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Fasting plasma ghrelin levels in subtypes of anorexia nervosa

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Abstract

Ghrelin has a role in regulating eating behavior and energy metabolism in the central nervous system, and has been reported to play an important role in the pathophysiology of anorexia nervosa (AN). The aim of the present study was to compare fasting plasma ghrelin levels in different subtypes of untreated AN patients. The subjects included 39 female AN patients and 11 female controls. The patients were then divided into two subtypes as follows: 19 AN patients with restricting (AN-R) and 20 AN patients with binge-eating/purging (AN-BP) form of the illness. Blood samples from subjects after an overnight fast were used to analyze plasma ghrelin concentrations. Plasma ghrelin concentrations in both AN-R and AN-BP were negatively correlated with body mass index (BMI). The mean plasma ghrelin levels in both AN-R and AN-BP were significantly higher than that in controls. The mean ghrelin level in AN-BP was significantly higher than that in AN-R. However, mean BMI and serum potassium in both groups were not significantly different. These results suggest that both BMI and the presence of binge-eating/purging may have some influence on fasting plasma ghrelin levels in patients with AN.

Keywords: Ghrelin; Body mass index; Anorexia nervosa; Restricting; Habitual binge-eating/purging

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1. Introduction

Ghrelin was originally discovered in the rat and human stomach (Kojima et al., 1999), and increases food intake and body weight when administered centrally and peripherally and stimulates growth hormone secretion in rodents (Tschöp et al., 2000). Ghrelin-producing endocrine cells account for about 20% of the oxyntic gland endocrine cell population (Dornonville de la Couret al., 2001). This peptide is an orexigenic peptide that increased the hypothalamic neuropeptide Y (NPY) mRNA expression and abolished leptin-induced feeding reduction (Shintani et al., 2001). Antibodies and antagonists of NPY and agouti-related protein (AGRP) blocked ghrelin-induced feeding (Nakazato et al., 2001). These findings suggest a role for ghrelin in the regulation of feeding behavior and energy metabolism in the central nervous system. Additionally, it has been reported that fasting plasma ghrelin concentrations in humans are negatively correlated with BMI (Shiiya et al., 2002), and fasting leptin concentrations (Tschöp et al., 2001).

Alternations in the release of gastrointestinal peptides (Baranowska et al., 2000) and increased fasting plasma ghrelin levels (Otto et al., 2001) have been reported in patients with anorexia nervosa (AN). AN is subdivided into two subtypes: the restricting form, characterized by dietary restriction, and the binge-eating/purging form, which includes the presence of binge-eating and/or purging, according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV; American Psychiatric Association, 1994). Research examining the interactions between appetite control mechanisms and AN subtype may assist in our understanding of the diagnosis and treatment of these illnesses. Thus in this study, we measured the fasting plasma concentrations of ghrelin from AN patients and compared the mean ghrelin levels among the subtypes of AN to sex- and age-matched healthy controls.

2. Methods

2.1. Subjects

Nineteen female AN-R patients (20.1±4.9 years, mean age±SD) and twenty female AN-BP patients (21.9±4.7 years) defined in DSM-IV and eleven healthy female volunteers (control) (21.0±1.9 years) were the subjects in this study. The mean values of BMI in the subjects were as follows: AN-R (13.6±1.5 kg/m^2, mean±SD); AN-BP (13.7±1.9 kg/m^2); control (21.4±1.2 kg/m^2). Duration of illness was as follows: AN-R (2.5±1.5 years, mean±SD); AN-BP (3.3±3.1 years). Controls had no history of psychiatric illness. Patients were excluded if they had a history of alcohol or drug abuse, or gastrointestinal disease and were assayed before the initiation of active treatment. AN-BP patients had a history of binge-eating/ purging at least twice a week over the preceding 3 months. The Institutional Committee of Kagoshima University approved the protocol, and all subjects provided written informed consent before participation.

2.2. Protocol

We collected blood samples from subjects at 0800 h after an overnight fast. Body weight was measured on the day when samples were obtained. Blood was drawn into chilled tubes containing EDTA·2Na (1 mg/ml) and aprotinin (500 U/ml). Plasma ghrelin was measured using a RIA as described elsewhere (Shiiya et al., 2002). In brief, antiserum against the C-terminal region of human ghrelin was raised in New Zealand white rabbits immunized against synthetic human ghrelin[13-28] that had been coupled with maleimide-activated mariculture keyhole limpet hemocyanin. The antiserum recognized acylated ghrelin and non-acylated ghrelin equally on a molar basis. Human Tyr^0-ghrelin[13-28] was radioiodinated by the lactoperoxidase method for use in the assay. Inter- and intra-assay variation was <8 and <6%, respectively. The limit of detection of this assay is 12 fmol/tube of human ghrelin. Two milliliters of plasma was diluted with 2 ml of 0.9% saline and applied to a Sep-Pak C-18 cartridge (Waters, Milford, MA) pre-equilibrated with 0.9% saline. The cartridge was washed first with saline and then with a 0.1% trifluoroacetic acid (TFA) solution, and peptides were eluted with a 60% acetonitrile (CH_3CN) solution containing 0.1% TFA. The eluate was evaporated, reconstituted with RIA buffer, and subjected to RIA analysis. A diluted sample or a standard peptide solution (100 μl) was incubated for 24 h with 100 μl of the antiserum diluent (final dilution 1/20,000). The tracer solution (16,000 cpm/100 μl) was added, and the mixture incubated for 24 h. Bound and free ligands were separated by the second antibody method. All procedures were done at 4 °C. Recovery of human ghrelin added to the plasma was 90.7±4.0% (n=6).

2.3. Statistical analyses

Correlation coefficients were calculated by linear regression analysis. The subject groups (mean±SD) were compared using ANOVA and a post-hoc Scheffe's test. The p value of <0.05 was considered statistically significant.

3. Results

The mean values of serum potassium in the subjects were as follows: AN-R (4.1±0.2 mmol/l, mean±SD); AN-BP (4.0±0.6 mmol/l); control (4.2±0.2 mmol/l). Fasting plasma ghrelin concentrations were negatively correlated with BMI in both AN-R (r=−0.47, p<0.05) [Fig. 1(A)] and AN-BP (r=−0.51, p<0.05) [Fig. 1(B)]. There was no significant correlation between plasma ghrelin concentration and BMI in controls (r=−0.28, p=0.42) (data not shown).

The mean fasting plasma ghrelin levels in both AN-R and AN-BP were significantly higher (p<0.01) than that in controls (Fig. 2). The mean plasma ghrelin level in AN-BP was higher than that in AN-R (p<0.01) (Fig. 2), although the mean serum potassium and BMI levels between the AN-BP and AN-R groups did not differ significantly (p>0.2).

