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Ghrelin: Structure and Function

MASAYASU KOJIMA AND KENJI KANGAWA

Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Fukuoka; Department of Biochemistry, National Cardiovascular Center Research Institute, Suita, Osaka; and Translational Research Center, Kyoto University Hospital, Kyoto, Japan

Kojima, Masayasu, and Kenji Kangawa. Ghrelin: Structure and Function. *Physiol Rev* 85: 495-522, 2005; doi:10.1152/physrev.00012.2004.—Small synthetic molecules called growth hormone secretagogues (GHSs) stimulate the release of growth hormone (GH) from the pituitary. They act through the GHS-R, a G protein-coupled receptor whose ligand has only been discovered recently. Using a reverse pharmacology paradigm with a stable cell line expressing GHS-R, we purified an endogenous ligand for GHS-R from rat stomach and named it "ghrelin," after a word root ("ghre") in Proto-Indo-European languages meaning "grow." Ghrelin is a peptide hormone in which the third amino acid, usually a serine but in some species a threonine, is modified by a fatty acid; this modification is essential for ghrelin's activity. The discovery of ghrelin indicates that the release of GH from the pituitary might be regulated not only by hypothalamic GH-releasing hormone, but also by ghrelin derived from the stomach. 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 bloodstream 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 GH release and energy homeostasis in vertebrates.

I. INTRODUCTION

Growth hormone (GH), a multifunctional hormone secreted from somatotrophs of the anterior pituitary, regulates overall body and cell growth, carbohydrate-protein-lipid metabolism, and water-electrolyte balance (10, 34). Production and release of GH are controlled tightly but occasionally fall into imbalance; GH excess results in acromegaly and gigantism, whereas its deficiency in children results in impaired growth and short stature. GH is controlled by many factors, in particular by two hypothalamic neuropeptides; GH release is stimulated by hypothalamic GH-releasing hormone (GHRH) and inhibited by somatostatin (5, 165). Recently, however, a third independent pathway regulating GH release has been identified from studies of GH secretagogues (GHSs) (26, 66, 87, 126, 222). GHSs are synthetic compounds that are potent stimulators of GH release, working through a G protein-coupled receptor (GPCR), the GHS-receptor (GHS-R) (111, 138, 186). Because GHSs are a group of artificial compounds and do not exist naturally, it was postulated that there must exist an endogenous ligand that binds to GHS-R and carries out similar functions to GHSs in situ (198, 218, 220, 221).

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 (157, 195), orexin/hypocretin (206), prolactin-releasing peptide (105), apelin (237), metastin (178), neuropeptide B (81, 232), and neuropeptide W (210, 232). Figure 1 describes this orphan-receptor strategy used to identify endogenous ligands (44). First, a cell line is established that stably expresses an orphan GPCR. Then, a peptide extract is applied to the cell 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. Under monitor of this assay system, the endogenous ligand can be purified through several chromatographic steps. In this way, orphan receptors represent important new tools for the discovery of novel bioactive molecules and in drug development (45, 112, 262).

Among the numerous orphan GPCR receptors awaiting study several years ago, GHS-R attracted the attention of many academic and industrial scientists, since its endogenous ligand could potentially be used directly for treatment of GH deficiency. Unlike other orphan GPCRs,

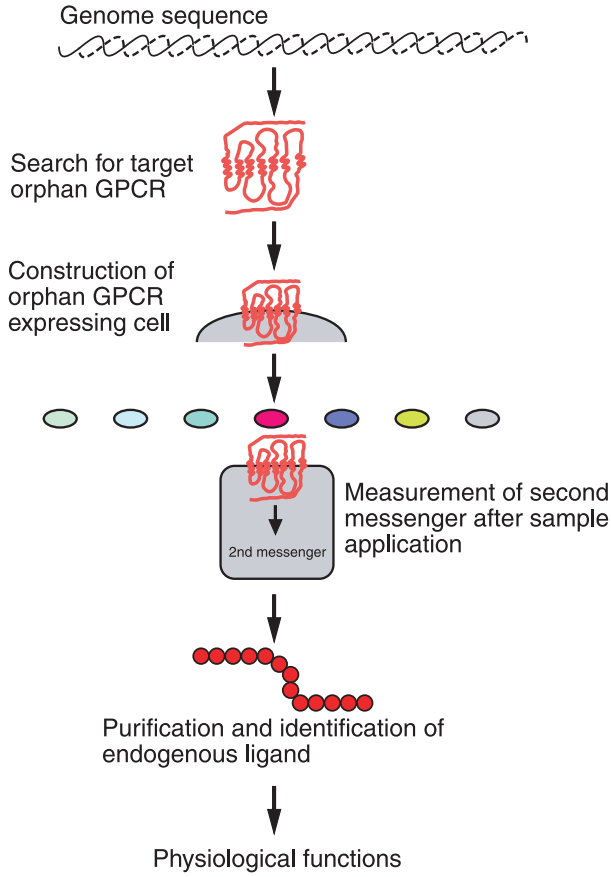


FIG. 1. Orphan receptor strategy. A number of G protein-coupled receptors (GPCRs) have been found in the genomes of mammals and fishes and even in *Caenorhabditis elegans*. These GPCRs can be expressed in cultured cells and their activation is monitored using an assay that measures second messenger changes. These assay systems can be used to purify endogenous ligands of these GPCRs, and the ligand structures are determined. After these steps, the physiological functions of these ligands are examined. Thus the orphan receptor strategy is the reverse of classical strategies in hormone research, in which physiological functions of a putative ligand are used to develop assays to purify them, after which they are used to identify their receptors.

GHS-R was known to bind artificial ligands, such as GHRP-6 or hexarelin, providing a convenient positive control for any screening assay (111, 186). 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 (24, 93). Unexpectedly, we succeeded in the purification and identification of the endogenous ligand for the GHS-R from the stomach and named it "ghrelin" (133, 135). Ghrelin is a GH-releasing and appetite-stimulating peptide (139). Here we review the purification, structure, distribution, and physiological functions of ghrelin (Table 1).

TABLE 1. *Essential chronology of ghrelin research*

Year	Research	Investigators	Reference No.
1954	Discovery of X/A-like cell	Davis	59
1976	First GHS	Bowers et al.	27
1982	Discovery of GHRH	Guillemin et al.	94
		Rivier et al.	197
1992–1993	Cloning of GHRH receptor	Mayo et al.	153
		Gaylinn et al.	84
1984	Development of potent peptidyl GHS, HPRP-6	Bowers et al.	28
1993	First nonpeptidyl GHS	Smith et al.	217
1996	Cloning of GHS receptor	Howard et al.	111
1999	Discovery of ghrelin	Kojima et al.	133
2000–2001	Orexigenic activity of ghrelin	Tschop et al.	246
		Nakazato et al.	173
		Shintani et al.	211
		Wren et al.	266
		Kamegai et al.	122
2002	High plasma ghrelin in anorexia nervosa	Ariyasu et al.	11
		Otto et al.	179
2003	High plasma ghrelin in Prader-Willi syndrome	Cummings et al.	49
2003	Hypothalamic circuit of ghrelin cell	Cowley et al.	48
200X	Discovery of ghrelin acyl-modifying enzyme?		
200X	Clinical use of ghrelin?		

GHS, growth hormone secretagogue; GHRH, growth hormone-releasing hormone.

II. HISTORY OF GROWTH HORMONE SECRETAGOGUE AND ITS RECEPTOR

In 1976, C. Y. Bowers and co-workers found that some opioid peptide derivatives that did not exhibit any opioid activity instead had weak GH-releasing activity, and were referred to as GHSs (27, 61). The structure of the first GHS was Tyr-D-Trp-Gly-Phe-Met-NH_2, which induced GH release by directly acting on the pituitary. This synthetic peptide was a methionine enkephalin derivative, in which the second Gly was replaced with a D-Trp, and the COOH terminus had an amide structure. After the discovery of ghrelin, it was revealed that bulky hydrophobic side-chain groups are important for its activity (161). Thus the D-Trp in the aforementioned GHS was probably a core structure mediating its binding to the GHS receptor, which had not been yet identified at that time. The GH-releasing activity of early GHSs was very weak and was only observed in vitro. However, their discovery led to the synthesis of many peptidyl derivatives, in a search for more GHSs with more potent activity.

In 1984, a potent GHS, GHRP-6, was synthesized based on conformational energy calculations in conjunction with peptide chemistry modifications and a biological activity assay (28). A hexapeptide, GHRP-6, was shown to be active both in vitro and in vivo, which suggested its possible application for clinical use (9, 88, 158).

In 1993, the first nonpeptide GHS, L-692,429, was synthesized by R. G. Smith and co-workers (43, 217). This nonpeptide GHS suggested a possibility for the clinical use of GHSs, and another nonpeptide GHS, L-163,191 (MK-0677), was practically applied for clinical studies, since it retained sufficient activity even when orally administered (183, 239).

During this period, researchers investigated the mechanisms of GHS action. Whereas GH release from the pituitary was known to be stimulated by hypothalamic GHRH, exogenous GHSs were thought to induce GH release through a pathway different from that of GHRH (4, 25, 41, 42, 187). GHRH acts on the GHRH receptor to increase intracellular cAMP, which serves as a second messenger. On the other hand, GHSs were found to act on a different receptor, increasing intracellular Ca^{2+} concentration via an inositol 1,4,5-trisphosphate (IP_3) signal transduction pathway (Fig. 2).

In 1996, the GHS-R was identified by expression cloning using a strategy based on the findings that GHSs stimulate phospholipase C, resulting in an increase in IP_3 and intracellular Ca^{2+} (111). *Xenopus* oocytes were injected with in vitro-transcribed cRNAs derived from swine pituitary, supplemented simultaneously with various Gα subunit mRNAs. MK0677-stimulated Ca^{2+} increase could be detected by bioluminescence of the jellyfish photoprotein aequorin, which was expressed by the *Xenopus* oocytes. The identified GHS-R is a typical GPCR. In situ hybridization analyses showed that GHS-R is expressed in the pituitary, hypothalamus, and hippocampus (24, 93, 111). This receptor was for some time an example of an orphan GPCR; that is, a GPCR with no known natural ligand. After identification of the GHS-R, a search for its endogenous ligand was actively undertaken, using the orphan receptor strategy (Fig. 1).

