl group attached to nascent ghrelin molecules corresponded to that of the ingested MCFAs or MCTs. Moreover, *n*-heptanoyl (C7:0) ghrelin, an unnatural form of ghrelin, was produced in the stomach of mice following ingestion of *n*-heptanoic acid or glyceryl triheptanoate. These findings indicate that ingested fatty acids are directly utilized for acyl-modification of ghrelin.

A number of acyltransferases have previously been identified in mammals; the only reported enzymes that use MCFAs as substrates are carnitine octanoyltransferases, which function in the β-oxidation of fatty acids (Ramsay and Naismith 2003). Members of the serine acyltransferase family that transfer acyl groups to serine residues of target molecules have been identified, including two serine palmitoyltransferases functioning in the biosynthesis of sphingolipids in mammals (Hanada 2003) and a plant Ser *O*-acetyltransferase gene family in *Arabidopsisthaliana* (Howarth et al. 2003). The putative ghrelin Ser *O*-acyltransferase may have structural homology with these acyltransferases. Further investigations characterizing the putative ghrelin Ser *O*-acyltransferase are required to elucidate the mechanism of the unique acyl modification seen in ghrelin.

6
Ghrelin Receptor Family

The ghrelin receptor, or GHS-R, is a typical G protein-coupled receptor with seven transmembrane domains (7-TM). Two distinct ghrelin receptor cDNAs have been isolated: GHS-R type 1a mRNA and type 1b mRNA (Howard et al. 1996). The first, GHS-R Type 1a, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor. This Type 1a receptor has features characteristic of a typical GPCR, including conserved cysteine residues in the first two extracellular loops, several potential sites for post-translational modifications (*N*-linked glycosylation and phosphorylation), and an aromatic triplet sequence (E/DRY) located immediately after TM-3 in the second intracellular loop. Another GHS-R cDNA, type 1b, is produced by an alternative splicing mechanism. The GHS-R gene consists of two exons; the first exon encodes TM-1 to 5, and the second exon encodes TM-6 to 7. Type 1b is derived from only the first exon and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a C-terminal truncated form of the type 1a receptor and is pharmacologically inactive.

The ghrelin receptor (GHS-R) has several homologues, whose endogenous ligands are gastrointestinal peptides or neuropeptides. This receptor superfamily contains receptors for ghrelin, motilin (Feighner et al. 1999),

neuromedin U (Howard et al. 2000; Kojima et al. 2000) and neurotensin (Vincent et al. 1999). All of these peptides are found in gastrointestinal organs and regulate gastrointestinal movement and other functions. This family also contains an orphan receptor, GPR39, whose endogenous ligand was identified as obestatin, a ghrelin precursor-derived peptide (Zhang et al. 2005). However, negative results have been reported against obestatin as the ligand for GPR39.

The ghrelin receptor is most homologous to the motilin receptor; the human forms share 52% identical amino acids (Inui 2001; Smith et al. 2001). Moreover, their ligands, ghrelin and motilin peptides, have similar amino-acid sequences. Preliminary studies have shown that motilin can stimulate the ghrelin receptor, albeit at a low level. In contrast, ghrelin does not activate the motilin receptor (Dass et al. 2003).

The ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, chicken, and pufferfish (Fugu) (Palyha et al. 2000; Smith et al. 2001). This strict conservation suggests that ghrelin and its receptor serve important physiological functions.

One case of familial short stature associated with a missense mutation in the ghrelin receptor has been reported (Pantel et al. 2006). This mutation changed a single amino acid, resulting in a charge change at a highly conserved extracellular position. This mutated ghrelin receptor shows severely impaired ghrelin binding.

7
Ghrelin and Motilin

The ghrelin receptor is most homologous to the motilin receptor (Feighner et al. 1999; Inui 2001). Accordingly, the amino-acid sequence of ghrelin has homology with that of motilin, another gastric peptide with gastric contractile activity (Asakawa et al. 2001). Alignment of the 28 amino-acid peptide ghrelin and the 19-amino-acid motilin reveal that they share eight identical amino acids. In fact, after our discovery of ghrelin, Tomasetto and coworkers reported the identification of a gastric peptide, the motilin-related peptide (MTLRP) (Tomasetto et al. 2000). They had tried to isolate new protein clones whose expression was restricted to the gastric epithelium using differential screening. The amino-acid sequence of MTLRP turned out to be identical to that of ghrelin[1-18]; however, the putative processing site of MTLRP, Lys–Lys, is not used in ghrelin in gastric cells. Moreover, the sequence data alone could not reveal any potential acyl-modifications (Del Rincon et al. 2001; Folwaczny et al. 2001).