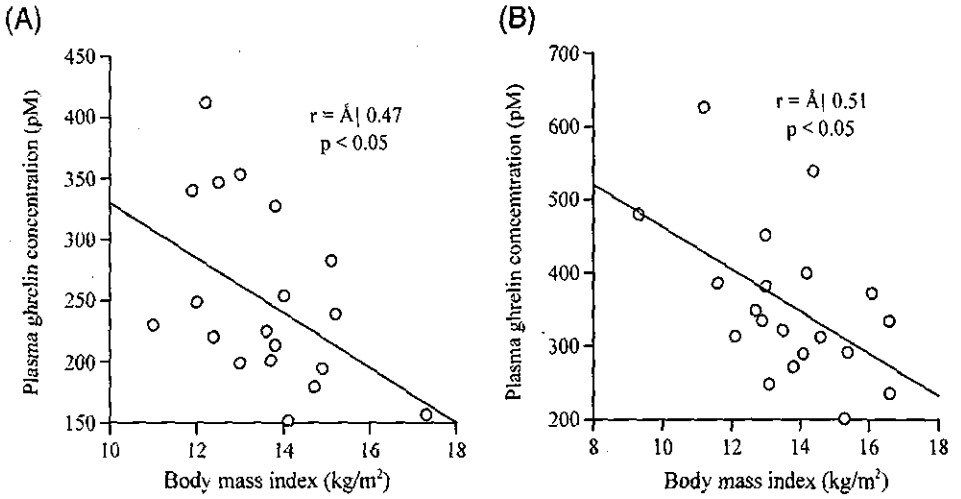

Fig. 1. (A) Relationship of plasma ghrelin concentration and body mass index for anorexia nervosa patients with restricting (AN-R). (B) Relationship of plasma ghrelin concentration and body mass index for anorexia nervosa patients with binge-eating/purging (AN-BP).

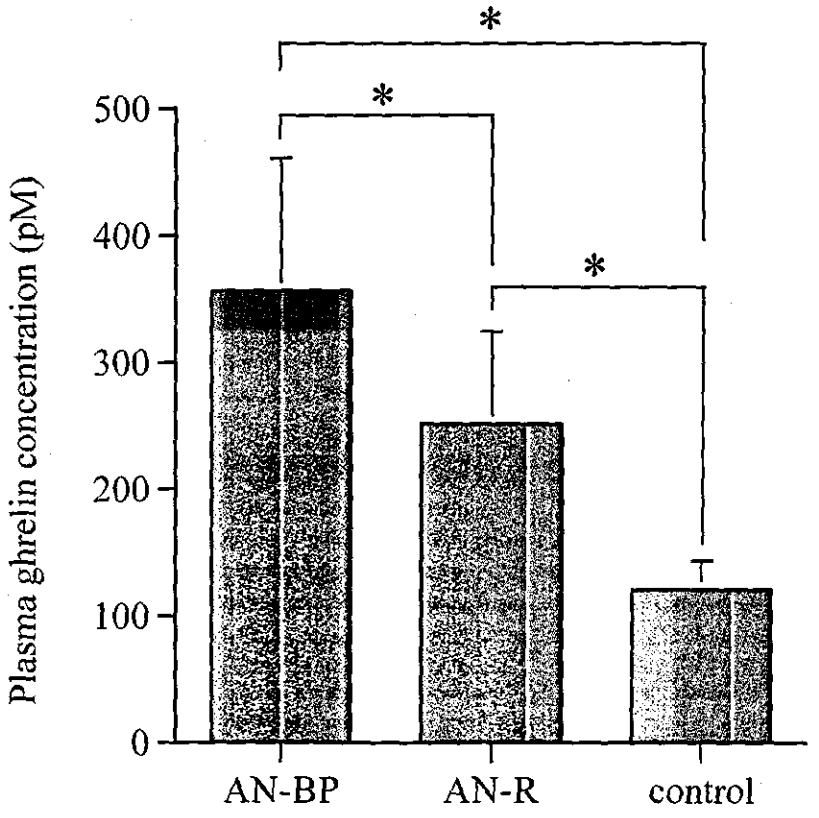

Fig. 2. Comparison of plasma ghrelin concentrations in anorexia nervosa patients with restricting (AN-R), anorexia nervosa patients with binge-eating/purging (AN-BP) and healthy volunteers (control). $*p<0.01$

4. Discussion

The fasting plasma ghrelin concentrations in both AN-R and AN-BP patients were negatively correlated with BMI. This finding is in agreement with previous human studies (Tschöp et al., 2001; Shiiya et al., 2002). Since data of BMI in the control group was distributed over a narrow range, the significant difference in correlation with ghrelin was statistically undetectable. Though the plasma ghrelin level in AN patients has been reported to be significantly higher than that found in control subjects (Otto et al., 2001), our study documents for the first time differences of the plasma ghrelin level between AN-R and AN-BP patients with similar BMI's. Our findings suggest that not only BMI but also the presence of binge-eating/purging may influence circulating ghrelin levels.

One of the reasons for the difference in the circulating ghrelin levels between the patients may relate to the condition of their stomach, which is one of the main sites of ghrelin secretion (Date et al., 2000). Vomiting is reported to induce several esophageal and gastric lesions, ranging from esophagitis to perforation of the esophagus or stomach (De Caprio et al., 2000) and increase the release of nitric oxide from gastric mucosa cells (Zicari et al., 2001). We therefore theorize that these mechanical stimulation and intracellular change might accelerate the release of ghrelin or cause damage to the ghrelin-producing endocrine cells.

Another possible reason for these findings is that the regulation of gastrointestinal peptides, which secretion may be affected by the vagus system (Uvnäs-Moberg, 1983). Ghrelin is also one of the gastrointestinal peptides which, when administered intracerebroventricularly, stimulates gastric acid secretion by activating the vagus system (Date et al., 2001). Since vagal afferent nerve endings in the alimentary canal (Fukui et al., 1994) and the nucleus tractus solitarius have serotonin-3 and serotonin-4 receptors whose activation has been implicated in nausea and vomiting (Andrews et al., 1990), self-induced vomiting may have some influence on circulating ghrelin levels via the vagal system.

We hypothesize that fasting plasma ghrelin levels may increase to a more food intake and energy saving metabolism in response to chronic change in negative energy balance in both of AN-R and AN-BP patients. Moreover, we speculate that binge-eating/purging episodes with associated self-induced vomiting may cause a more increase in circulating ghrelin levels, which may induce more binge-eating/purging cycles through the appetite control system in patients with AN-BP.

In the present study, plasma ghrelin was demonstrated to vary between subjects with AN-R and AN-BP even though weight and serum potassium did not differ. Until now, hypokalemia in eating disorders has been considered to be an important screening tool for covert binge/purge behavior because AN-BP patients tend to hide important negative prognostic factors such as their binge/purge behavior (Ostuzzi et al., 1999). However, a recent study concluded that routine electrolyte measurement was a poor screening method for binge/purge behavior as the frequency of hypokalemia in the patients with eating disorders was found to occur in only 4.6% of the subjects (Greenfeld et al., 1995). Therefore it is possible that plasma ghrelin might be a useful tool in identifying covert habitual binge/purge behavior in AN.

Finally we conclude, (1) there were significant correlations between plasma ghrelin and BMI in both subtypes of AN; and (2) the plasma ghrelin in AN-BP was elevated compared to AN-R despite of similar BMI. These findings suggest that both BMI and the presence of binge-eating/purging may have some influence on fasting plasma ghrelin levels in patients with AN.

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Ghrelin: A Gastric Peptide that Regulates Hypothalamic Control of Feeding

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Abstract: Ghrelin, a novel growth-hormone-releasing peptide isolated from human and rat stomach, is a 28-amino acid peptide with a posttranslational acylation modification that is indispensable for its activity. Ghrelin stimulates secretion of growth hormone, food intake, and body weight gain. This review will focus on the discovery, structural characteristics, tissue distribution, and physiological functions of ghrelin, as well as the regulation of its expression and secretion. Future directions of clinical application of ghrelin as an anabolic peptide are discussed.