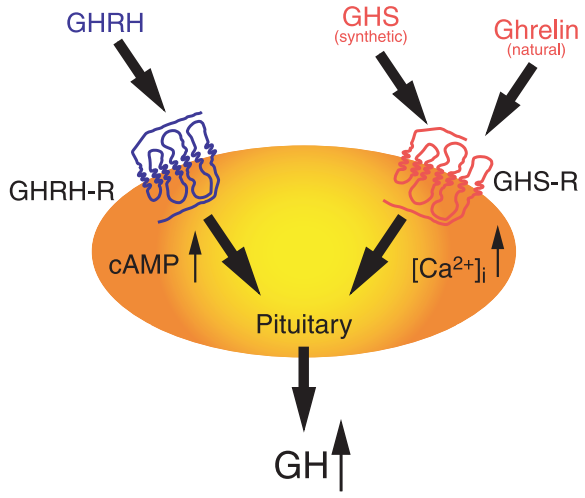


FIG. 2. Regulation of growth hormone release from the pituitary. In pituitary somatotroph cells, growth hormone (GH)-releasing hormone (GHRH) stimulates GH release through binding to the GHRH receptor and increasing cAMP levels. In contrast, GH secretagogues (GHSs) stimulate GH release through the GHS receptor (GHS-R or ghrelin receptor) to increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels. Because GHSs are artificial molecules and do not exist in nature, an endogenous ligand for the GHS-R was postulated but remained unknown until the discovery of ghrelin.

III. PURIFICATION AND IDENTIFICATION OF GHRELIN

A. Purification and Structure of Ghrelin

Because the ligands of most GPCRs are unknown, assays for their activity generally have no positive controls. GHS-R, however, was known to bind several artificial ligands, such as GHRP-6, hexarelin, or nonpeptide GHS MK-0677, providing a convenient positive control for constructing the assay system used to search for the endogenous ligand (111, 186). 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 (133).

Ghrelin 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 (108). 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. 3). Ghrelin is the first known case of a peptide hormone modified by a fatty acid. Rat and human ghrelins differ in only two amino acid residues (133). There is no structural homology between ghrelin and peptide GHSs such as GHRP-6 or hexarelin.

In rat stomach, a second type of ghrelin peptide has been purified and identified as des-Gln14-ghrelin (108). 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 addition, *n*-decenoyl (C10:1)-modified ghrelin exists in the stomach in small amounts.

In the course of purifying human ghrelin from the stomach, we also isolated several minor forms of the peptide (109). These could be classified into four groups by the type of acylation observed at Ser3: nonacylated,

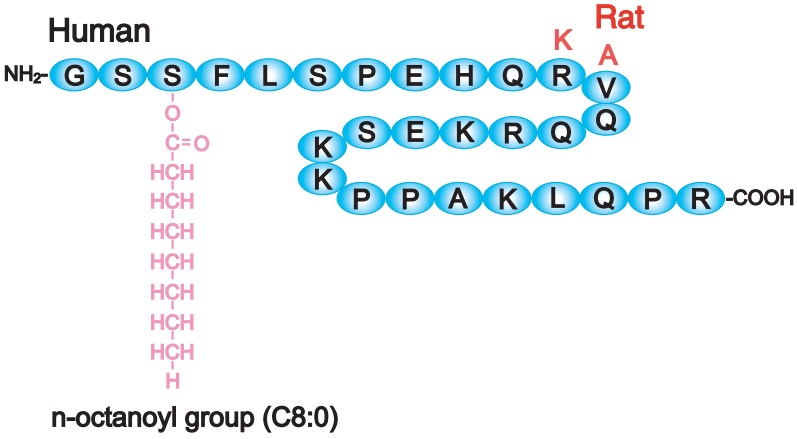


FIG. 3. Structures of human and rat ghrelins. Both human and rat ghrelins are 28-amino acid peptides, in which Ser3 is modified by a fatty acid, primarily *n*-octanoic acid. This modification is essential for ghrelin's activity.

octanoylated (C8:0), decanoylated (C10:0), and possibly decenoylated (C10:1). All peptides found were either 27 or 28 amino acids in length, the former lacking the COOH-terminal Arg28, and are derived from the same ghrelin precursor through two alternative pathways. As was the case in the rat, the major active form of human ghrelin is a 28-amino acid peptide with octanoylated Ser3. Synthetic octanoylated and decanoylated ghrelins stimulate the increase of intracellular Ca^{2+} in GHS-R-expressing cells and stimulate GH release in rats to a similar degree.

B. Des-acyl Ghrelin

The nonacylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in both stomach and blood (107). In blood, des-acyl ghrelin circulates in amounts far greater than acylated ghrelin. It is often observed that not only active, but also inactive, forms of peptide hormones exist in our body. Because the clearance rates of inactive forms of peptide hormones are often reduced, their half-lives are often longer than those of their respective active forms. For example, a preform of adrenomedullin exists in the bloodstream that retains a COOH-terminal Gly that is used for amidation in active adrenomedullin (131).

Ghrelin in the plasma binds to high-density lipoproteins (HDLs) that contain a plasma esterase, paraoxonase, and clusterin (21). Because a fatty acid is attached to the Ser3 of ghrelin via an ester bond, paraoxonase, a potent esterase, may be involved in deacylation of acyl-modified ghrelin. Thus des-acyl ghrelin may represent either a preform of acyl-modified ghrelin or the product of its deacylation.

Des-acyl ghrelin does not replace radiolabeled 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 human. Thus one question is whether there is a specific receptor for des-acyl ghrelin and whether des-acyl ghrelin has specific functions distinct from those of acyl-modified ghrelin. Baldanzi et al. (15) have suggested the existence of another ghrelin receptor in the cardiovascular system. 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. Moreover, it has been reported that des-acyl ghrelin shares with active acyl-modified ghrelin some nonendocrine actions, including the modulation of cell proliferation and, to a small extent, adipogenesis (35). Further study is required to determine whether des-acyl ghrelin is biologically active and binds to an as-yet-unidentified receptor.

C. Mammalian Ghrelins

In mammals, ghrelin homologs have been identified in human (133), rhesus monkey (8), rat (133), mouse (233), mongolian gerbil (GenBank accession no. AF442491), cow (GenBank accession no. AB035702), pig (GenBank accession no. AB035703), sheep (GenBank accession no. AB060699), and dog (241) (Fig. 4). The amino acid sequences of mammalian ghrelins are well conserved; in particular, the 10 amino acids in their NH_2 termini are identical. This structural conservation and the universal requirement for acyl-modification of the third residue indicate that this NH_2-terminal region is of central importance to the activity of the peptide.

Bovine and ovine ghrelins are 27-amino acid peptides that, like rat des-Gln14 ghrelin, lack the Gln14 residue. In the genes encoding these ghrelins, there is only one AG splice acceptor site between exons 2 and 3, resulting in the production of only one mRNA that gives rise the 27-residue ghrelin.

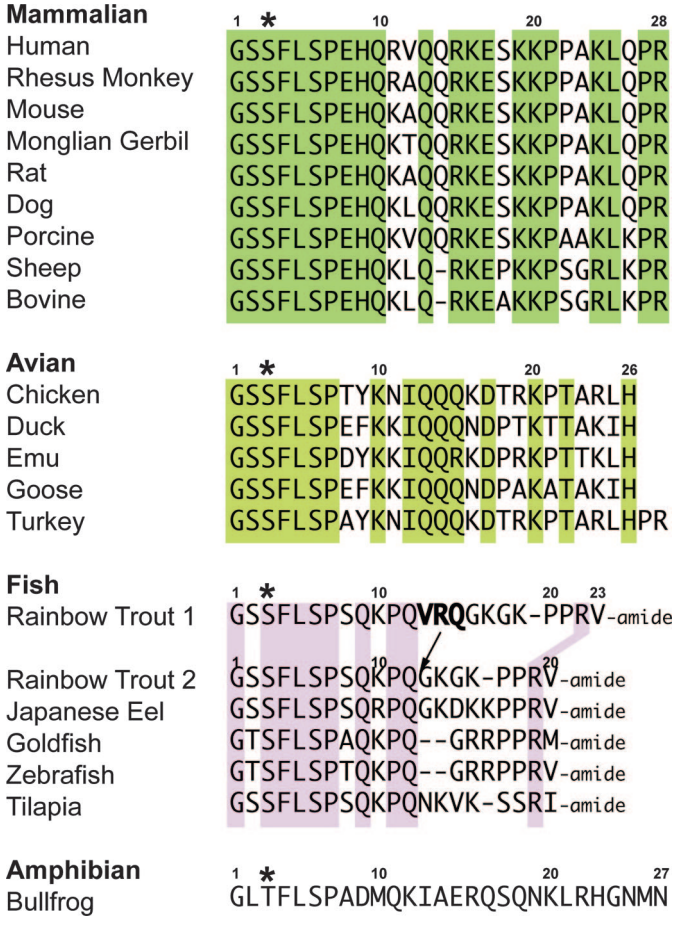


FIG. 4. Sequence comparison of vertebrate ghrelins. Identical amino acids in each species of mammal, bird, and fish are colored. The asterisks indicate acyl-modified third amino acids. NH_2-terminal cores with acyl-modification sites are well conserved among all vertebrate ghrelins.

D. Ghrelin and Motilin Family

As described in section vA, the ghrelin receptor is most homologous to the motilin receptor (74, 156). Accordingly, the amino acid sequence of ghrelin has homology with that of motilin, another gastric peptide with gastric contractile activity (14, 65). 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 et al. (240) reported the identification of a gastric peptide, motilin-related peptide (MTLRP). 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 (47, 64, 78).

Interestingly, the region of homology between ghrelin and motilin lies not near the NH_2 terminus, where ghrelin's acyl-modification occurs, but in their respective central regions.

Ghrelin and motilin play similar roles in the stomach. Both peptides stimulate gastric acid secretion and gastric movement (149).

Thus ghrelin and motilin are structurally and functionally considered to compose a peptide superfamily and may have evolved from common ancestral gene (64, 78).

E. Gene and Precursor Structures of Ghrelin

Figure 5 describes the processing from the ghrelin gene to the active ghrelin peptide. 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 (222).