Interestingly, the region of homology between ghrelin and motilin lies not near the N-terminus, where ghrelin's acyl-modification occurs, but in their respective central regions. Ghrelin and motilin play similar roles in the stom-

ach. Both peptides stimulate gastric-acid secretion and gastric movement (Masuda et al. 2000). Thus, ghrelin and motilin are structurally and functionally considered to compose a peptide superfamily, and may have evolved from a common ancestral peptide.

8
Distribution of Ghrelin

8.1
Plasma Ghrelin

Two major forms of ghrelin are found in plasma: *n*-octanoyl-modified and des-acyl ghrelin (Hosoda et al. 2000a). The normal ghrelin concentration of plasma samples in humans is 10–20 fmol/ml for *n*-octanoyl ghrelin and 100–150 fmol/ml for total ghrelin, including both acyl-modified and des-acyl ghrelins. Plasma ghrelin concentration is increased in fasting conditions and reduced after habitual feeding, suggesting that ghrelin may be an initiation signal for food intake or ghrelin secretion is controlled by some nutritional factors in blood (Cummings et al. 2001; Tschop et al. 2001a).

It is not clear what factors are involved in the regulation of ghrelin secretion. Blood glucose level may be critical: oral or intravenous administration of glucose decreases plasma ghrelin concentration (McCowen et al. 2002; Shiiya et al. 2002). Since gastric distention by water intake does not change ghrelin concentration, mechanical distention of the stomach alone clearly does not induce ghrelin release. Plasma ghrelin concentration is sensitive, however, to the makeup of a meal; it is decreased by a high lipid meal and increased by a low protein one.

Plasma ghrelin concentration is low in obese people and high in lean people, indicating that plasma ghrelin concentration is in inverse proportion to BMI (Hanada 2003; Hansen et al. 2002; Shiiya et al. 2002; Tschop et al. 2001b). Related to this fact, the plasma ghrelin level is highly increased in anorexia nervosa patients and returns to normal levels upon weight gain and recovery from the disease (Ariyasu et al. 2001; Cuntz et al. 2002; Otto et al. 2001). Ghrelin concentration is also increased in cachexia due to cancer, heart failure, and chronic fasting disease, etc.

8.2
Gastric and Intestinal Ghrelin

In all vertebrate species, ghrelin is mainly produced in the stomach. In the stomach, ghrelin-containing cells are more abundant in the fundus than in the pylorus (Date et al. 2000a; Yabuki et al. 2004) (Fig. 3A). In situ hybridization and immunohistochemical analyses indicate that ghrelin-containing cells