INTRODUCTION

Obesity is closely linked to disability, disease, and death [1-3]. Public health officials and organizations have issued warnings regarding the dangers of obesity, reporting the years of life lost due to it [4, 5]. Food intake and energy homeostasis are tightly and redundantly regulated by neurohumoral factors in the central nervous system, especially in the hypothalamus [6-8], but the critical mechanisms underlying this regulation have yet to be understood. In order to develop effective treatments for obesity, it is necessary to clarify the system by which energy homeostasis is regulated.

Ghrelin is a growth hormone-releasing peptide initially isolated from human and rat stomach as a result of intensive screening using "reverse pharmacology". It is a novel orexigenic peptide and an important endogenous regulator of energy homeostasis. Ghrelin is the first example of a bioactive peptide modified by *n*-octanoic acid; indeed, this modification is unprecedented in mammals. The discovery of ghrelin provides new evidence that the stomach, where ghrelin is discovered, plays an important role not only in digestion of food but also in regulating feeding and controlling the secretion of growth hormone from the pituitary gland.

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

DISCOVERY OF GHRELIN

Growth hormone (GH) regulates body size and cell growth, carbohydrate-protein-lipid metabolism, and water-electrolyte balance [9]. During the 1970s, when enkephalins were identified as the endogenous ligands for morphine receptors, Bowers and co-workers found that some opioid peptide derivatives had weak GH-releasing activity [10]. The discovery that met-enkephalin stimulates GH release from the anterior pituitary *in vitro* [11-13] has led to the development of small synthetic peptidyl and non-peptidyl molecules, called growth-hormone secretagogue (GHS) [14-19].

Groups continued to generate artificially modified enkephalin derivatives with increasingly potent and long-acting GHS activity [20]. Two such GHSs, GH-releasing peptide 6 (GHRP-6) and hexarelin, were found to stimulate GH release when added to cultured pituitary cells, injected intravenously into rats and humans, and even when administered orally [13-16, 21]. Co-administration of growth hormone-releasing hormone (GHRH) and GHS stimulated GH secretion synergistically, suggesting that GHS operate through a unique pathway to stimulate GH secretion [22]. GHRH was found to stimulate GH release through its binding to the GHRH receptor (GHRH-R), which leads to increased intracellular cAMP (Fig. 1). In contrast, GHS was observed to stimulate GH release by increasing intracellular calcium levels through the activation of the phospholipase-IP3 pathway [15, 23, 24]. In 1993, Smith and co-workers

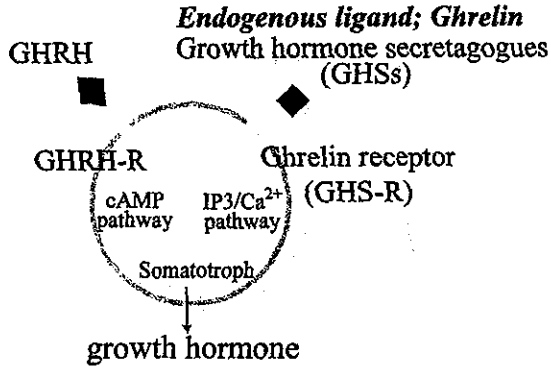

Fig. (1). Pathways of growth hormone release from a somatotroph. Growth hormone-releasing peptide (GHRH) binds to the GHRH receptor (GHRH-R) and increases the intracellular cAMP level. Ghrelin and growth hormone secretagogues (GHSs) bind to the ghrelin receptor (formally called the growth hormone secretagogue receptor, GHS-R) and increase the intracellular calcium level.

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found a non-peptidyl GHS, L-692,429 [20], and, thereafter, a more potent non-peptidyl GHS, MK-0677, was synthesized [25]. In 1996, the ghrelin receptor (formerly known as the GHS-R), a G-protein-coupled seven-transmembrane receptor, was identified using an expression cloning method [26-28]. After the discovery of the ghrelin receptor, a search for its natural endogenous ligand was undertaken using the orphan receptor strategy. The natural endogenous ligand was thought to be a peptide, because the ghrelin receptor shared structural similarities to peptide receptors such as the neurotensin receptor [26, 29]. A cultured cell line transfected with the ghrelin receptor was used to identify a natural endogenous ligand from tissue extracts that stimulate the ghrelin receptor, as monitored by increases in intracellular calcium influx. As it was known that the ghrelin receptor was expressed in the pituitary, hypothalamus, and hippocampus, its ligand was thought to exist also in the brain [30]. However, weak activation of the ghrelin receptor by brain extracts indicated that the endogenous ligand might be present at low abundance in the brain. Several other tissue extracts were screened for activation of ghrelin receptor artificially expressed in CHO cells; unexpectedly, a strong response was found to stomach tissue extract. Active peptide was purified from the extract by gel filtration, ion exchange, and reverse-phase chromatography, and they were named "ghrelin", a word root in Proto-Indo-European languages for "grow". Ghrelin is a 28-amino acid peptide modified at its third residue, a serine (Ser3), by a middle-chain fatty acid, *n*-octanoic acid (Fig. 2). The Ser3-acylation is essential for its biological activity, the binding and activation of the ghrelin receptor [31]. More recently, a motilin-related peptide (MTLRP), or m46, was identified using COS cells by a conventional molecular biological technique; its amino acid sequence was found to be identical to that of ghrelin [32]. MTLRP has no biological activity because it is not acylated, underscoring a general limitation in the ability of molecular biology to detect moieties whose activity depends on post-translation modification.

STRUCTURAL CHARACTERISTICS OF GHRELIN AND GHRELIN RECEPTOR

Ghrelin is a 28-amino acid bioactive peptide modified by *n*-octanoic acid. This unique modification seems to increase the hydrophobicity of the peptide. Acyl modifications have been reported in integral membrane proteins, but not in bioactive secreted peptides. At present, ghrelin has been found in fishes, amphibians, birds and many mammals. There is no structural homology between ghrelin and peptidyl GHS (GHRP-6 and hexarelin). Both rat and human ghrelin peptides are 28 amino acids in length, differing only in two residues [31]. The acylated serine is changed to a threonine in Amphibian (bullfrog) ghrelin [33]. The seven-amino-acid sequence at the N-terminal and the acyl modification of the third residue are well conserved in multiple species [34, 35], suggesting that these features are important for ghrelin's biological activity. The human ghrelin gene is located on chromosome 3p25-26 and consists of 4 exons and 3 introns. The structure of the rat ghrelin gene is identical to that of the human gene, but the mouse ghrelin gene consists of 5 exons and 4 introns [36-38]. Analysis of the ghrelin genomic structure has shown that the mature peptide is encoded in exons 1 and 2. The rat and human ghrelin precursors both comprise 117 amino acids