The 5′-flanking region of the human ghrelin gene contains a TATA box-like sequence (TATATAA; −585 to −579), as well as putative binding sites for several transcription factors, such as AP2, basic helix-loop-helix (bHLH), PEA-3, Myb, NF-IL6, hepatocyte nuclear factor-5, and NF-κB, and half-sites for estrogen and glucocorticoid response elements (124, 130, 233). However, neither mutation nor deletion of the TATA box-like element de-

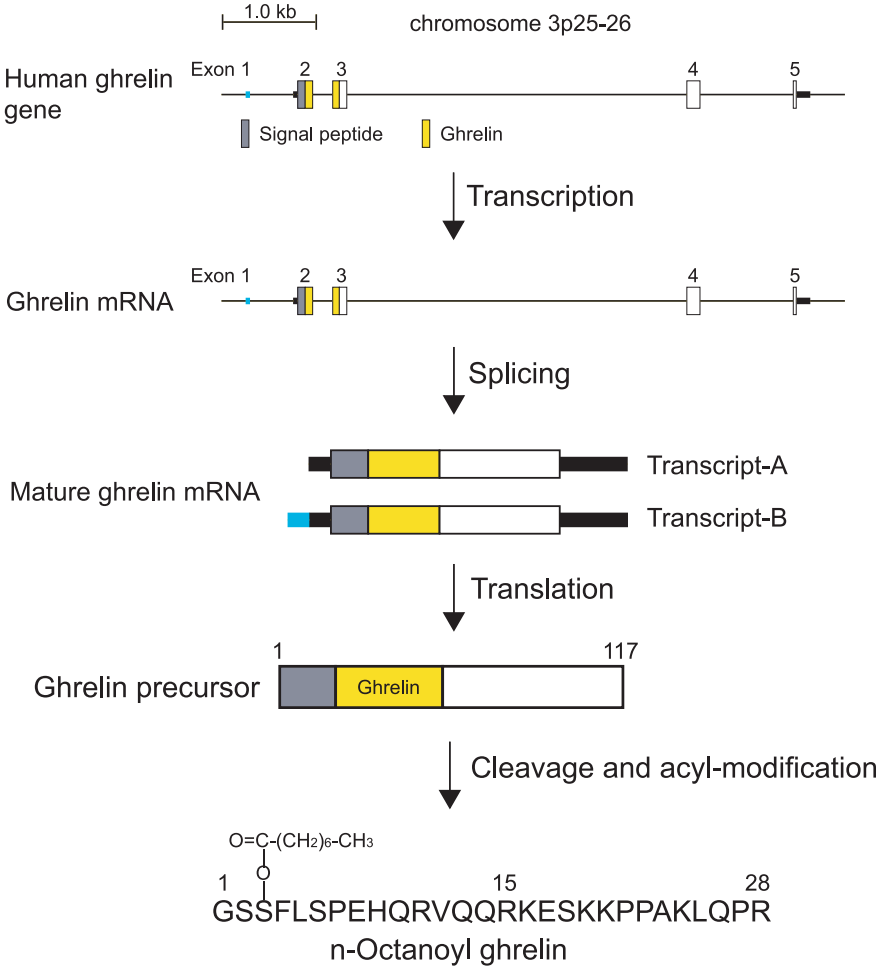


FIG. 5. From the human ghrelin gene to an active peptide. The human ghrelin gene comprises five exons. The first exon encodes the 5′-untranslated region and is very short. cDNA analyses of human ghrelin have revealed that transcript A, an alternative splicing product from exon 2 to exon 4, is the main form of human ghrelin mRNA in vivo. This mRNA is translated into a 117-amino acid ghrelin precursor (preproghrelin). Protease cleavage and acyl-modification of the ghrelin precursor result in the production of a 28-amino-acid-long active acyl-modified ghrelin peptide. In rat, mouse, and pig, another splicing variant encoding des-Gln14-ghrelin is produced by alternative splicing at the end of intron 2.

creased the promoter activity, suggesting that this element is not used. There was neither a typical GC nor a CAAT box.

Studies of ghrelin promoter activity in TT cells, a human thyroid medullary carcinoma cell line, revealed the presence of activating sequences within −1509 to −1110 and −349 to −193 in the 5′-flanking region of ghrelin gene (124). Another report by Nakai et al. (172) using TT cells showed that significant level of promoter activity was observed in the 1107–1225 bp upstream region of the translation initiation site, and specific protein binded to the promoter region of −1129 to −1100. Furthermore, a study by Kishimoto et al. (130) using ECC10 cells, a human stomach-derived cell line, indicated that −2000 to −605 in the 5′-flanking region of the ghrelin gene contains an activating sequence (130). These results suggest that ghrelin gene expression may be cell-type specific.

In the 5′-flanking region of the ghrelin gene, several E-box consensus sequences exist (124). Destruction or site-directed mutagenesis of these sites decreased the promoter activity in TT cells, implicating them in promoter activation. Upstream stimulatory factors (USF), members of the bHLH-LZ family of transcription factors, bind to these E-box elements and may thus regulate human ghrelin gene expression.

Ghrelin promoter activity in ECC10 cells was stimulated by glucagon and its second messenger cAMP (130). These results suggest that in fasting conditions, a high level of ghrelin production may be related to increased glucagon.

The human ghrelin gene, like the mouse gene, comprises five exons (124, 233). 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) (124).

The 28 amino acids of the functional ghrelin peptide are encoded in exons 1 and 2. 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 (108). 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.

Complementary DNA analyses indicated that des-Gln14-ghrelin cDNA also exists in human stomach (GenBank accession no. AB035700). However, the number of human des-Gln14-ghrelin cDNA clones is low, and des-Gln14-ghrelin peptides have not yet been isolated from stomach tissue. Moreover, two cDNA clones from *Homo sapiens* fetus library that code for human des-Gln14-ghrelin are deposited in the NCBI nucleotide data base (AI338429 and BY149645). There are two types of porcine ghrelin cDNA, which encode ghrelin and des-Gln14-ghrelin, that are present at an approximate ratio of 1:1 (GenBank accession nos. AB035703 and AB035704). In the cow, only one ghrelin mRNA exists, and it encodes a 27-amino acid ghrelin.

Moreover, another splicing variant was expressed in the mouse testis (234). This variant, a ghrelin gene-derived transcript (GGDT), comprises the 68-bp 5′-unique sequence and the exons 4 and 5 of mouse ghrelin gene. GGDT encodes 12 amino acid residues, which is an unrelated sequence to the mouse ghrelin precursor, and the COOH-terminal 42-amino acid sequence of mouse ghrelin precursor. The 5′-unique sequence of GGDT is located between exons 3 and 4 of the ghrelin gene, indicating that GGDT is generated by alternative usage of the 68-bp exon as the testis-specific first exon. Because GGDT does not encode the ghrelin sequence, its function is not clear.

The amino acid sequences of mammalian ghrelin precursors are well conserved (Fig. 6). 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 (208, 225), the COOH terminus of the ghrelin peptide sequence is processed at an uncommon Pro-Arg recognition site.

F. Putative Ghrelin Acyl-Modifying Enzyme

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.

Our group has reported recently that 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. 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 (Kojima, unpublished data).

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 (192, 193). Members of the serine acyltransferase family

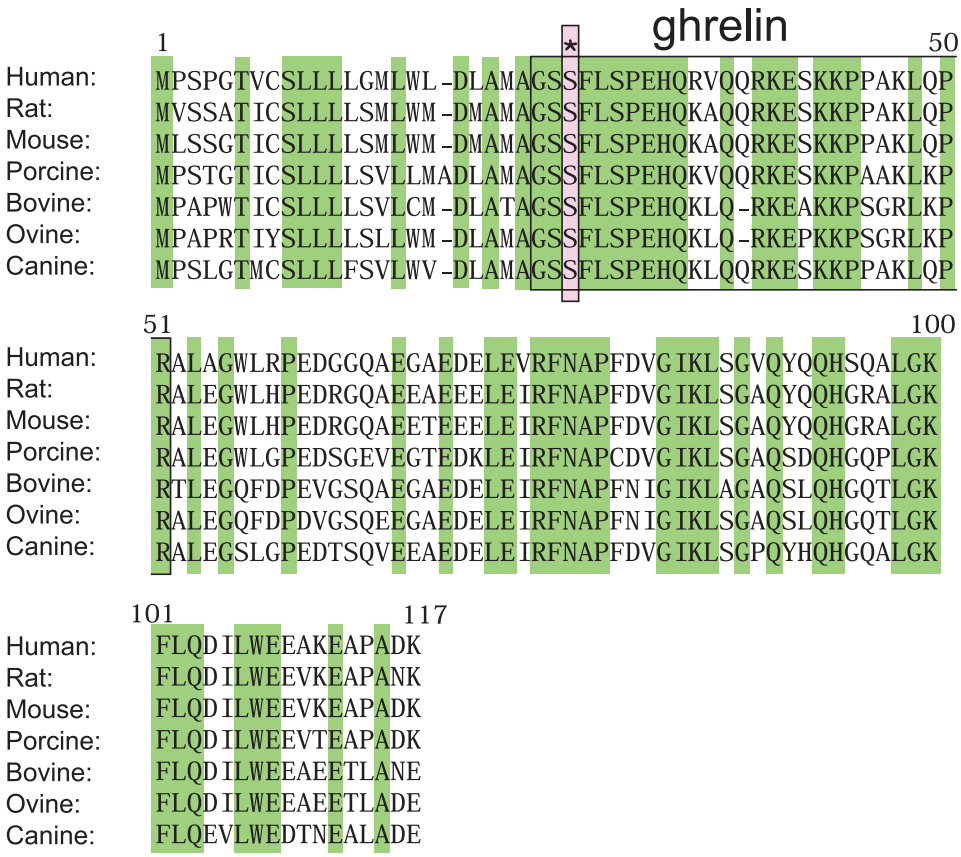


FIG. 6. Amino acid sequences of mammalian ghrelin precursors. A sequence comparison between mammalian ghrelin precursors is shown. Identical amino acids are colored. The asterisk shows the position of the acyl-modified Ser3. Note that the amino acid sequences of mammalian ghrelin precursors are well conserved; in particular, the NH_2-terminal 10 amino acids of all of these active ghrelin peptides, each of which contains an acyl-modified serine in its active core, are identical.

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 (95) and a plant Ser *O*-acetyltransferase gene family in *Arabidopsis thaliana* (114). An acyl transferase has also been purified from the gastric mucosa of rat (127, 215). This enzyme is an integral rough microsomal protein, catalyzing the transfer of acyl-CoA to mucosal proteins. 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 modifcation seen in ghrelin.

G. Ghrelin Derivatives

Chemical synthesis of ghrelin derivatives revealed that bulky hydrophobic groups attached to the side chain of the third amino acid residue are essential for maximum activity of ghrelin (22, 150). Elongation by two carbons in the acyl modification of ghrelin, the maximum response was observed when ghrelin was modified by the *n*-octanoyl group. Substantial activity was retained when ghrelin was modified by *n*-lauroyl or palmitoyl groups. Modification of ghrelin Ser3 by an unsaturated or a branched fatty acid, such as 3-octenoyl (C8:1) or 4-methylpentanoyl, respectively, also retained activity. Moreover, a ghrelin derivative in which the third amino acid residue was replaced with an aromatic amino acid, Trp, still retained weak activity. Interestingly, alignment of the amino acid sequences of GHRP-6 and ghrelin revealed three-dimensional structural similarity between Trp4 in the active core of GHRP-6 and the acyl-modified Ser3 of ghrelin (151).

Short peptides derived from the first four residues of ghrelin, Gly-Ser-Ser(n-octanoyl)-Phe-NH_2, could activate the ghrelin receptor, but the first three alone could not, indicating that the four-residue peptide is the minimum segment necessary for receptor activation (22, 150, 151). One of the smallest molecules that retains almost full ghrelin activity is Ape-Ser(Octyl)-Phe-Leu-aminoethylamide (mol wt 618.9), in which Ape is 5-aminopentanoic acid (151).

The ester bond between the side chain of Ser3 and *n*-octanoic acid is not essential for ghrelin's activity (150). Activity was retained in ghrelin derivatives in which the ester bond between octanoic acid and the Ser3 side chain was changed to a more chemically stable thioether [Cys3(octyl)] or ether [Ser3(octyl)] bond, as well as in a derivative containing 2,3-diaminopropionic acid in the

third position, to which an *n*-octanoyl group was attached through an amide bond.