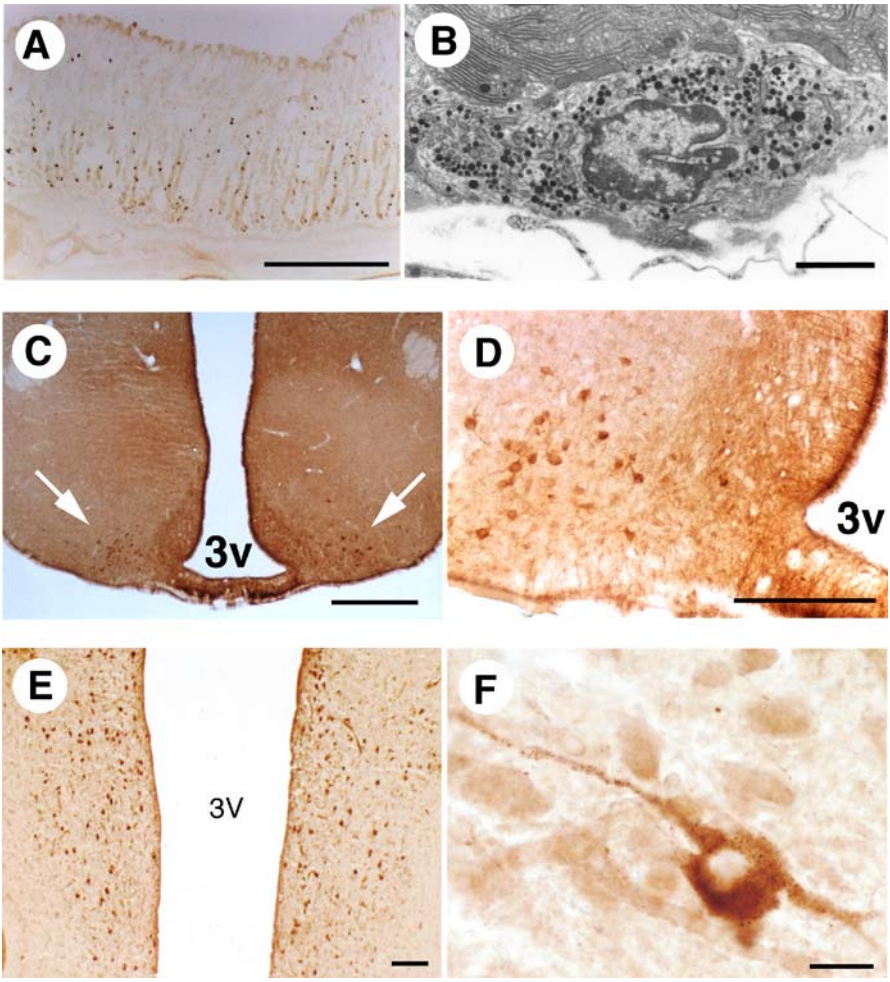

Fig. 3 Ghrelin cells in the stomach and hypothalamus. **A** Ghrelin-immunoreactive cells in the stomach are found from the neck to the base of the oxyntic gland. *Scale bar*: 400 μm. This distribution pattern is typical for gastric endocrine cells. **B** Ghrelin-producing cell has many round, compact, electron-dense granules in its cytoplasm. *Scale bar*: 500 nm. **C** *Arrows* indicate ghrelin neurons in the hypothalamic arcuate nucleus. *Scale bar*: 500 μm. **D** High magnification of (**C**). *Scale bar*: 200 μm. **E** Localization of ghrelin-immunoreactive neurons in the porcine hypothalamic paraventricular nucleus. *Scale bar*: 200 μm. **F** A ghrelin-producing neuron in the paraventricular nucleus. *Scale bar*: 20 μm

are a distinct endocrine cell type found in the mucosal layer of the stomach (Date et al. 2000a; Rindi et al. 2002).

Four types of endocrine cells have been identified in the oxyntic mucosa: ECL, D, enterochromaffin (EC), and X/A-like cells and they show the following relative abundances. The rat oxyntic gland contains approximately

60–70% ECL cells, 20% X/A-like cells, 2–5% D cells, and 0–2% EC cells; in humans, the corresponding percentages are 30%, 20%, 22%, and 7%. The major products in the granules have been identified as histamine and uroguanylin in ECL cells, somatostatin in D cells, and serotonin in EC cells. However, the granule contents of X/A-like cells were unknown until the discovery of ghrelin. The X/A-like cells contain round, compact, electron-dense granules that are filled with ghrelin (Date et al. 2000a; Dornonville de la Cour et al. 2001) (Fig. 3B). These X/A-like cells account for about 20% of the endocrine cell population in adult oxyntic glands. However, the number of X/A-like cells in the fetal stomach is very low and increases after birth (Hayashida et al. 2002). As a result, the ghrelin concentration of fetal stomach is also very low and gradually increases after birth until five weeks of age.

The gastric X/A-like cells can be stained by an antibody that is specific to the N-terminal, acyl-modified portion of ghrelin, indicating that ghrelin in the secretory granules of X/A-like cells has already been acyl-modified. Two major forms of ghrelin are found in the stomach as in plasma: *n*-octanoyl-modified and des-acyl ghrelin.

Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum, and colon (Date et al. 2000a; Hosoda et al. 2000a; Sakata et al. 2002). In the intestine, ghrelin concentration gradually decreases from the duodenum to the colon. As in the stomach, the main molecular forms of intestinal ghrelin are *n*-octanoyl ghrelin and des-acyl ghrelin.

8.3 Pancreatic Ghrelin

The pancreas is a ghrelin-producing organ. Analyses combining HPLC and ghrelin-RIA revealed that ghrelin and des-acyl ghrelin both exist in the rat pancreas (Date et al. 2002b). However, the cell type that produces ghrelin in the pancreatic islets remains controversial, whether it be the α cells, β cells, the newly identified islet epsilon (ε) cells, or a unique novel islet cell type (Prado et al. 2004).

The homeodomain protein Nkx2.2 is essential for the differentiation of islet β cells and α cells, and lack of Nkx2.2 in mice results in replacement of pancreatic endocrine cells by cells that produce ghrelin (Prado et al. 2004). Normal murine pancreas also contains a small number of the newly identified islet cell type, ε cells.

The pancreatic ghrelin profile changes dramatically during fetal development (Chanoine and Wong 2004); pancreatic ghrelin-expressing cells are numerous from midgestation to the early postnatal period, comprising 10% of all endocrine cells, and decrease in number after birth. Ghrelin mRNA expression and total ghrelin concentration are markedly elevated in the fetal pancreas, 6–7 times greater than in the fetal stomach. Thus, the onset of islet ghrelin expression precedes that of gastric ghrelin. Pancreatic ghrelin

expression is highest in the prenatal and neonatal periods. In contrast, gastric ghrelin levels are low during the prenatal period and increase after birth (Hayashida et al. 2002). Moreover, pancreatic ghrelin levels are not affected by fasting.