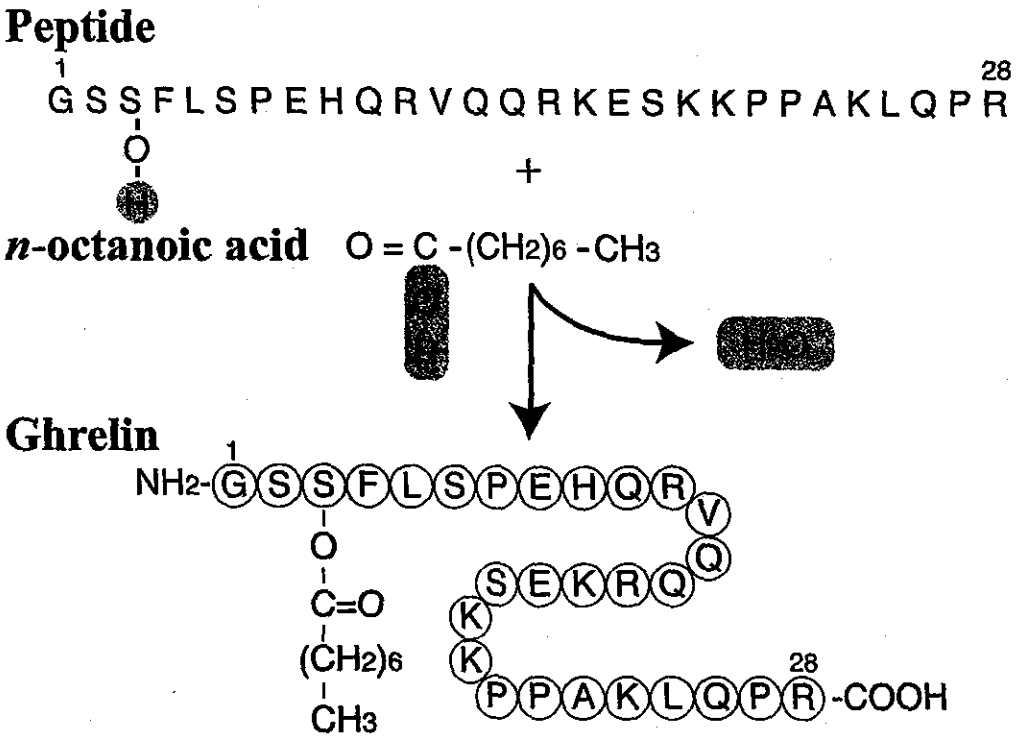

Fig. (2). Amino acid sequence of human ghrelin. The hydroxyl group of the Ser3 residue is esterified by *n*-octanoic acid.

[31]. In this 117-residue prepro-ghrelin, the mature ghrelin sequence in rat follows directly after a 23-residue signal peptide. Two isoforms of ghrelin precursor mRNA are produced in rat stomach cells from the same gene by an alternative splicing mechanism [39]. One encodes the ghrelin precursor, while the other codes for des-Gln14-ghrelin precursor, which lacks a glutamine (Gln) at position 14. Des-Gln14-ghrelin is another endogenous ligand for the ghrelin receptor; it is a 27-amino acid peptide with an *n*-octanoyl modification at Ser3 [39]. Des-Gln14-ghrelin comprises around 10% of all immunoreactive ghrelin and ghrelin-type molecules in the rat stomach and stimulates increases in intracellular calcium concentration and growth hormone secretion to the same degree as the 28-amino acid ghrelin [39]. These results indicate that the 28-amino acid isoform of ghrelin is the major bioactive form. A structure-function analysis showed that the des-*n*-octanoyl form of ghrelin does not induce changes in intracellular calcium concentration of target cells [40]. Identification of the acyl modifying enzyme and factors involved in its regulation will be invaluable in furthering our understanding of ghrelin's biosynthetic processing *in vivo*. The initial 4-5 amino acids of ghrelin (octanoylated ghrelin-(1-4) or octanoylated ghrelin-(1-8)) alone, which includes the acylated Ser3, can bind to the ghrelin receptor [41], but cannot stimulate secretion of GH [42, 43], suggesting that full-length acylated ghrelin is necessary for its biological activity. Genetic analysis of the human ghrelin gene has just begun. A twin study of ghrelin plasma levels showed that it is genetically determined [44], and polymorphisms of the prepro-ghrelin gene might contribute to obesity [45].

Testis-specific expression of a ghrelin gene transcript has been reported in mice; this mRNA has been named the ghrelin gene-derived transcript (GGDT) [37]. The sequence is derived by alternative splicing within intron 3 of the murine ghrelin gene and encodes a deduced 12-amino-acid peptide [38]. The function of GGDT remains to be elucidated.

Ghrelin receptor (GHS-R) is a G-protein coupled receptor (GPCR); the gene encoding it is located at 3q26.2. The ghrelin receptor exists in two isoforms, 1a and 1b [26, 27, 29, 46], the former of which is the functional receptor for ghrelin. The ghrelin receptor has 52% amino acid identity to the motilin receptor [29, 47, 48]. Moreover, the ghrelin and motilin peptides share 36% sequence homology [31, 32, 49, 50]. Motilin can not activate the GHS 1a receptor; however, like ghrelin, central administration of motilin stimulates GH secretion and increases food intake [47, 49, 50]. These findings suggest that ghrelin and motilin may have evolved from a common ancestral peptide.

TISSUE DISTRIBUTION OF GHRELIN AND GHRELIN RECEPTOR

Ghrelin mRNA is predominantly expressed in the stomach [31, 51, 52], lower amounts have been reported in the bowel [51], pituitary gland [53], kidney (rat mesangial cells and mouse podocytes) [54], lung [55], placenta [56], testis (Leydig cells) [57], pancreas [58], leukocytes (T cells, B cells, neutrophils) [59], hypothalamus [31], and small amounts have been detected by real-time PCR in the adrenal gland, adipocytes, gall bladder, skeletal muscle, myocardium, skin, spleen, liver, ovary, and prostate [60]. Biologically active ghrelin is present in various rat tissues in the following concentrations: 377 ± 55 fmol/mg in the stomach, 20 ± 0.6 fmol/mg in the duodenum, and <0.05 fmol/mg in the hypothalamus [61]. Plasma levels of ghrelin are decreased by 80% after gastrectomy in rats [51, 52], suggesting that the stomach is a major source of plasma ghrelin. Ghrelin is not detectable in the fetal stomach but appears after birth and increases progressively, especially during the second and third postnatal weeks. Plasma levels of ghrelin also increase in parallel with stomach ghrelin levels postnatally [62, 63].

In the rat stomach, ghrelin is abundant in the oxyntic mucosa of gastric fundus, and a double staining study showed that ghrelin-positive cells account for 20% of chromogranin A-immunoreactive endocrine cells [51]. These findings indicate that ghrelin is produced in the endocrine cells of the oxyntic gland. Four types of endocrine cells-enterochromaffin-like (ECL) cells, Detla (D) cells, enterochromaffin (EC) cells, and X/A-like cells- have been identified in the oxyntic mucosa by means of light- and electron-microscopic immunohistochemistry [64, 65]. These four cells are present in the rat oxyntic gland at the following relative percentages: 60 – 70% ECL cells, 20% X/A-like cells, 2 – 5% D cells, and 0 – 2% EC cells. In humans, the oxyntic gland is composed of 30% ECL cells, 20% X/A-like cells, 22% D cells, and 7% EC cells [66]. X/A-like cells are round to ovoid with compact, electron-dense granules [65, 67, 68]. Although they represent a major endocrine cell population in the oxyntic mucosa of both rats and humans, their products and functions have not previously been characterized. Morphological analysis shows that ghrelin-immunoreactive cells share many characteristics with X/A-like cells, such as its localization, abundance, and ultrastructure [51]. These data indicate that ghrelin cells are X/A-like cells. Ghrelin cells therefore can be abbreviated as Gr cells according to the precedent system of nomenclature for other enteroendocrine cells. Ghrelin cells in the stomach are the second-most abundant endocrine cells, after ECL cells [51], and are "closed-type" cells that have no physical connection to the lumen. Electron-microscopic analysis has shown that they are closely associated with the capillary network running through the lamina propria [51, 52]. In the rat gastrointestinal tract, "open-type" ghrelin cells increase in abundance along a gradient from the stomach to the lower gastrointestinal tracts [69]. Ghrelin circulates in the plasma, suggesting that ghrelin cells may function in an endocrine fashion [51, 52].