H. Nonmammalian Ghrelin

1. Amphibian ghrelin

A) BULLFROG GHRELIN. Three molecular forms of ghrelin have been identified in the bullfrog stomach (117) (Fig. 4). They contain either 27 or 28 amino acids and possess 29% sequence identity to human ghrelin. The difference in amino acid length is not due to alternative splicing, but to differential processing of the COOH-terminal Asn residue. A unique third residue (Thr3) in bullfrog ghrelin differs from the Ser3 in the mammalian ghrelins. Because serine and threonine both possess hydroxyl groups on their side chains, they can both be modified by fatty acids. Indeed, the bullfrog Thr3 is modified by either *n*-octanoic or *n*-decanoic acid.

Northern blot analysis demonstrated that bullfrog ghrelin mRNA is predominantly expressed in the stomach. Low levels of gene expression were observed in the heart, lung, small intestine, gallbladder, pancreas, and testis. Brain distribution of ghrelin was investigated in detail in the frog *Rana esculenta* (83). In the brain, sparse ghrelin-positive cells were detected in three nuclei of the diencephalon: the suprachiasmatic nucleus and the posterior tuberculum in the hypothalamus and the posterodorsal aspect of the lateral nucleus in the thalamus. A few ghrelin-immunoreactive neurons were also found in the mesencephalon, in the pretoral gray and the anterodorsal tegmental nucleus. Ghrelin-containing fibers are widely distributed in the frog brain. In particular, diffuse networks of immunoreactive processes were observed in various regions of the telencephalon, including the medial pallium, the striatum, the nucleus of the diagonal band of Broca, the nucleus accumbens, and the amygdala.

Bullfrog ghrelin stimulated the secretion of both GH and prolactin in dispersed bullfrog pituitary cells with a potency two to three orders of magnitude greater than that of rat ghrelin (117). These results indicate that although the ability of ghrelin to induce GH secretion is evolutionary conserved, the structural differences between the different ghrelins result in species-specific receptor binding.

2. Bird ghrelin

A) CHICKEN GHRELIN. Chicken (*Gallus gallus*) ghrelin is 26 amino acids long and possesses 54% sequence identity with human ghrelin (Fig. 4) (121). The serine residue at position 3 (Ser3) is conserved between the chicken and mammalian species, as is its acylation by either *n*-octanoic or *n*-decanoic acid. Chicken ghrelin mRNA is predominantly expressed in the stomach, where it is present in the proventriculus but absent in the gizzard. RT-PCR analysis revealed low levels of expression in the brain, lung, and intestine. Administration of chicken ghrelin increased plasma GH levels in both rats and chicks, with a potency similar to that of rat or human ghrelin (3, 19, 121). In addition, chicken ghrelin also increased plasma corticosterone levels in growing chicks at a lower dose than in mammals (121).

Ghrelin stimulates feeding in rats; however, intracerebroventricular injection of ghrelin strongly suppressed feeding in neonatal chicks (82). This anorexic effect was almost identical when chicken or rat ghrelin was administered. Intracerebroventricular injection of GHRP-2 (KP-102), a synthetic GHS, also inhibited feeding (202). These results indicate that food intake of neonatal chicks is inhibited by GHS-R agonists. Why ghrelin suppresses rather than stimulates food intake in neonatal chicks remains to be elucidated.

B) OTHER AVIAN GHRELINS. Other avian ghrelins have been identified in duck, goose, emu, and turkey (Fig. 4). The precursors of all avian ghrelins except that in turkey possess a pair of basic amino acids, Arg-Arg, for the COOH-terminal processing site of the mature ghrelin peptide. Turkey ghrelin has a Pro-Arg processing signal at this location, similar to its mammalian homologs.

3. Fish ghrelins

Fish ghrelins have been identified either by purifying peptides from stomachs or by cDNA cloning analyses in rainbow trout (118), eel (119), tilapia (120, 182), and goldfish (Fig. 4) (254). Fish ghrelins exist in multiple forms that vary in amino acid length and their specific acyl-modifications.

A) RAINBOW TROUT. Rainbow trout ghrelin was purified and identified from the stomach (118) (Fig. 4). Four isoforms of ghrelin peptide were isolated: a 24-amino-acid-long COOH-terminal amidated form (rt ghrelin 1)(GSSFLSPSQKPQVRQGKGKPPRV-amide); des-VRQ-rt ghrelin (rt ghrelin 2), in which three amino acids (V13R14Q15) are deleted; and two other forms that retain an additional glycine residue at their COOH termini, rt ghrelin-Gly, and des-VRQ-rt ghrelin-Gly. The third serine residue was modified by octanoic acid, decanoic acid, or unsaturated forms of those fatty acids. In agreement with the isolated peptides, two cDNAs of different lengths were isolated. The rt ghrelin gene has five exons and four introns, and two different mRNA molecules are produced by alternative splicing of the gene. A high level of ghrelin mRNA expression was detected in the stomach, and moderate levels were detected in the brain, hypothalamus, and intestinal tracts. Des-VRQ-rt ghrelin stimulated the release of GH but not of prolactin and somatolactin in rainbow trout in vivo and in vitro.

B) EEL GHRELIN. Eel ghrelin was purified from stomach extracts of a teleost fish, the Japanese eel (*Anguilla japonica*), and was found to contain an amide structure at its COOH-terminal end (119) (Fig. 4). Two molecular forms of ghrelin, each containing 21 amino acids, were identified by cDNA and mass spectrometric analyses. Northern blot and RT-PCR analyses revealed high gene expression in the stomach. Additionally, RT-PCR analysis revealed low levels of expression in the brain, intestines, kidney, and head kidney. Eel ghrelin-21 at a dose of 0.1 nM stimulated the release of GH and prolactin (PRL) from organ-cultured tilapia pituitary.

C) TILAPIA GHRELIN. Tilapia ghrelin was identified from the stomach of a euryhaline tilapia, *Oreochromis mossambicus* (120) (Fig. 4). The sequence of the 20-amino acid tilapia ghrelin is GSSFLSPSQKPQNKVKSSRI. The third serine residue is modified by *n*-decanoic acid. The COOH-terminal end of the peptide possesses an amide structure. RT-PCR analysis revealed high levels of gene expression in the stomach and low levels in the brain, kidney, and gill. Tilapia ghrelin stimulated GH and PRL release from organ-cultured tilapia pituitary at a dose of 10 nM.

D) GOLDFISH GHRELIN. Goldfish ghrelin was identified by cDNA analyses using rapid amplification of cDNA ends (RACE) and reverse transcription (RT)-polymerase chain reaction (PCR) (254) (Fig. 4). The 490-bp cDNA encodes a 103-amino acid preproghrelin comprising a 26-amino acid signal region, a 19-amino acid mature peptide sequence, and a 55-amino acid COOH-terminal region. The mature goldfish ghrelin peptide has two putative cleavage sites and amidation signals (GRR), one after 12 amino acids and the other after 19 residues. Structurally, the goldfish ghrelin gene resembles that in the human, with four exons and three short introns. Ghrelin mRNA expression was detected in the brain, pituitary, intestine, liver, spleen, and gill by RT-PCR followed by Southern blot analysis and in the intestine by Northern blot. Intracerebroventricular injection of *n*-octanoylated goldfish ghrelin-(1–19) stimulated food intake in goldfish (253).

E) ZEBRAFISH GHRELIN. A BLAST search of the zebrafish genomic database identified a zebrafish ghrelin with a sequence of GTSFLSPTQKPQGRRPPRV (GenBank accession no. AL918922) (Fig. 4). The 19-amino acid zebrafish ghrelin is most homologous to goldfish ghrelin, with which it shares a COOH-terminal valine amide structure and a putative cleavage site for amidation signals (GRR) after 12 amino acids.

IV. DISTRIBUTION OF GHRELIN

A. Measurement of Ghrelin Concentration

The active form of ghrelin is acyl-modified; this modification is easily cleaved during sample extraction. Moreover, peptide samples are easily digested by many proteases in cells. Thus, to measure ghrelin concentrations correctly in plasma and tissues, we have to inhibit protease digestion of the ghrelin peptide and cleavage of its acyl-modification.

To measure the plasma concentration of ghrelin, it is necessary to use EDTA and aprotinin when collecting blood samples (106, 107). After the samples are centrifuged, the plasma fraction should be collected and treated with 1/10 volume of 1 N HCl. The treated plasma should be kept in the freezer (−20 to −80°C). These samples are stable for at least 6–12 mo.

To measure the tissue concentration of ghrelin, it is sufficient to inactivate proteases by boiling the tissues in water for 5–10 min (125, 226). This simple method is sufficient to keep active ghrelin intact.

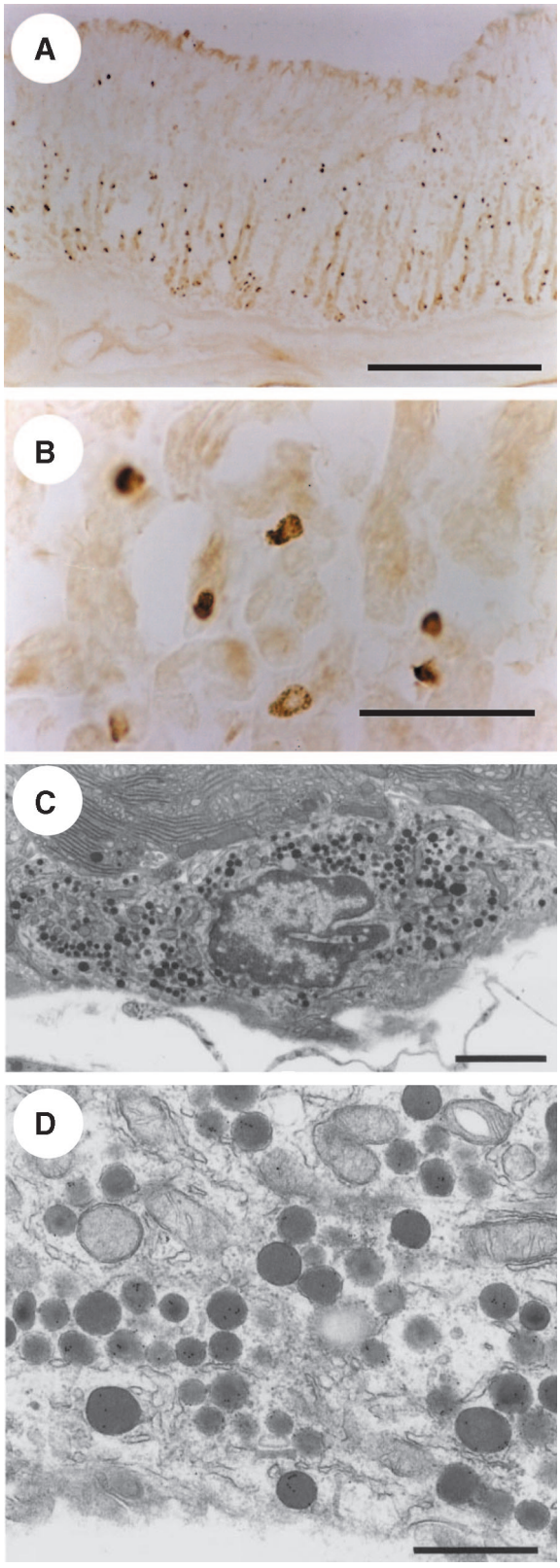
Two major forms of ghrelin are found in tissues and plasma: *n*-octanoyl-modified and des-acyl ghrelin (107). The normal ghrelin concentration of plasma samples in humans is 10–20 fmol/ml for *n*-octanoyl ghrelin and 100–150 fmol/ml for total ghrelin, including both acyl-modified and des-acyl ghrelins. Plasma ghrelin concentration is increased in fasting conditions and reduced after habitual feeding (50, 247), suggesting that ghrelin may be as an initiation signal for food intake or ghrelin secretion is controlled by some nutritional factors in blood. Plasma ghrelin levels were lower in obese subjects than the age-matched lean controls (98, 209, 248). Moreover, plasma ghrelin concentrations were significantly lower in Pima Indians, who are prone to develop insulin resistance and obesity, than in Caucasians (87 ± 28 vs. 129 ± 34 fmol/ml; $P < 0.01$) (248). However, it is unclear whether changes in plasma ghrelin concentration can influence the characteristics of obese people or Pima Indians.