8.4 Pituitary Ghrelin

GH-releasing somatotrophs in the pituitary gland are the target cells of ghrelin. In an in vivo assay, ghrelin stimulated primary pituitary cells and increased their intracellular Ca^{2+} concentration, indicating that the ghrelin receptor, GHS-R, is expressed in pituitary cells (Bennett et al. 1997; Guan et al. 1997; McKee et al. 1997a). Also, ghrelin has been found in the pituitary gland itself (Korbonits et al. 2001a; Korbonits et al. 2001b), where it may influence the release of GH in an autocrine or paracrine manner. Pituitary tumors, such as adenomas, corticotroph tumors, and gonadotroph tumors contain ghrelin peptides.

8.5 Ghrelin in the Brain

Since the ghrelin receptor, GHS-R, is mainly expressed in the hypothalamus and pituitary, its endogenous ligand is thought to exist mainly in the hypothalamic regions. This is supported by the finding that another growth-hormone-releasing peptide, GHRH (growth-hormone-releasing hormone) is produced in the hypothalamus and is secreted into the hypophyseal portal system to stimulate GH release from the pituitary somatotrophs. However, the ghrelin content of the brain was found to be very low (Hosoda et al. 2000a; Kojima et al. 1999). Ghrelin has been found in the hypothalamic arcuate nucleus, an important region for controlling appetite (Fig. 3C,D). In addition, it has been reported that ghrelin is found in previously uncharacterized hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (Cowley et al. 2003; Sato et al. 2005) (Fig. 3E,F). Two major ghrelin peptides are identified in the rat hypothalamus: *n*-octanoyl-modified and des-acyl ghrelins (Sato et al. 2005). Thus, in a manner similar to ghrelin in the stomach, the two major forms of ghrelin are also found in the hypothalamus.

9 Physiological Functions of Ghrelin

Ghrelin exerts two main physiological functions: growth hormone releasing activity from the pituitary and increase of food intake by stimulation of

the hypothalamic appetite regulatory region. Ghrelin also shows many other physiological functions.

9.1 Growth Hormone Releasing Activity of Ghrelin

Ghrelin has been shown to induce GH release not only in rats and humans (Kojima et al. 1999), but also in non-mammalian vertebrates, including chicken (Kaiya et al. 2002), fish (Kaiya et al. 2003a,b), and frog (Kaiya et al. 2001). Ghrelin stimulates growth-hormone release both in vitro and in vivo in a dose-dependent manner (Fig. 4). Figure 4A shows the increase of GH

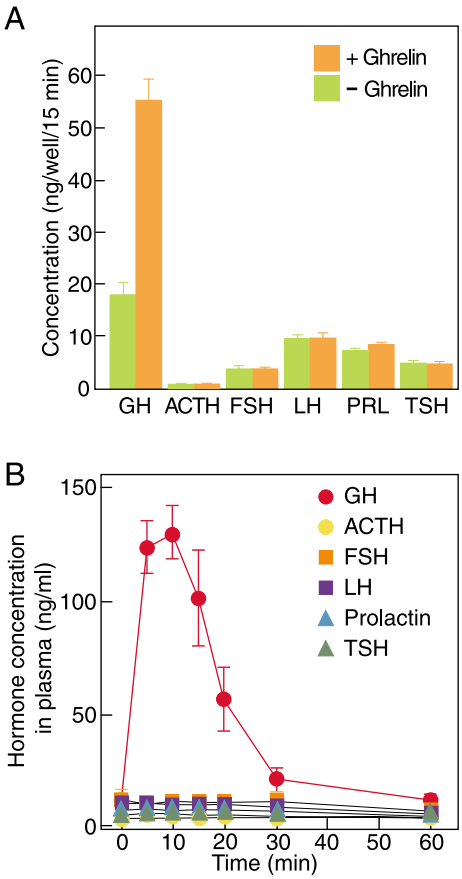

Fig. 4 Effects of ghrelin on pituitary hormone secretion in vitro and in vivo. **A** Effects of a high dose (10^{-6} M) of ghrelin on hormone secretion from rat primary pituitary cells in vitro. ACTH, adrenocorticotropin; FSH, follicle-stimulating hormone; LH, lutenizing hormone; PRL, prolactin; and TSH, thyroid-stimulating hormone. **B** Time courses of plasma hormone concentrations after IV injection of ghrelin into anesthetized male rats in vivo

concentration that was secreted from primary pituitary cultured cells into medium after ghrelin addition (Kojima et al. 1999). Moreover, intravenous injection of ghrelin induces potent GH release in many species. Thus, ghrelin is a potent GH-releasing peptide.