In the central nervous system, ghrelin mRNA and immunoreactive peptide levels are very low. Immunohistochemical analysis showed that ghrelin-positive neuronal cells are localized to a limited region of the hypothalamic arcuate nucleus [31, 70], where is known to be rich in GHRH neurons and involved in the regulation of food intake. Ghrelin is synthesized in the hypothalamic arcuate nucleus [31], but the number of ghrelin-positive neurons is small. Hypothalamic ghrelin might act on the anterior pituitary via the portal vein to release GH. On the other hand, hypothalamic ghrelin may also be involved in feeding behavior and energy homeostasis via neural pathways.

The ghrelin receptor is distributed among the hypothalamic-pituitary areas, other brain areas, stomach, intestine [51], kidney [54], pancreas [30], heart, and aorta [71] of rodents and humans [26, 30, 60, 72, 73]. It is most abundant in the hippocampus [74], suggesting a potential role in memory formation. In pathophysiological conditions, ghrelin receptor is expressed in gastric [75], lung [76], and pancreatic tumors [77, 78]. The wide distribution of ghrelin receptor in both physiological and pathophysiological conditions may explain the multifaceted roles of ghrelin and GHS [15, 59, 79-87].

GHRELIN ACTIONS

Growth Hormone Secretion

Ghrelin stimulates GH secretion from rat anterior pituitary cells *in vitro* in a dose-dependent manner; however, unlike GHS, ghrelin does not affect the secretion of other pituitary hormones *in vitro* [20, 31, 88]. The GH-releasing potency of ghrelin is comparable to that of GHRH *in vitro*, and is higher in freely moving animals [31]. Intravenous (IV) administration of ghrelin specifically stimulates GH secretion in rats [31]. Ghrelin also stimulates GH release *in vivo* upon intracerebroventricular (ICV) administration [89]. IV and ICV injection of ghrelin stimulates GH release in rats *in vivo* with minimum doses of 1.5nmol and 10 pmol [90], showing that ICV administration is more potent. IV injection of ghrelin also induces GH secretion in healthy humans in a dose-dependent manner, with a minimum dose of 0.2mg/kg [91-94]. Ghrelin activates pituitary-specific transciption factor-1 (Pit-1) in time- and dose-dependent manners in cultured rat anterior pituitary cells [95]. On the other hand, chronic ICV administration of ghrelin in rats for 12 days reduces plasma GH levels [89]. Frequent exposure of ghrelin to isolated pituitary cells *in vitro* inhibits GH secretion [96], indicating that desensitization of the ghrelin receptor might occur after chronic administration of the peptide.

Co-administration of ghrelin and GHRH elicits a significantly synergistic effect on growth hormone secretion [94, 97]. Furthermore, infusion of GHRH in freely moving rats results in a significant increase in the expression of ghrelin and ghrelin receptor genes in the pituitary gland [98]. Finally, GHRH antisera and GHRH antagonists attenuate the GH-secreting activity of ghrelin [86]. These results indicate that GHRH may modulate ghrelin's role in GH secretion [98].

Recent data have shown that the vagus nerve is involved in GH secretion from the pituitary gland. The vagus nerve is a cranial nerve that contains both efferent and afferent fibers. The afferent fibers of the vagus nerve form a neuroanatomical link between the alimentary tract and the nucleus of the solitary tract (NTS) in the hindbrain [7, 99-101]. Approximately 90% of vagus nerve fibers in the subdiaphragma are afferent and are composed of unmyelinated, thin, capsaicin-sensitive fibers. Some of these afferent endings present within the gastro-intestinal mucosa and submucosa are optimally positioned to detect luminal substances. Selective chemical and surgical deafferentation of the gastric vagal nerve can attenuate the GH secretion induced by peripheral administration of ghrelin, suggesting that stomach-derived ghrelin physiologically stimulates GH release *in vivo* via the vagus nerve (Fig. 3) [90].

Other Hormonal Effects of Ghrelin

IV injection of ghrelin stimulates secretion *in vivo* of adrenocorticotropic hormone (ACTH), cortisol, prolactin, and aldosterone [97], but not lutenizing hormone (LH), follicular stimulating hormone, or thyroid stimulating hormone. No synergistic effect of co-administration of ghrelin and GHRH on the secretion of any hormones other than GH has been observed [94, 97]. The physiological significance of ghrelin's ability to induce the secretions of these anterior pituitary hormones has not yet been elucidated. *In vivo* ghrelin-induced ACTH secretion is thought to be mediated by vasopressin secretion [102]. Peripheral administration of ghrelin also increases CRH expression in the hypothalamus in mice [84]. IV administration of ghrelin suppresses pulsatile LH secretion in 17β-estradiol-treated ovarectomized rats [87], and also suppresses testosterone secretion in normal rats[57], suggesting that ghrelin may participate in the regulation of gonadal functions *in vivo*.

Feeding Regulation and Energy Homeostasis

Ghrelin is a strong orexigenic and adipogenic molecule in mammals [42, 74, 86, 103, 104]. The hypothalamus is a center for the control of energy homeostasis [8, 105], and the gastrointestinal tract and brain are closely linked in the regulation of food intake [106]. The discovery of ghrelin implicated the stomach as an endocrine organ that links the central and peripheral regulation of feeding in mammals.

Feeding regulation by GHS and GHS-R (ghrelin receptor) has been studied extensively. GHS increases feeding when administered both ICV and IV [107-109]. The ghrelin receptor is widely expressed throughout the central nervous system [30, 110, 111], including the piriform cortex, dentate gyrus, hippocampus, granular cell layer of the olfactory bulb, and supraoptic, paraventricular, suprachiasmatic, tuberomammalliary and dorsal raphe nuclei [30]. After ICV administration of ghrelin, Fos protein, a marker of neuronal activation, can be detected in areas where the ghrelin receptor is distributed [74, 112]. Fos is especially highly expressed in the dentate gyrus and hippocampus, where the ghrelin receptor is abundant [74]. However, des-octanoyl ghrelin does not induce Fos expression [112]. Inhibiting ghrelin receptor expression in the hypothlamus of transgenic rats by expressing anti-sense ghrelin receptor mRNA decreases GH secretion, food intake, and body fat mass [113], suggesting that in the hypothalamus, the ghrelin receptor is important in GH secretion and energy homeostasis.