B. Stomach and Gastrointestinal Organs

In all vertebrate species, ghrelin is mainly produced in the stomach (11). In the stomach, ghrelin-containing cells are more abundant in the fundus than in the pylorus (54, 241, 268). In situ hybridization and immunohistochemical analyses indicate that ghrelin-containing cells are a distinct endocrine cell type found in the mucosal layer of the stomach (Fig. 7) (54, 196).

Four types of endocrine cells have been identified in the oxyntic mucosa with the following relative abundances: ECL, D, enterochromaffin (EC), and X/A-like cells (32, 59, 91, 223). The rat oxyntic gland contains ~60–70% ECL cells, 20% X/A-like cells, 2–5% D cells, and 0–2% EC cells; in the human, 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 (Fig. 7) (54, 67, 268). These X/A-like cells account for ~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 (103). As a result, the ghrelin concentration of fetal stomach is also very low and gradually increases after birth until 5 wk of age.



The gastric X/A-like cells can be stained by an antibody that is specific to the NH_2-terminal, acyl-modified portion of ghrelin, indicating that ghrelin in the secretory granules of X/A-like cells has already been acyl-modified. Ghrelin concentration in rat stomach is 377.31 ± 55.83 fmol/ml (*n*-octanoyl ghrelin) and 1,779.8 ± 533.9 fmol/ml (total ghrelin) (107).

Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum, and colon (54, 107, 203). 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 (54).

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 (57). 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 (57, 190, 259, 260).

The pancreatic ghrelin profile changes dramatically during fetal development (38a, 262); 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, six to seven 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 (103). Moreover, pancreatic ghrelin levels are not affected by fasting.

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 (190). Normal murine pancreas also contains a small number of this new islet cell type, epsilon cells.

FIG. 7. Ghrelin cells in the stomach. *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*: high magnification of *A*. Scale bar, 40 μm. *C* and *D*: representative immunoelectron photographs of a ghrelin-producing cell in the oxyntic gland. *C*: this ovoid cell has many round, compact, electron-dense granules in its cytoplasm. Scale bar, 2 μm. *D*: high magnification of *C*. Scale bar, 500 nm. Granules in the cytoplasm are labeled with immunogold staining for ghrelin. [Adapted from Kojima et al. (133) and Date et al. (54).]

C. Brain and Pituitary

Since the ghrelin receptor GHS-R is mainly expressed in the hypothalamus and pituitary, its endogenous ligand has been thought to exist mainly in the hypothalamic regions (93, 111). This is supported by the finding that another GH-releasing peptide, GHRH, is produced in the hypothalamus and is secreted into the hypophysial portal system to stimulate GH release from the pituitary somatotrophs. However, the ghrelin content of the brain is found to be very low (107, 133).

Ghrelin has been found in the hypothalamic arcuate nucleus, an important region for controlling appetite (133, 148). In addition, a recent study has reported the presence of ghrelin in previously uncharacterized hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (48). These ghrelin-containing neurons send efferent fibers to neurons that contain neuropeptide Y (NPY) and agouti-related protein (AgRP) and may stimulate the release of these orexigenic peptides. These localization patterns of ghrelin suggest a role in controlling food intake. In fact, injection of ghrelin into the cerebral ventricles of rats potently stimulates food intake.

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 GHS-R is expressed in pituitary cells (24, 93, 155). Also, ghrelin has been found in the pituitary gland itself (137, 140), where it may influence the release of GH in an autocrine or paracrine manner. The expression level of ghrelin in the pituitary is high after birth and declines with puberty. Pituitary tumors, such as adenomas, corticotroph tumors, and gonadotroph tumors contain ghrelin peptides.

D. Other Tissues

Ghrelin mRNA is expressed in the kidney, especially in the glomeruli (89, 162). Moreover, peptide extracts from mouse kidney contain both *n*-octanoyl and des-acyl ghrelin peptides in significant amounts. The plasma ghrelin concentration was significantly correlated with the serum creatinine level and was increased 2.8-fold in patients with end-stage renal disease compared with those in patients with normal renal function (271). This result suggests that the kidney is an important site for clearance and/or degradation of ghrelin.

Ghrelin-immunoreactive cells were detectable in cytotrophoblast cells in first-trimester human placenta but were undetectable in third-trimester placenta (92). Ghrelin-containing cells were also detected in syncytiotrophoblast cells of the human placenta and in the cytoplasm of labyrinth trophoblasts of the rat placenta. Placental ghrelin mRNA was undetectable during early pregnancy, with a sharp peak of expression at *day 16* that decreases in the later stages of gestation.

Ghrelin immunoreactive cells have been identified in interstitial Leydig cells and in Sertoli cells (18, 238). However, ghrelin levels in Sertoli cells are very low. Moreover, the ghrelin receptor has been detected in germ cells, mainly in pachytene spermatocytes, as well as in somatic Sertoli and Leydig cells (85).

E. Ghrelin-Producing Cells

Several cultured cell lines express ghrelin. Ghrelin is produced in TT cells, a human thyroid medullary carcinoma cell line (123). TT cells express ghrelin mRNA, and both conditioned medium and cellular extracts of TT cells contain ghrelin peptides. As in the stomach, cellular extracts of TT cells contain both *n*-octanoyl ghrelin and des-acyl ghrelin. Other cultured cells that express ghrelin include the kidney-derived cell line NRK-49F (162), gastric carcinoid ECC10 cells (130), and the cardiomyocyte cell line HL-1 (115).

Corebetta et al. (46) reported a patient with a malignant neuroendocrine pancreatic tumor with ghrelin immunoreactivity and a high circulating ghrelin level. A patient with a metastasizing gastric neuroendocrine tumor was also reported to have extremely high circulating levels of ghrelin (249). In the latter case, the patient developed diabetes mellitus and hypothyroidism. However, it is not clear whether high ghrelin level had the pathophysiological role in these symptoms. In both cases, GH and IGF-I levels were within the normal range, and the patients had no clinical features of acromegaly.

V. GHRELIN RECEPTOR

A. The Ghrelin Receptor Family

Ghrelin receptor, or GHS-R, is a typical GPCR with seven transmembrane domains (7-TM) (111, 155, 218). Two distinct ghrelin receptor cDNAs have been isolated (111). 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 posttranslational 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 (111). The GHS-R gene consists of two exons; the first exon encodes TM-1 to

TM-5, and the second exon encodes TM-6 to TM-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 COOH-terminal truncated form of the type 1a receptor and is pharmacologically inactive.

The GHS-R has several homologs, whose endogenous ligands are gastrointestinal peptides or neuropeptides. Figure 8 shows a dendrogram alignment of the ghrelin receptor superfamily. This superfamily contains receptors for ghrelin, motilin, neuromedin U (80, 110, 113, 132), and neurotensin (257). 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 ligand is also likely to be a gastrointestinal peptide (156).

The ghrelin receptor is most homologous to the motilin receptor; the human forms share 52% identical amino acids (74, 116, 219). 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 motilin receptor (53).

The ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, chicken, and pufferfish (Fugu) (180, 218, 219). This strict conservation suggests that ghrelin and its receptor serve important physiological functions.

It is suggested that a novel unidentified subtype of ghrelin receptor exists. Ghrelin binding activity is demonstrated in 3T3-L1 cells by radiolabeled ghrelin, although RT-PCR detected no signal for the ghrelin receptor (273). Moreover, both ghrelin and des-acyl ghrelin bind to H9c2 cardiomyocytes, which do not express the ghrelin receptor (15). However, BLAST searches of the human genome using ghrelin receptor (GHS-R) cDNA as a search sequence have not revealed any ghrelin receptor homologs. Further study is required to search for an as-yet-unidentified ghrelin receptor subtype.

One case of familial short stature associated with a missense mutation in the ghrelin receptor has been reported. 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 (181).

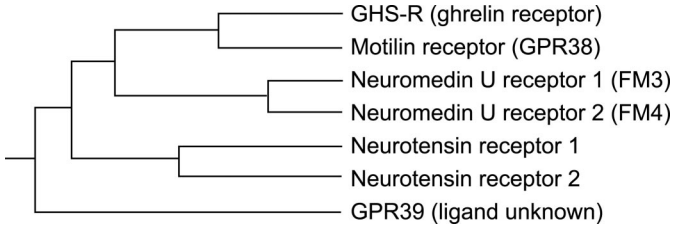


FIG. 8. Dendrogram alignment of ghrelin receptor (GHS-R) and other GPCRs. The ghrelin receptor is part of a GPCR superfamily that contains the motilin, neuromedin U and neurotensin receptors, and is most homologous to the motilin receptor. Because their endogenous ligands, ghrelin and motilin, have partly homologous amino acid sequences, the ghrelin and motilin systems may have evolved from a common ancestral system. This superfamily also contains an orphan receptor, GPR39, whose endogenous ligand is expected to be a peptide.

B. Ghrelin Receptor Activation and Downstream Signal Transduction Pathways

Two endogenous GH-releasing peptides have been identified, ghrelin and GHRH. GHRH acts on the GHRH receptor to activate adenylate cyclase and increase intracellular cAMP, which serves as a second messenger to activate protein kinase A. This indicates that the GHRH receptor is coupled to a G_s subunit. On the other hand, ghrelin acts on the GHS-R and activates phospholipase C to generate IP_3 and diacylglycerol, resulting in an increase of intracellular Ca^{2+}, indicating that the ghrelin receptor is coupled to a G_q subunit.