A single intracerebroventricular administration of ghrelin also increased rat plasma GH concentration in a dose-dependent manner, with a minimum dose of only 10 pmol (Date et al. 2000b). Thus, ICV injection appears to be a more potent route of delivery than IV administration.

Co-administration of ghrelin and GHRH had a synergistic effect on GH secretion; that is, co-administration results in more GH release than does either GHRH or ghrelin alone (Hataya et al. 2001). This finding implies that GHRH is necessary for GH release to be maximally effective.

9.2 Appetite Stimulating Activity of Ghrelin

Recent identification of appetite-regulating humoral factors reveal regulatory mechanisms not only in the central nervous system, but also mediated by factors secreted from peripheral tissues (Coll et al. 2007; Stanley et al. 2005). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain, while ghrelin, produced in the stomach, is an appetite-stimulating factor that transmits hunger signals to the brain. Ghrelin, thus, is functionally a natural antagonist to leptin.

Ghrelin is produced primarily in gastrointestinal organs in response to hunger and starvation, and circulates in the blood, serving as a peripheral signal telling the central nervous system to stimulate feeding. When ghrelin is injected into the cerebral ventricles of rats, their food intake is potently stimulated (Nakazato et al. 2001; Shintani et al. 2001; Tschop et al. 2000; Wren et al. 2000) (Fig. 5A). Furthermore, chronic ICV injection of ghrelin increases cumulative food intake and decreased energy expenditure, resulting in body weight gain (Fig. 5B). Ghrelin-treated mice also increase their fat mass, both absolutely and as a percentage of total body weight. Not only ICV injection, but also IV and subcutaneous injection of ghrelin have been shown to increase food intake. IV injection of ghrelin (5.0 pmol/kg/min) into human volunteers increased food intake by an average of 28% in every individual (Wren et al. 2001).

The hypothalamic arcuate nucleus is the main site of ghrelin's activity in the central nervous system. The arcuate nucleus is also a target of leptin, an appetite-suppressing hormone produced in adipose tissues, and NPY and AgRP, which are both appetite-stimulating peptides (Morton and Schwartz, 2001). NPY and AgRP are produced in the same population of neurons in the arcuate nucleus, and their appetite-stimulating effects are inhibited directly by leptin. At least part of the orexigenic effect of ghrelin is mediated by upregulating the genes encoding these potent appetite stimulants (Fig. 6).

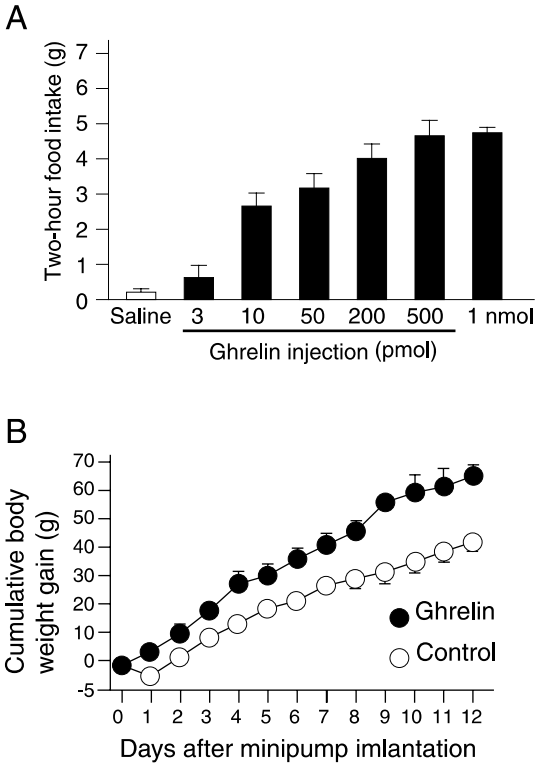

Fig. 5 Stimulation of feeding by ICV administration of ghrelin. **A** Two-hour food intake of free-feeding rats injected with various doses of ghrelin. Control rats were given 0.9% saline. **B** Effect of chronic ghrelin ICV administration on rats. Cumulative body weight gain during an ICV infusion of 250 pmol/day for 12 days