Ghrelin induces weight gain and adiposity [74, 103, 104, 114-116]. ICV administration of ghrelin to free-feeding rats during light and dark phases increases food intake in a dose-dependent manner [74]. Neutralization of ghrelin with anti-ghrelin immunoglobulin G suppresses starvation-induced feeding in a dose-dependent manner [74], suggesting that endogenous ghrelin is a strongly orexigenic. ICV administration of ghrelin to genetically GH-deficient rats

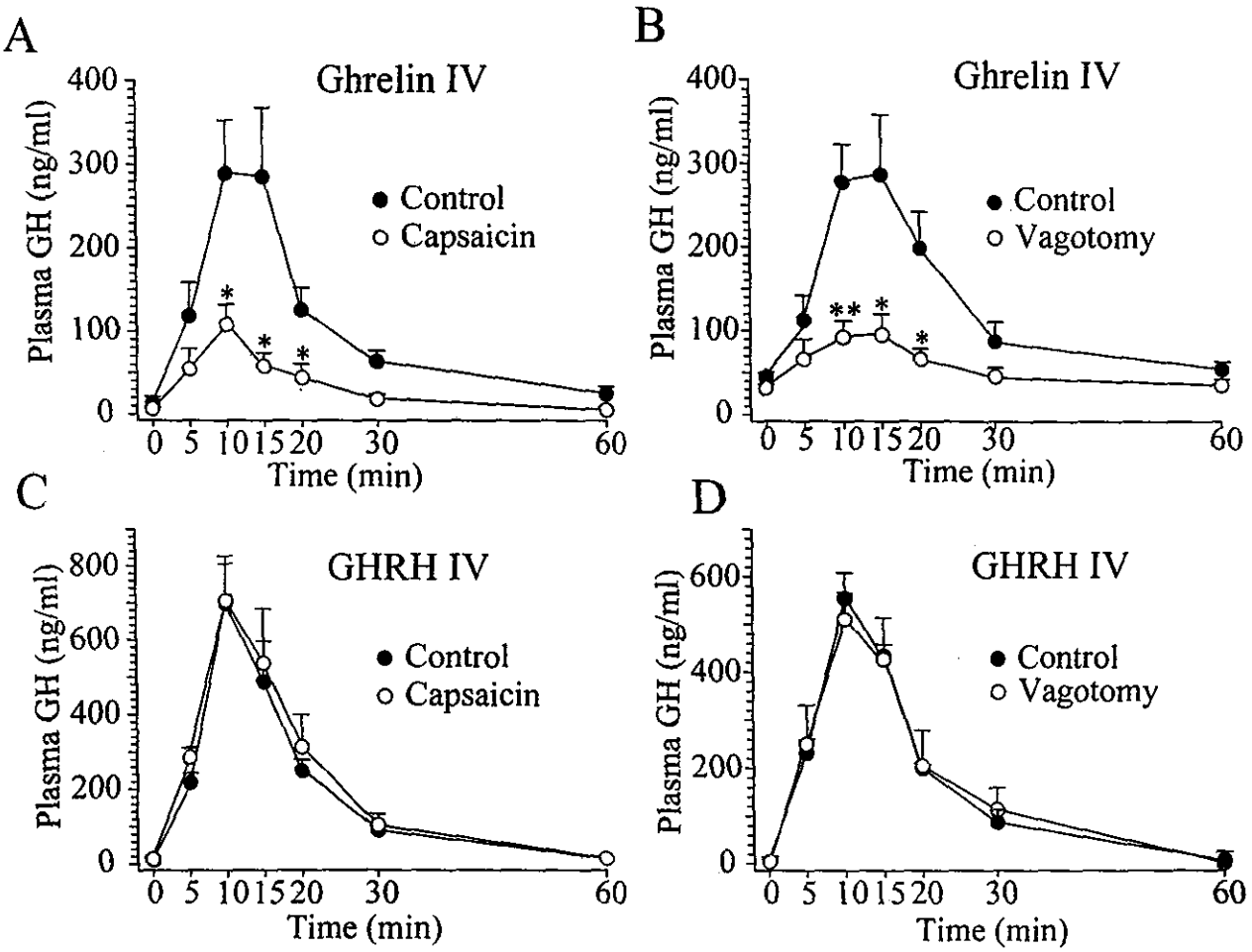

Fig. (3). Gastric afferent nerve effects on ghrelin-induced growth hormone secretion. (*A*) (*B*) Plasma GH response (mean ± SEM) after intravenous (IV) administration of ghrelin to rats that had undergone perivagal capsaicin application (*A*) or vagotomy (*B*). (*C*) (*D*) Plasma GH response after IV administration of GHRH to rats that had undergone perivagal capsaicin application (*C*) or vagotomy (*D*). **P*<0.01; ***P*<0.001 vs. control. Redrawn from [90].

stimulates food intake, indicating that ghrelin's orexigenic activity is independent of the GH signaling pathway [74]. Continuous ICV administration of ghrelin induces food intake and an increase in fat mass by selectively utilizing carbohydrates, leading to weight gain [103]. IV administration of ghrelin to healthy humans increases the visual analogue score for appetite [116]. In another study, ghrelin strongly induced the sensation of hunger in 75% of treated humans [92, 97, 117]. These data indicate that ghrelin is a peripheral orexigenic and adipogenic peptide.

Ghrelin-producing neurons are present in the arcuate nucleus of the hypothalamus, which is also a target for leptin. Leptin is a strong peripheral anorectic protein secreted from adipose tissues [8, 118] that reduces food intake and fat mass without altering lean body mass [119]. Microinjection of ghrelin into the arcuate nucleus in rats induced food intake [120]. Neuropeptide Y (NPY) and agouti-related protein (AgRP), both leptin-responsive orexigenic peptides, are produced in the same neurons of the arcuate nucleus. NPY/AgRP-producing neurons express the leptin receptor. ICV administration of ghrelin induces Fos expression in 39% of NPY neurons [74] and increased both NPY and AgRP mRNA in the arcuate nucleus [98, 115, 121, 122]. Ghrelin-induced feeding was suppressed by a receptor antagonists and specific antisera against NPY and AgRP [74, 115]. Ghrelin-immunoreactive axonal terminals made direct synaptic contacts with NPY/AgRP-producing neurons in the arcuate nucleus and orexin-(hypocretin-)producing neurons in the lateral hypothalamus (Toshinai K, date Y, Murakami N, Shimada M, Mondal SM, Shimbara T, Guan JL, Wang QP, Funahashi H, Sakurai, T, Shioda S, Matsukura S, Kangawa K, Nakazato M, in press). Moreover, plasma ghrelin concentration and hypothalamic NPY mRNA level in streptozotocin (STZ)-induced diabetic rats are significantly higher than those in control rats and were normalized by insulin treatment [123]. Furthermore, hyperphagia in STZ-induced rats is partially reversed by the administration of a ghrelin-receptor antagonist. These results indicate that ghrelin is an upstream regulator of the orexigenic peptide NPY/AgRP and antagonizes leptin's effect on NPY/AgRP neurons, resulting in an increase in feeding and body weight. Ghrelin is a natural antagonist to leptin through the activation of the NPY/Y1 receptor signaling pathway [8, 74, 114, 115, 124, 125].