The signal transduction pathway following ghrelin receptor activation was investigated using HepG2, a hepatoma cell line that responds to ghrelin (166). Ghrelin upregulates several activities that are also potentiated by insulin, including tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), association of the adaptor-molecule growth factor receptor-bound protein 2 with IRS-1 and stimulates mitogen-activated protein kinase activity. However, unlike insulin, ghrelin inhibits Akt kinase and partially reverses the downregulating effect of insulin on phospho*enol*pyruvate carboxykinase (PEPCK) mRNA expression, a rate-limiting enzyme of gluconeogenesis.

C. Ghrelin Receptor Distribution

Ghrelin receptor mRNA is prominently expressed in the arcuate (ARC) and ventromedial nuclei (VMN) and in the hippocampus (93, 111, 173). The ghrelin receptor is highly sensitive to GH; its expression is increased in GH-deficient *dw*/*dw* dwarf rats, and treatment of these rats with GH decreases ghrelin receptor expression (24). GHS-R mRNA is also detected in multiple hypothalamic nuclei and in the pituitary, as well as the dentate gyrus, CA2, and CA3 regions of the hippocampus, the substantia nigra, the ventral tegmental area, and the dorsal and median raphe nuclei.

RT-PCR analyses demonstrated ghrelin receptor mRNA expression in many peripheral organs, including heart, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissue, and immune cells (89, 93, 102, 134), indicating that ghrelin has multiple functions in these tissues (30).

The existence of ghrelin and its receptor in the hippocampus (24, 93), a region that is associated with learning and memory, suggest the role of ghrelin in memrory formation. In fact, intracerebroventricular injection of ghre-

lin induced c-Fos expression in the hippocampal C1, CA2, and C3 regions, indicating that the ghrelin receptor is active in that region (173). The involvement of ghrelin in memory was investigated using open-field, plus-maze, and step-down tests of inhibitory avoidance (33). Ghrelin administration increased freezing in the open field and decreased the number of entries into open spaces and the time spent on the open arms in the plus-maze, indicating that ghrelin has an anxiogenic effect. Moreover, ghrelin increased in a dose-dependent manner the latency time in the step-down test, suggesting that it increases memory retention.

VI. PHYSIOLOGICAL FUNCTIONS OF GHRELIN

A. GH-Releasing Activity

Ghrelin is a multifaceted peptide hormone (see Table 2). Ghrelin acts on the GHS-R, increasing intracellular Ca^{2+} concentration via IP_3 to stimulate GH release. In terms of both the area under the curve and mean peak GH levels, the GH-releasing activity of ghrelin is similar to that of GHRH when injected intravenously into rats (12, 133, 184, 230). However, the maximal stimulation effected by ghrelin is two to three times greater than that of GHRH (12).

Ghrelin stimulates GH release both in vitro and in vivo in a dose-dependent manner (Fig. 9) (12, 13, 55, 133, 184, 230). Intravenous injection of ghrelin induces potent GH release both in rats and in humans. When anesthetized rats were injected intravenously with ghrelin, an increase in plasma GH concentration was observed [basal level: 12.0 ± 5.4 ng/ml; after ghrelin injection: 129.7 ± 11.3 (SE) ng/ml] (133). GH release peaks at ~5–15 min after ghrelin injection and returns to basal levels 1 h later. 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 (55). Thus intracerebroventricular injection appears to be a more potent route of delivery than intravenous administration. Ghrelin has also been shown to induce GH release in nonmammalian vertebrates, including chicken (19, 121), fish (118–120, 254), and frog (117). Together, these in vivo assays confirmed that ghrelin is a potent GH-releasing peptide. In addition, high doses of ghrelin in humans increase ACTH, prolactin, and cortisol levels (13, 230).

Ghrelin stimulates GH release from primary pituitary cells, which indicates that ghrelin can act directly on the pituitary (133). However, the involvement of the hypothalamus in ghrelin-mediated stimulation of GH release has been strongly suggested. Patients with organic lesions in the hypothalamic region showed insufficiency of GH release even when stimulated by ghrelin (188). Moreover, when using primary pituitary cells, the ghrelin treatment only increased GH release by two to three times above the basal level (117, 133), which is lower than the level of induction seen when ghrelin is administered to rats in

TABLE 2. *Effects of ghrelin*

Hormone secretion	
Growth hormone release	↑
ACTH release (weak)	↑
Cortisol release (weak)	↑
Prolactin release (weak)	↑
Insulin release	↑ ? ↓
Anabolic effects	
Appetite	↑
Adipocity	↑
Blood glucose	↑
Gastric function	
Gastric acid secretion	↑
Gastric movement	↑
Turnover of gastric and intestinal mucosa	↑
Cardiovascular function	
Cardiac output	↑
Blood pressure	↓

↑, Stimulate; ↓, decrease.

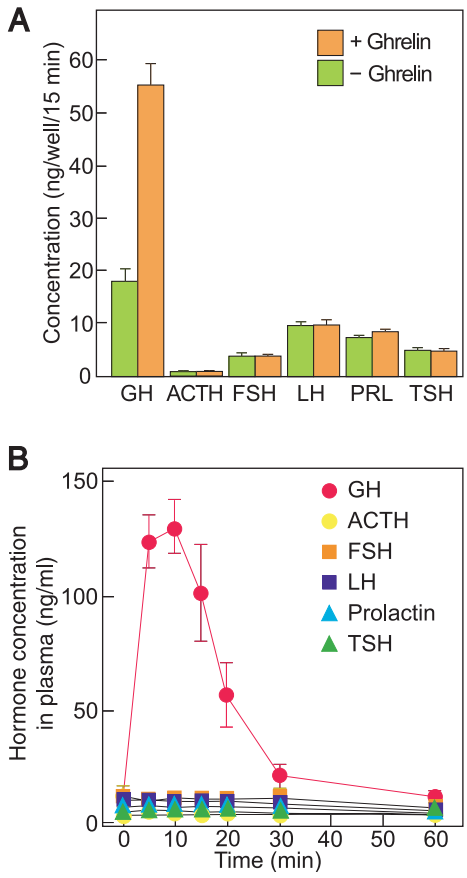


FIG. 9. Effects of ghrelin on pituitary hormone secretion in vitro and in vivo. *A*: effects of a high dose (10^{-6} M) of ghrelin on hormone secretion from rat primary pituitary cells in vitro. *B*: time courses of plasma hormone concentrations after intravenous injection of ghrelin into male rats in vivo. ACTH, adrenocorticotropin; FSH, follicle-stimulating hormone; LH, lutenizing hormone; PRL, prolactin; TSH, thyroid-stimulating hormone. [Adapted from Kojima et al. (133).]

vivo. These facts suggest that other factors are involved in vivo in order for this maximal level of GH release to be achieved by ghrelin administration. One possibility is transmission via the vagus nerve. When the vagus nerve is cut, the induction of GH release after ghrelin injection is dramatically decreased (56, 261), indicating that the vagus nerve is needed for the maximal stimulatory effects of ghrelin. Another possibility is the lack of GHRH in primary pituitary cells. Coadministration of ghrelin and GHRH had a synergistic effect on GH secretion; that is, coadministration results in more GH release than does either GHRH or ghrelin alone (13, 101). Synergistic effect on GH release was also observed by coadministration of GHSs, synthetic ghrelin agonists, and GHRH (25, 41, 42). This finding implies that GHRH is necessary for GH release to be maximally effective in inducing GH release.

B. Appetite Regulation

1. *Hypothalamic appetite regulation*

Feeding is a basic behavior that is necessary for life. Long-term lack of food results in death. It is well accepted that appetite is controlled by the brain and that feeding behavior is regulated by complex mechanisms in the central nervous system, in particular the hypothalamus (70, 207, 256). Removal of the lateral hypothalamus causes hypophagia (decreased feeding), leading to death due to severe weight loss. On the other hand, removal of the ventromedial hypothalamus causes hyperphagia (increased feeding); treated animals increase both feeding amount and frequency, leading to weight gain and severe obesity. Thus feeding is regulated by a balance of stimulating and inhibiting forces in the hypothalamus.

Recent identification of appetite-regulating humoral factors reveals regulatory mechanisms not only in the central nervous system, but also mediated by factors secreted from peripheral tissues (174, 216, 250, 267). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain (79). Hunger signals from peripheral tissues, however, had remained unidentified until the recent discovery of ghrelin.

2. *Ghrelin neurons in the hypothalamic appetite regulatory region*

Immunohistochemical analyses indicate that ghrelin-containing neurons are found in the arcuate nucleus of the hypothalamus, a region involved in appetite regulation (133, 148). This localization suggests a role of ghrelin in controlling food intake. Moreover, a recent report has indicated that ghrelin is also expressed in previously uncharacterized hypothalamic neurons that are adjacent to the third ventricle between the dorsal, ventral, paraventricular (PVN), and arcuate (ARC) hypothalamic nuclei (48). In the ARC, these ghrelin-containing neurons send efferent fibers onto NPY- and AgRP-expressing neurons to stimulate the release of these orexigenic peptides and onto POMC neurons to suppress the release of this anorexigenic peptide (Fig. 10). Neural network of ghrelin in the PVN is more complex. In the PVN, ghrelin neurons also send efferent fibers onto NPY neurons, which in turn suppress GABA release, resulting in the stimulation of corticotrophin-releasing hormone (CRH)-expressing neurons, leading to ACTH and cortisol release (Fig. 10).

3. *Ghrelin is a potent appetite stimulant*

When ghrelin is injected into the cerebral ventricles of rats, their food intake is potently stimulated (122, 173, 211, 246, 266). Among all discovered orexigenic peptide, ghrelin has been found to be the most powerful. Chronic intracerebroventricular injection of ghrelin increases cumulative food intake and decreases energy expenditure, resulting in body weight gain. Ghrelin-treated mice also increase their fat mass, both absolutely and as a percentage of total body weight.