ICV injection of ghrelin induces c-Fos expression in NPY-expressing neurons and increases the amount of NPY mRNA in the arcuate nucleus (Kamegai et al. 2001; Nakazato et al. 2001; Shintani et al. 2001). Moreover, ICV ghrelin injection increases the AgRP mRNA level in the hypothalamus. The appetite-stimulating effects of ghrelin are blocked by an antagonist of NPY receptor 1. ICV injections of an AgRP inhibitor, anti-NPY IgG, and anti-AgRP IgG inhibits the appetite-stimulating effects of ghrelin. Intravenous injection of ghrelin also stimulates NPY/AgRP neurons in the hypothalamus. Immunohistochemical analysis indicated that ghrelin neuron fibers directly contact NPY/AgRP neurons (Cowley et al. 2003). These results indicate that ghrelin exerts its feeding activity by stimulating NPY/AgRP neurons in the hypothalamus to promote the production and secretion of NPY and AgRP peptides. Studies with knockout mice of NPY, AgRP or both confirms these results. Although deletion of either NPY or AgRP caused a modest or no effect on the

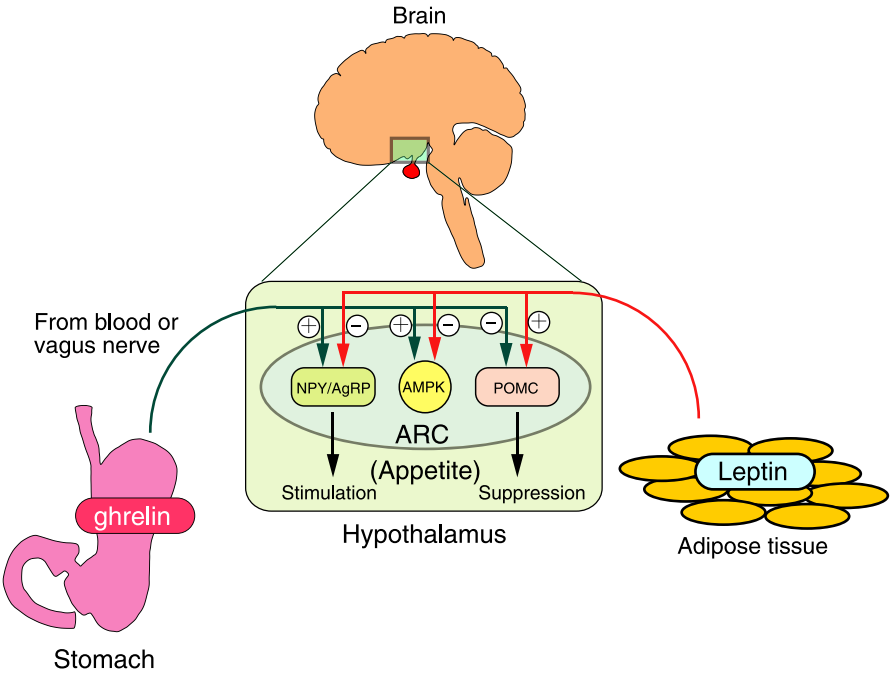

Fig. 6 Hypothalamic appetite regulation by ghrelin and leptin. The arcuate nucleus (ARC) of the hypothalamus is the main target of ghrelin and leptin. Ghrelin is a peripheral orexigenic signal secreted from the stomach, whereas leptin is a peripheral anorexigenic signal secreted from adipose tissue. The effects of ghrelin are opposite to those of leptin. In the ARC, ghrelin stimulates NPY/AgRP neurons and suppresses POMC neurons. On the other hand, leptin suppresses NPY/AgRP neurons and stimulates POMC neurons. Moreover, ghrelin increases AMPK activity in the hypothalamus, whereas leptin decreases AMPK activity

orexigenic action of ghrelin, the double knockout mice lacked the action of ghrelin completely (Chen et al. 2004).

Recently, AMP-activated protein kinase (AMPK) has been shown to be involved in hypothalamic appetite regulation (Minokoshi et al. 2004). Injection of 5-amino-4-imidazole carboxamide riboside, an activator of AMPK, significantly increases food intake. Administration of ghrelin in vivo increases AMPK activity in the hypothalamus (Andersson et al. 2004). By contrast, injection of leptin decreases hypothalamic AMPK activity.

9.3 Pathway of the Ghrelin Signal; from Peripheral Tissues to the Central Nervous System

Peripherally injected ghrelin stimulates hypothalamic neurons and stimulates food intake. In general, peptides injected peripherally do not pass the blood–

brain barrier. Indeed, the rate at which peripheral ghrelin passes the barrier has shown to be very low. Thus, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway.

The detection of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (Date et al. 2002a; Zhang et al. 2004). Moreover, the observation that ICV administration of ghrelin induces c-Fos in the dorsomotor nucleus of the vagus and stimulates gastric-acid secretion indicates that ghrelin activates the vagus system (Date et al. 2001).