Ghrelin is the most powerful orexigenic peptide produced in the gastrointestinal tract. Plasma ghrelin concentration increases two-fold before each meal and decreases back to baseline within 1 hour after eating [126, 127]. Ghrelin levels are lower in obese humans [128] and higher in patients with anorexia nervosa [129], and correlate negatively with body mass index [128-130]. Meal-related metabolites such as monoamines and peptides, as well as mechanical and chemical stimuli, transmit satiety signals to the nucleus of solitary tract (NTS) via vagal afferent fibers or to the hypothalamus via the bloodstream. Ghrelin had been thought to enter the brain across the blood-brain barrier, but a study showed that an intraperitoneal injection of ghrelin to totally vagotomized mice does not stimulate food intake [49]. Recent data show that blockade of the gastric vagal afferent fibers by vagotomy or perivagal application of caspsaicin abolishes ghrelin-induced feeding and activation of NPY- and GHRH-producing neurons (Fig. 4) [90]. The ghrelin receptor is synthesized in vagal afferent neurons in the nodosa ganglion and transported to their afferent terminals in the stomach. IV administration of ghrelin suppresses firing of the vagal afferents [90]. These findings together suggest that the gastric vagal afferents comprise the major pathway conveying ghrelin's starvation signals to the brain. Conversely, the electrical activity of efferent fibers of the vagus nerve is stimulated by ghrelin administration. Both IV and ICV administrations of ghrelin in rodents stimulate gastric acid secretion and gastric contraction and emptying [49, 81, 131]. These data indicate that the vagus nerve is important for conveying ghrelin's signals not only from stomach to the central nervous system but also *vice versa* (Fig. 5).

Ghrelin and the Cardiovascular System

GHSs have been reported to have protective properties for cardiac dysfunction in rats and injured endothelial cells [132-135]. GHSs increase cardiac index, cardiac output, and stroke volume index in healthy subjects and patients with GH deficiency [136-138]. Ghrelin, like GHSs, protects against cardiac dysfunction but also possesses other cardiovascular activities. IV infusion of human ghrelin significantly decreases mean arterial pressure without changing heart rate and also increases cardiac output in healthy volunteers [71] and patients with chronic heart failure [139]. Infusion of human ghrelin into the forearm artery of normal subjects increases blood flow in a dose-dependent manner. Plasma levels of ghrelin are significantly higher in chronic heart failure patients with cachexia than in those without cachexia [130]. Chronic administration of ghrelin for 3 weeks improved left ventricular dysfunction concomitant with elevations in plasma GH and IGF-I; it also attenuated the development of left ventricular remodeling and cardiac cachexia in rats with chronic heart failure [140]. These results suggest that ghrelin may be used potentially as a new therapeutic tool for cachectic patients with chronic heart failure in order to improve cardiac function. Ghrelin receptor expression was found in rat heart and aorta [71] and is

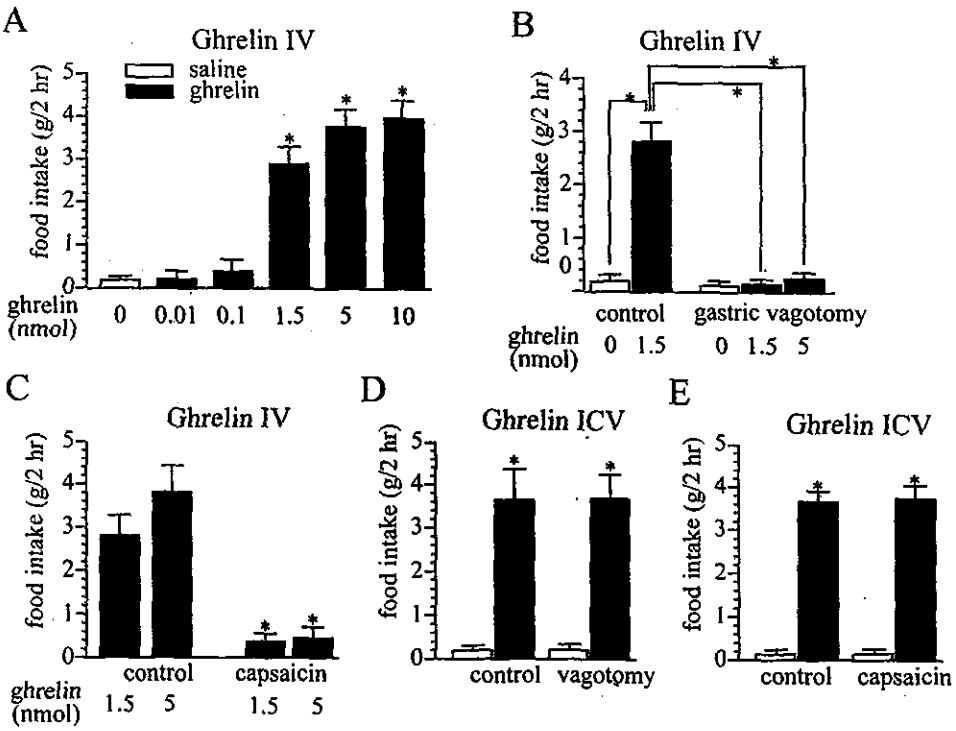

Fig. (4). Gastric afferent nerve effects on ghrelin-induced feeding. (*A*) Two-hour food intake (mean ± SEM) of rats after a single intravenous (IV) administration of ghrelin. (*B*) (*C*) Food intake after ghrelin IV in rats treated with vagotomy (B) and perivagal capsaicin (C). (*D*) (*E*) Food intake after intracerebroventricular (ICV) administration of ghrelin in rats treated with gastric branch (D) and perivagal capsaicin (E). **P*<0.01vs. control. Redrawn from [90].

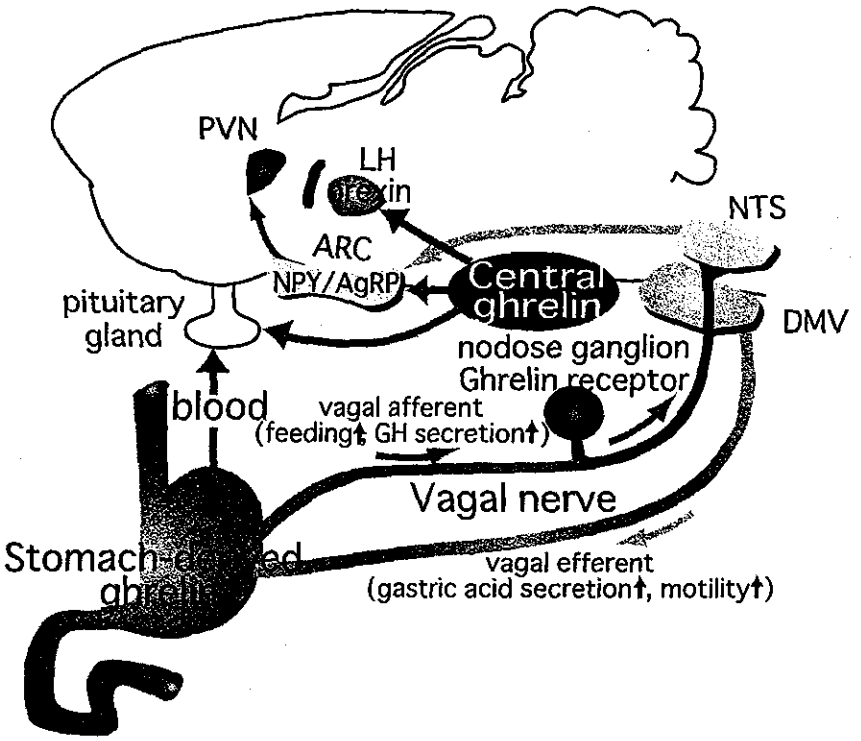

Fig. (5). Pathways of ghrelin action in the regulation of feeding and growth hormone release. Ghrelin's signal for starvation and GH secretion to the brain is conveyed via gastric vagal afferent nerves. AgRP; agouti-relate peptide protein, ARC; arcuate nucleus, DMV; dorsomotor nucleus of the vagus, LH; lateral hypothalamus, NPY; neuropeptide Y, NTS; nucleus of solitary tract, PVN; paraventricular nucleus.

upregulated in atherosclerotic coronary arteries [141, 142], suggesting that ghrelin might be involved in the pathological process of atherosclerosis.