Not only intracerebroventricular injection, but also intravenous and subcutaneous injection of ghrelin have been shown to increase food intake (173, 246, 265). Ghrelin is produced primarily in gastrointestinal organs in

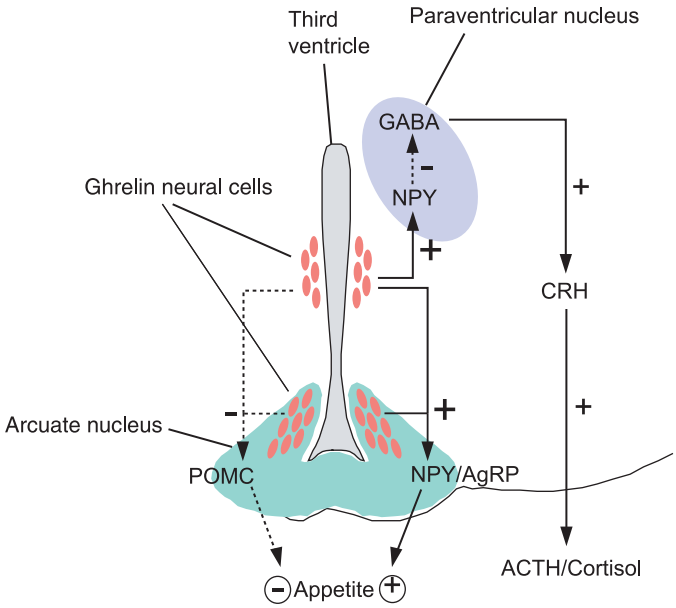


FIG. 10. Hypothalamic neural networks involving appetite-regulating peptides. Ghrelin-producing neurons in the arcuate nucleus (ARC) presynaptically induce neuropeptide Y (NPY) neurons to release NPY, a potent orexigenic neuropeptide, thus stimulating food intake. These ghrelin-producing neurons in the ARC also increase the rate of secretion of GABA, which may postsynaptically modulate the release of POMC, an anorexigenic neuropeptide. In the paraventricular nucleus (PVN), ghrelin stimulates NPY release, which in turn suppresses GABA release, resulting in the simulation of corticotropin releasing hormone (CRH)-expressing neurons, leading to ACTH and cortisol release.

response to hunger and starvation, and circulates in the blood, serving as a peripheral signal telling the central nervous system to stimulate feeding.

4. Mechanism of appetite stimulation by ghrelin

The hypothalamic ARC is the main site of ghrelin's activity in the central nervous system. The ARC is also a target of leptin, an appetite-suppressing hormone produced in adipose tissues, and NPY and AgRP, which are both appetite-stimulating peptides (76, 163). NPY and AgRP are produced in the same population of neurons in the ARC, 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. 10).

As suggested by the distribution of ghrelin-containing neurons in the hypothalamus (Fig. 10), intracerebroventricular injection of ghrelin induces Fos expression in NPY-expressing neurons and increases the amount of NPY mRNA in the ARC (122, 173, 211). Moreover, intracerebroventricular 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. Intracerebroventricular injection of an AgRP inhibitor, anti-NPY IgG, or 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 (48). 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 confirm these results. Although deletion of either NPY or AgRP caused a modest or no effect on the orexigenic action of ghrelin, the double knockout mice lacked the action of ghrelin completely (40). Ghrelin, thus, is functionally a natural antagonist to leptin.

Recently, AMP-activated protein kinase (AMPK) has been shown to be involved in hypothalamic appetite regulation (159). 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 (6). In contrast, injection of leptin decreases hypothalamic AMPK activity.

5. Vagus nerve and appetite regulation by ghrelin

Peripherally injected ghrelin stimulates hypothalamic neurons (104, 201, 258) and stimulates food intake (56, 265). 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 (56, 204, 272). Moreover, the observation that intracerebroventricular 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 (58).

In contrast, vagotomy inhibits the ability of ghrelin to stimulate food intake and GH release (7, 56). 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 after vagotomy. On the other hand, fasting-induced elevation of plasma ghrelin is completely abolished by subdiaphragmatic vagotomy or atropine treatment (261).

6. Ghrelin and orexin

Orexin, an orexigenic hypothalamic neuropeptide, is involved in the regulation of food intake and arousal (39, 206). Its intracerebroventricular injection stimulates food intake, and its expression correlates negatively with blood glucose, leptin, and food intake levels (199, 205, 214). Ghrelin stimulates isolated orexin neurons, whereas glucose and leptin inhibit them (269). Intracerebroventricular injection of ghrelin induces Fos expression in orexin-producing cells (242). The appetite-stimulating activity of ghrelin is reduced in orexin-null mice. Pretreatment with anti-orexin IgG, but not with anti-MCH IgG, attenuates ghrelin-induced feeding. Moreover, coinjection of ghrelin with NPY-Y1 antagonist and anti-orexin IgG was shown to suppress food intake by 87% compared with injection of ghrelin alone. These results indicate that feeding behavior is regulated in part by cooperative activity between ghrelin and orexin.

7. Meals and ghrelin

Plasma ghrelin levels increase immediately before each meal and fall to minimum levels within 1 h after eating (50, 247). The clear preprandial rise and postprandial fall in plasma ghrelin levels support the hypothesis that ghrelin is an initiation signal for meal consumption.

The preprandial increase in ghrelin levels was observed in humans that initiated meals voluntarily without any time- and food-related cues (49a). Indeed, ghrelin levels and hunger scores have shown to be positively correlated. Furthermore, the postprandial suppression of plasma ghrelin level is proportional to the ingested caloric

load (31), further reinforcing the hypothesis that ghrelin is a hunger signal.

8. Ghrelin gene expression and appetite

Ghrelin gene expression in the stomach is increased by fasting and decreased by administration of leptin and interleukin (IL)-1β (14, 129, 243). Ghrelin produces a positive energy balance by promoting food intake and decreasing energy expenditure and blocks IL-1β-induced anorexia. This fact suggests a possible clinical use of ghrelin for pathological anorexia that occurs as a side effect of some drugs and surgical operations and as a symptom of cancer and AIDS.

9. Gastric bypass

To treat severe obesity, gastric bypass operations are often performed (75, 77). The purpose of this procedure is to reduce the space for food in the gastric cavity and hence reduce total caloric intake. In the United States, a total of 40,000 people are estimated to have been treated with a gastric bypass in 2000, and 75,000 in 2001. However, the exact mechanism of action of this operation is unknown.

Recent research has revealed that ghrelin may contribute to the body weight reduction that occurs following gastric bypass. Patients receiving such a procedure were examined for ghrelin content after successful weight loss (51, 86, 146). Total ghrelin secretion was found to be reduced by up to 77% compared with normal-weight control groups and by up to 72% compared with matched obese groups (51). 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.

The mechanism for decreasing plasma ghrelin level in gastric bypass patients is not known. One hypothesis is that direct contact between gastric mucosa and food is important for the production and secretion of ghrelin (1).

C. Gastrointestinal Functions

Intravenous administration of ghrelin dose-dependently increases gastric acid secretion and stimulates gastric motility (68, 149). The maximum 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 H2-receptor antagonist. Intracerebroventricular administration of ghrelin also increases gastric acid secretion in a dose-dependent manner (58).

Intracerebroventricular 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 (58), indicating that ghrelin's ability to stimulate gastric acid secretion is mediated through activation of the vagus nerve.

D. Cardiovascular Functions

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 (89, 169), and intravenous injection of ghrelin into human volunteers induces a decrease in blood pressure (169). In addition, a radiolabeled ghrelin, [^{125}I-His9]ghrelin, was shown to bind to heart and to peripheral vascular tissue (128). The signal attributed to this radiolabeled molecule is augmented in atherosclerotic regions, suggesting that ghrelin receptor expression is upregulated in such areas and implicating ghrelin in the development of atherosclerosis.

An intravenous bolus of human ghrelin decreased mean arterial pressure without changing the heart rate (169, 170). Ghrelin also 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 left ventricular dP/d*t*-[max] compared with afflicted, but placebo-treated controls (167). Furthermore, ghrelin increased the diastolic thickness of the noninfarcted posterior wall, inhibited left ventricle enlargement, and increased left ventricular fractional shortening in these CHF rats (171). Ghrelin, thus, improves left ventricle dysfunction and attenuates the development of left ventricular remodeling and cardiac cachexia (168).

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 (147, 152). 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 (15, 185). These effects are regulated through activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases. Des-acyl ghrelin is similarly active, even though it does not bind to and activate the ghrelin receptor. Moreover, H9c2 cardiomyocytes do not express the ghrelin receptor, indicating that another unidentified ghrelin receptor-related receptor may be involved.

E. Ghrelin and Insulin Secretion

The identification of the pancreatic ghrelin-expressing cells is a matter of controversy, as described in the section on ghrelin distribution. 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 (2, 29, 57, 144, 194).

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 et al. (57) 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. In contrast, ghrelin had no effect on insulin release in the context of a basal level of glucose (2.8 mM).

Hepatic and renal gluconeogenesis is crucially important in maintaining glucose homeostasis. The rate-limitinig enzyme of gluconeogenesis, PEPCK, is downregulated by insulin at the transcriptional level (166). With the use of a rat hepatoma cell line, H4-II-E cells, ghrelin reversed the downregulating effect of insulin on PEPCK mRNA levels. Because the ghrelin receptor mRNA is detected in liver and kidney tissues by RT-PCR method (89), ghrelin may be concerned in the regulation of gluconeogenesis in vivo.

F. Life Without Ghrelin

1. Total gastrectomy

The stomach is the major source of circulating ghrelin (11). Total gastrectomy, as is performed in the treatment of gastric cancer or sever gastric ulcers, was shown to decrease the plasma concentrations of ghrelin to ~30–50% of those of pregastrectomy when measured at 30 min after the operation (109, 145, 146). This concentration gradually increased to ~70% of the level before the operation. These results indicate that gastric ghrelin production accounts for ~50–70% of circulating ghrelin but that this percentage is subject to compensatory production possibly by the intestines and pancreas.

It has been suggested that gastric factor(s) may control bone formation (244), since total gastrectomy sometimes induces osteopenia (145). Synthetic ghrelin agonists, GHSs, have been shown to directly stimulate osteocyte growth (229). Thus ghrelin may be involved in the gastric regulation of bone formation.

2. Ghrelin knockout mouse

A ghrelin knockout mouse was produced, and its phenotype was examined (227, 263, 264). Ghrelin knockout mice showed normal size, growth rate, food intake, body composition, reproduction, and gross behavior, without any pathological changes. Because survival is more acutely threatened by starvation than obesity, it may be no surprise that an orexigenic-peptide-null mouse showed no change in food intake and body weight.

However, the ghrelin-null mouse showed a significant reduction in respiratory quotient and a trend for lower body fat mass when the mouse was fed with a high-fat diet (263, 264). These results indicate that ghrelin is not a critically required orexigenic factor but may function in nutrient sensing and switching of metabolic substrates.

3. GHS-R knockout mouse

Mice lacking GHS-R (Ghsr-null mice) do not show the typical increases in GH release and food intake upon ghrelin administration, indicating that GHS-R is indeed the primary biologically relevant ghrelin receptor (228). Growth and development of Ghsr-null mice are normal, and their appetite and body composition are not different from those of their wild-type littermates. Thus ghrelin and its receptor are not critical for growth and appetite regulation.

However, serum insulin-like growth factor I (IGF-I) levels and body weights of Ghsr-null mice are modestly decreased compared with those of their wild-type littermates. These results suggest that ghrelin sets the IGF-I level for the maintenance of an anabolic state.

4. Anti-GHS-R transgenic rat

A transgenic rat expressing an antisense GHS-R mRNA in the hypothalamus to block the signal pathway of ghrelin was expected to show the same phenotype as the ghrelin-null mouse, but in fact their phenotypes are clearly different. The transgenic rat has a lower body weight and less adipose tissue compared with wild-type rats and consumes less food (212). The difference in phenotypes between the ghrelin-null mouse and the anti-GHS-R mRNA-expressing transgenic rats may be due to the presence of a compensatory mechanism existing in the former, but has not been fully addressed.