In contrast, vagotomy inhibits the ability of ghrelin to stimulate food intake and GH release (Date et al. 2002a). A similar effect was also observed when capsaicin, a specific afferent neurotoxin, was applied to vagus nerve terminals to induce sensory denervation. However, the basal level of ghrelin concentration is not affected and a decrease of ghrelin levels is not observed after vagotomy. On the other hand, fasting-induced elevation of plasma ghrelin is completely abolished by subdiaphragmatic vagotomy or atropine treatment (Williams et al. 2003).

Moreover, peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus (Date et al. 2006). Bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in the hindbrain that express dopamine β-hydroxylase (an NA synthetic enzyme), abolished ghrelin-induced feeding. Thus, the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin. These results indicate that the response of ghrelin to fasting is transmitted through vagal afferent transmission.

9.4 Ghrelin and Eating Disorders

Anorexia nervosa (AN) is a syndrome often seen in young women characterized by a combination of weight loss, amenorrhea, and behavioral changes. Some of these changes are reversible with weight gain. Plasma ghrelin levels in AN patients are high and return to control levels after weight gain by renutrition (Ariyasu et al. 2001; Cuntz et al. 2002; Otto et al. 2001). AN patients often show markedly elevated GH levels, which may be due to high circulating levels of ghrelin. Moreover, high ghrelin increases ACTH, prolactin, and cortisol levels in humans (Takaya et al. 2000), which may explain the amenorrhea and behavioral changes observed in AN patients.

High plasma ghrelin concentration is observed in Prader–Willi syndrome (PWS) (Cummings et al. 2002a), cachexia with cancer or chronic diseases. PWS is a complex genetic disorder characterized by mild mental retardation, hyperphagia, short stature, muscular hypotonia, and distinctive behavioral features. Excessive appetite in PWS causes progressive severe obesity. The

PWS genotype is characterized by a loss of one or more paternal genes in region q11–13 on chromosome 15 (Nicholls and Knepper 2001). It has been suggested that this genetic alteration leads to dysfunction of several hypothalamic areas, including appetite regulatory regions.

To treat severe obesity, gastric bypass operations are often performed (Fobi 2004). Recent research has revealed that ghrelin may contribute to the body-weight reduction that occurs following gastric bypass. Total ghrelin secretion was found to be reduced by up to 77% compared to normal-weight control groups and by up to 72% compared to matched obese groups (Cummings et al. 2002b). Furthermore, the normal meal-related fluctuations and diurnal rhythm of ghrelin level were absent in these patients. Thus, the mean plasma ghrelin concentration decreased significantly after gastric bypass surgery, which may have been responsible for their lack of hyperphagia and contributed to their weight loss.

9.5 Cardiovascular Function of Ghrelin

Evidence for a cardiovascular function of ghrelin has been found: expression of mRNA encoding both ghrelin and its receptor has been observed in the heart and aortas (Gnanapavan et al. 2002; Nagaya et al. 2001a). Moreover, an intravenous bolus of human ghrelin decreased mean arterial pressure without changing the heart rate (Nagaya et al. 2001a). Ghrelin increased the cardiac index and stroke volume indices. Rats with chronic heart failure (CHF) that were treated with ghrelin showed higher cardiac output, stroke volume, and LV dP/dt[max] when compared to afflicted, but placebo-treated controls (Nagaya and Kangawa 2003). Furthermore, ghrelin increased the diastolic thickness of the non-infarcted posterior wall, inhibited LV enlargement, and increased LV fractional shortening in these CHF rats (Nagaya et al. 2001b). Ghrelin, thus, improves LV dysfunction and attenuates the development of LV remodelling and cardiac cachexia.

The decrease in mean arterial pressure induced by ghrelin seems not to occur through its direct action on the circulatory system, but through its action on the nucleus of the solitary tract (Lin et al. 2004; Matsumura et al. 2002). Microinjection of ghrelin into this nucleus significantly decreased the mean arterial pressure and heart rate. This injection also suppressed sympathetic activity. It has been reported that ghrelin inhibits apoptosis of primary adult and H9c2 cardiomyocytes and endothelial cells in vitro.

9.6 Gastrointestinal Function of Ghrelin

Intravenous administration of ghrelin dose-dependently increases gastric-acid secretion and stimulates gastric motility (Masuda et al. 2000). The max-

imum response to ghrelin in terms of gastric-acid secretion is almost as high as that elicited by subcutaneous treatment with histamine (3 mg/kg). These responses to ghrelin were abolished by pretreatment with either atropine or bilateral cervical vagotomy, but not by a histamine H_2-receptor antagonist. ICV administration of ghrelin also increases gastric-acid secretion in a dose-dependent manner (Date et al. 2001).

ICV administration of ghrelin was shown to induce c-Fos expression in the nucleus of the solitary tract and the dorsomotor nucleus of the vagus nerve (Date et al. 2001), indicating that ghrelin's ability to stimulate gastric-acid secretion is mediated through activation of the vagus nerve.