VII. REGULATION OF GHRELIN SECRETION AND ASSOCIATED DISEASES

A. Regulation of Ghrelin Secretion

The most important factor for the regulation of ghrelin secretion is feeding. Plasma ghrelin concentration is increased when fasting and decreased after food intake (50, 247). 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 (154, 209). Because gastric distension by water intake does not change ghrelin concentration, mechanical distension 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-fat meal (72, 90).

Plasma ghrelin concentration showed a nocturnal increase (71, 270). Ghrelin levels increased during sleep, and this increase was blunted in obese subjects or by sleep deprivation.

Plasma ghrelin concentration is low in obese people and high in lean people (23, 51, 98, 99, 200, 209, 248). Related to this fact, plasma ghrelin level is highly increased in anorexia nervosa patients and returns to normal levels upon weight gain and recovery from the disease (11, 52, 179, 224, 236). Ghrelin concentration is also increased in bulimia nervosa patients (235). Patients with gastric bypass lose their weight, and their ghrelin levels decrease (51, 86, 146). Changes in ghrelin concentration associated with food intake are diminished in these patients, confirming that the stomach is the main site of ghrelin production. Plasma ghrelin concentration also decreases in patients with short bowel syndrome (143), probably due to the loss of ghrelin-producing tissues.

Exogenous treatment with somatostatin and its analogs, such as octreotide, as well as infusion of urocortin-1, a potent anorexigenic peptide, suppress plasma ghrelin concentration (17, 60, 100, 177, 231). However, administration of leptin does not modify ghrelin levels (36).

Exogenous GH decreases stomach ghrelin mRNA expression and plasma ghrelin concentration, but does not affect stomach ghrelin stores (191). These results suggest that pituitary GH exhibits a feedback regulation on stomach ghrelin production. Moreover, relatedness between ghrelin and GH pulsatility has been demonstrated, suggesting either that ghrelin participates in the pulsatile GH release or that the two hormones are simultanously coregulated (141).

B. Polymorphisms in the Ghrelin Gene and Obesity

The relationship between genomic variants of the ghrelin gene and obesity has been suggested. In humans, two polymorphisms have been reported: Arg51Gln and Leu72Met (160, 189, 251, 252). For both polymorphisms, allelic frequencies are similar between obese patients and controls. However, it has been reported that obese patients with the Met72 allele became obese earlier than patients homozygous for the wild-type Leu72 allele, suggesting that the polymorphism may affect ghrelin's activity.

The Arg51Gln mutation results in a change in the COOH-terminal processing site of the ghrelin peptide within its precursor protein from Pro-Arg to Pro-Gln, resulting in the failure of the normal cleavage necessary to produce the 28-amino acid ghrelin. A 94-amino-acid-long pro-ghrelin peptide may still be produced, although its biological activity has not been assessed. Interestingly, Ukkola et al. (252) reported that in 96 nonobese female controls [mean BMI 23.0 $\pm$ 1.4 (kg/m^2) of the Swedish Obese Subjects cohort], the Arg51Gln mutation was identified in six (all heterozygotes) obese subjects (6.3%) but not among controls ($P < 0.05$) (252). However, it is not clear that this mutation in fact changes the activity or biological properties of ghrelin.

C. Feeding 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 (11, 52, 179, 224, 236). 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 (13, 230), which may explain the amenorrhea and behavioral changes observed in AN patients.

Prader-Willi syndrome (PWS) is a complex genetic disorder characterized by mild mental retardation, hyperphagia, short stature, muscular hypotonia, and distinctive behavioral features (176). Excessive appetite in PWS causes progressive severe obesity, which in turn leads to an increase of cardiovascular morbidity and mortality. The PWS genotype is characterized by a loss of one or more paternal genes in region q11–13 on chromosome 15 (176). It has been suggested that this genetic alteration leads to dysfunction of several hypothalamic areas, including appetite regulatory regions. Moreover, GH deficiency is common in PWS.

High plasma ghrelin concentration is observed in PWS patients (49, 63). The mean plasma concentration of ghrelin was higher by three- to fourfold in PWS than in a reference population. Thus ghrelin may be responsible, at least in part, for the hyperphagia seen in PWS. It is unclear, however, what underlies the increased ghrelin levels in these patients. Imprinting of paternal genes in region q11–13 on chromosome 15 may induce the production of excessive amounts of transcription factors that increase ghrelin expression or, alternatively, a loss of a transcription inhibitory factor that normally suppresses ghrelin expression. Elucidation of the precise mechanism by which ghrelin gene expression is regulated may reveal the genetic cause of hyperphagia in PWS.

TABLE 3. *Possible clinical applications of ghrelin*

GH deficiency
Diagnosis of pituitary function
Child and adult GH deficiency
Eating disorder
Anorexia nervosa
Bulimia nervosa
Prader-Willi syndrome
Gastrointestinal disease
Cardiovascular disease
Heart failure
Dilated cardiomyopathy
Osteoporosis
Aging
Catabolic state or chronic wasting syndrome
Cachexia (cancer, cardiac chachexia)
AIDS
Postoperative patients

GH, growth hormone.

VIII. CLINICAL APPLICATION OF GHRELIN

The diverse functions of ghrelin raise the possibility of its clinical application (73, 255) (Table 3).

Because of its potent GH-releasing activity and specificity, ghrelin may be applied to the diagnosis and treatment of GH deficiency (16, 62, 239). To diagnose GH deficiency, the most common GH stimulus used is insulin-induced hypoglycemia, in which blood glucose levels decrease to <40 mg/dl. This test can evaluate both GH and ACTH release in patients with pituitary disease. However, the hypoglycemic action of insulin may sometimes cause side effects. At present, intravenous injection of ghrelin into humans does not show any side effects, suggesting that ghrelin may be useful for diagnosing GH deficiency.

Adult and child GH deficiency may be benefitted by ghrelin treatment. The GH-releasing activity of ghrelin is comparable to that of GHRH (12, 133, 184, 230). In addition, coadministration of ghrelin and GHRH has a synergistic effect on GH secretion, and their combined administration is the most potent inducer of GH release yet identified (101). At small doses of 0.08 or 0.2 μg/kg ghrelin, the combined administration of ghrelin and GHRH significantly stimulated GH release in a synergistic manner. This synergy was observed even at a high dose of 1.0 μg/kg ghrelin, although the comparison was not statistically significant.

At present, ghrelin is only a peripheral orexigenic signal that is effective upon its intravenous injection (69, 250). Thus blocking or neutralizing ghrelin's action may be a reasonable approach to reversing a chronic obese state. However, appetite is regulated by numerous factors that may interact with and compensate for each other (20); thus a ghrelin antagonist might only have a limited effect on obesity. Indeed, ghrelin-null mice showed no obvious abnormalities in feeding behavior (227).

In contrast, ghrelin may be useful as an orexigenic agent for the treatment of eating disorders such as AN (164). Injection of ghrelin can stimulate appetite and improve the nutritional state of these patients. However, plasma ghrelin concentration in AN patients is very high. This result indicates that sensitivity to ghrelin is severely disturbed in these individuals.

Ghrelin stimulates gastric motility (68, 149), which makes it a candidate for the treatment of postoperative gastric ileus. Ghrelin administration has been shown to have a strong prokinetic effect, accelerating gastric emptying and the small intestinal transit of liquid meals and reversing delayed gastric evacuation, thus counteracting gastric ileus (245).

Central and intraperitoneal administrations of ghrelin reduced ethanol-induced gastric ulcers in a dose-dependent manner (136, 213). This effect is prevented by N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthesis, and by capsaicin, indicating that the gastroprotective effect of ghrelin is mediated by nitric oxide and requires capsaicin-sensitive sensory nerve activity.

Ghrelin has positive cardiovascular effects, as indicated by the presence of its receptor in blood vessels and the cardiac ventricles. In vitro, ghrelin inhibits apoptosis of cardiomyocytes and endothelial cells (15). In humans, infusion of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with heart failure. Administration of ghrelin improves cardiac structure and function, and attenuates the development of cardiac cachexia in rats with heart failure (37, 38, 168). These results suggest that ghrelin has cardiovascular protective effects and regulates energy metabolism through GH-dependent and -independent mechanisms. Thus ghrelin may be a new therapeutic agent for the treatment of severe chronic heart failure.

Other potential clinical applications of ghrelin are in osteoporosis, aging, and catabolic states including those seen in postoperative patients and in AIDS- and cancer-associated wasting syndromes (96, 97, 175). For example, in human immunodeficiency virus (HIV)-lipodystrophy patients GH and ghrelin levels are both reduced (142). Reduced ghrelin level may be in part cause to decrease GH level. Ghrelin therefore may be useful to treat HIV-lipodystrophy by its GH releasing activity as well as its anabolic effect.

IX. EPILOGUE

The discovery of ghrelin occurred against a backdrop of a long history of peptide research. A small opioid peptide derivative with weak GH-releasing activity opened a wide new field in endocrinology and metabolism. The story of ghrelin, from the initial development of

an artificial GHS to the identification of the endogenous ligand, is a typical example of the general paradigm of reverse pharmacology. This story also makes us reevaluate the importance of purification of natural substances. By scanning genomic databases, we can identify amino acid sequences; however, natural substances sometimes escape our notice. Ghrelin is such a case.

About 25 years ago, the intitial report of a GHS introduced a new regulatory pathway for GH release to accompany the known GHRH pathway. Since then, many potent GHSs have been developed, and the structure of the GHS-R has been identified. However, despite intensive research, the identity of the endogenous ligand of the GHS-R had remained elusive until the discovery of ghrelin. This finding has launched a whole new field of research in GH and appetite regulation. Growing evidence supports the notion that GH release from the pituitary is controlled not only by GHRH from the hypothalamus, but also by ghrelin from the stomach and hypothalamus. In addition, ghrelin is a peripheral fasting signal and stimulates food intake, in contrast to the functions of leptin, a peripheral satiety signal from adipose tissues.

The structure of ghrelin, and the tissue in which it is produced, is unprecedented in the fields of bioactive peptides and endocrinology. Ghrelin is the first case of a bioactive peptide that is modified by a fatty acid, possessing primarily an *n*-octanoyl modification that is essential for its activity. Ghrelin exists not only in mammalian species, but also in nonmammals such as frog, chicken, and fish. Ghrelin, thus, may be an essential hormone for maintaining GH release and energy homeostasis in vertebrates. Moreover, the acyl-modified structure of ghrelin reveals an unknown pathway for pro-peptide processing.

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.

We express our gratitude to students, collaborators, and fellow scientists who shared their scientific interests with us and obtained the findings we have reviewed in this article.

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Address for reprint requests and other correspondence: M. Kojima, Molecular Genetics, Institute of Life Science, Kurume University, Hyakunenkouen 1–1, Kurume, Fukuoka 839–0864, Japan (E-mail: mkojima@lsi.kurume-u.ac.jp).

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