9.7 Ghrelin and Pancreatic Function

The role of ghrelin in insulin secretion is likewise under debate. Ghrelin has been shown to inhibit insulin secretion in some experiments and stimulate insulin release in others (Adeghate and Ponery 2002; Broglio et al. 2001; Date et al. 2002b; Lee et al. 2002). These discrepancies may be due to species differences and/or experimental design. Plasma ghrelin and insulin levels are affected by blood glucose level; high glucose suppresses ghrelin secretion and stimulates insulin secretion. Thus, the glucose level in experiments may be important. Date and colleagues reported that ghrelin stimulates insulin release in the presence of high levels of glucose (8.3 mM) that could release insulin from cultured islet cells (Date et al. 2002b). In contrast, ghrelin had no effect on insulin release in the context of a basal level of glucose (2.8 mM). In contrast, Dezaki and colleagues reported that ghrelin inhibits insulin secretion, while administration of ghrelin-receptor antagonists or anti-ghrelin antibodies increases insulin secretion that was induced by glucose injection (Dezaki et al. 2006). Moreover, they reported that an increase of glucose-induced insulin secretion was observed in ghrelin-null mice. Ghrelin knockout mice showed no change in density, size, insulin level and insulin mRNA of pancreatic island.

9.8 Ghrelin and the Process of Learning and Memory

Ghrelin may be involved in the process of learning and memory. Diano et al. reported that circulating ghrelin entered the hippocampus and bound to the hippocampal neurons to promote synapse formation of the dendritic spines and generate long-term potentiation (Diano et al. 2006). This synapse formation may be paralleled by enhanced spatial learning and memory after ghrelin injection. In contrast, ghrelin knockout mice had a decreased number of dendritic spine synapses in the hippocampal CA1 region and were impaired in behavioral memory in the novel object recognition test. Moreover, the de-

crease in synapse formation and impairment of memory test were promptly recovered by ghrelin administration. Further studies are needed to confirm that ghrelin directly acts on the hippocampal cells to enhance learning and memory processes.

10 Obestatin, a Ghrelin Precursor-Derived Peptide?

In November 2005 Zhang and colleagues from Stanford University reported a novel peptide hormone called "obestatin" from the Latin "obedere", meaning to devour, and "statin", meaning suppression, because it suppressed food intake (Zhang et al. 2005). An interesting fact is that obestatin is processed from the ghrelin precursor; this means that the two peptide hormones with opposing action on food intake, orexigenic ghrelin and anorectic obestatin, are derived from the same hormone precursor. They proposed that no obvious phenotypes in ghrelin knockout mice were due to the lack of both ghrelin and obestatin. Moreover, obestatin is the endogenous ligand for GPR39, an orphan GPCR that shows amino-acid sequence homology to ghrelin, motilin, neurotensin and neuromedin U receptors (McKee et al. 1997b).

However, several reports that followed raised objections to obestatin in its action and the matched receptor (Chartrel et al. 2007; Gourcerol et al. 2007; Holst et al. 2007; Lauwers et al. 2006; Nogueiras et al. 2007; Seoane et al. 2006).

The amino acid sequences of mammalian obestatins are well conserved. However, in non-mammalian species the obestatin parts are not conserved, while the ghrelin parts are well conserved. Moreover, the original paper on obestatin reported that the C-terminal amide structure is essential for obestatin to bind and activate GPR39, however, the precursor parts that seem to contain non-mammalian obestatins lack the Gly residue for the amide formation. Thus, non-mammalian obestatins, if they were contained in the stomach, are not of C-terminal amide structure. Furthermore, the general processing sites for the prohormone convertases, such as Arg-Arg or Lys-Arg, were not found in the non-mammalian obestatin parts. In addition, if both ghrelin and obestatin are processed from the same ghrelin precursor protein, the amount and secretion of both ghrelin and obestatin should be of a similar level and manner. However, the plasma content of ghrelin is higher than that of obestatin and after fasting plasma obestatin concentration did not change while ghrelin concentration was increased. Thus, it is likely that obestatin is not produced by a proper processing of the ghrelin precursor, but by a non-specific protease digestion. Future studies are needed to elucidate the role of obestatin.

11 Epilogue

Seven years have past since the discovery of ghrelin and since that time intensive research has been carried out on ghrelin. However, there remain many interesting questions regarding ghrelin-related biology. These include the identification of the pathways regulating ghrelin's production and release from the stomach, the enzyme that catalyzes its acyl-modification, as well as the continuing search for its physiological actions. Further research will answer these questions and elucidate the biochemical and physiological characteristics of this unique hormone.

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