Ghrelin's Role in Gastric and Pancreatic Function and Glucose Homeostasis

IV administration of ghrelin in rats stimulates gastric acid secretion and gastric motility in a dose-dependent manner [74, 131, 143, 144]. This effect is cancelled by both vagotomy and administration of atropine, but not by administration of histamine H2-receptor antagonist, suggesting that ghrelin affects gastric function via the vagus nerve [131]. ICV administration of ghrelin in anesthetized rats also stimulates gastric acid secretion, an effect that is abolished by both vagotomy and pre-treatment with atropine [81]. However, centrally administered GHS and ghrelin inhibit gastric acid secretion in conscious rats [145] These conflicting effects might be due to the state of anesthesia of the rodents; however, they both suggest that ghrelin participates in the central regulation of gastric acid secretion via the vagus nerve and functions as a metabolic signaling molecule.

Like GHS, IV administration of ghrelin in humans induces hyperglycemia by reducing insulin secretion without affecting GH secretion [117] [146-148]. Ghrelin has been shown to co-localize with glucagon in rat islet alpha-cells. It also increases the cytosolic free calcium concentration in alpha-cells and stimulates insulin secretion [58, 62]. Ghrelin infusion in rats suppresses pancreatic protein stimulation by cholecystokinin-8 in a dose-dependent manner [149]. Subdiaphragmatic vagotomy does not affect this suppressive activity of ghrelin. Exposure of cultured acinar cells to ghrelin does not change basal amylase release [149], suggesting that the suppression of pancreatic secretion by ghrelin may be an indirect effect.

Ghrelin and Cell Proliferation

Ghrelin mRNA expression is upregulated in pituitary tumors. Expression of ghrelin is high in non-functioning adenomas and gonadotropin-producing adenomas, but low in prolactinomas [78, 150, 151], carcinoid tumors [75, 152], medullary thyroid carcinoma cell lines [153], islet cell tumors [154] and thyroid follicular tumors [155]. Ghrelin receptor is found in human breast carcinomas and lung cancers [156, 157]. Ghrelin significantly inhibits cell proliferation of human breast carcinoma cell lines that express the ghrelin receptor [157], but not in the human lung cancer cell line CALU-1 [156]. Ghrelin's activity in some respects counters that of insulin; it inhibits Akt (serine-threonine kinase) activity and upregulates gluconeogenesis in hepatoma cells [158]. On the other hand, ghrelin also exhibits insulin-like activities, stimulating the insulin receptor substrate (IRS)-1-growth factor receptor-bound protein2-mitogen-activated protein (MAP) kinase pathways, which activates cell proliferation in hepatoma cells [158].

Ghrelin stimulates proliferation of PC-3 prostate cancer cells *in vitro* [159]. These results suggest that local ghrelin in tumors might be involved in cell proliferation by acting in an autocrine and/or paracrine fashion; however, these diverse intracellular signaling pathways remain to be clarified.

REGULATION OF GHRELIN EXPRESSION AND SECRETION

In humans, the fasting plasma level of ghrelin was shown to be 140±14 fmol/ml, using a ghrelin-specific radioimmunoassay that recognizes the C-terminal region of ghrelin [130]. Immunoreactive acylated ghrelin exists human plasma at a concentration of 5.4±1.4 fmol/ml. In rats, the plasma concentration of immunoreactive ghrelin is 556±43 fmol/ml, and that of acylated ghrelin is 94±14 fmol/ml [51]. Plasma ghrelin in gastrectomized patients is reduced to 35% of the level in normal controls [160], suggesting that the stomach is a major source of plasma ghrelin in humans.

Plasma ghrelin concentration peaks in humans at 2 am. It increases two-fold before each meal and decreases back to baseline within 1 hour after eating, a pattern reciprocal to that of insulin. Plasma ghrelin concentration is not changed by water intake [44, 49, 126, 127, 160-163]. Administration of ghrelin in healthy subjects induces hunger sensations [92, 97, 117]. These results suggest that preprandial elevation of ghrelin is an initiation signal for food intake. Plasma ghrelin level is increased by a low-protein meal and decreased by a high-fat diet [164]. It is profoundly decreased after gastric bypass surgery [165], suggesting that ingested nutrients might be important in inducing ghrelin secretion from the stomach; however, a transient surge of ghrelin secretion just before a scheduled feeding in sheep could not be due to the ingestion of food [166]. In general, the mechanisms underlying the regulation of ghrelin secretion remain to be clarified.

Plasma ghrelin concentration is lower in obese humans [128, 165, 167, 168] and in Pima Indians who have obesity and type 2 diabetes mellitus [128]. On the contrary, plasma ghrelin is higher under fasting conditions [103, 160, 161] and in individuals with anorexia, bulimia nervosa, cachexia [129, 130, 160, 169-171] and Prader-Willi syndrome (PWS) [172, 173]. These results, except for the correlation with PWS, suggest that plasma ghrelin level correlates negatively with body mass index [128-130]. Increased plasma ghrelin levels in PWS patients might be involved in their insatiable appetite and obesity. Plasma ghrelin levels are increased in patients with anorexia nervosa, but normalize after weight gain [129]. In GH-related diseases, patients with GH deficiency exhibit no increase in plasma ghrelin levels [174]. Normalization of GH in acromegalic patients increases ghrelin levels [175, 176].

Ghrelin mRNA expression in the gastric fundus is increased after 48 hours of fasting and by administration of insulin and leptin [161]. Hyperinsulinemic euglycemic clamp studies in humans have shown that insulin regulates plasma levels of ghrelin [177, 178]. Ghrelin mRNA is increased in ob/ob mice, which are deficient in leptin [49], and in untreated STZ-induced rats [123]. It is decreased in db/db mice, which are deficit in the leptin receptor [49]. The increased ghrelin expression in ob/ob mice is downregulated by leptin administration. These results indicate that leptin is the upstream regulator of gastric ghrelin.

Ghrelin, unlike GH and IGF-I, does not exhibit any gender-based differences in its secretion and expression [129, 179]. Expression of stomach-derived ghrelin in rats does not differ between males and females, and is not influenced by gonadal steroids [179]. Interestingly, ghrelin in the placenta is expressed during the first half of the pregnancy and is not detected at term; the physiological significance of this finding remains to be determined [56].

In summary, regulation of ghrelin expression and secretion are influenced by energy balance and glucose homeostasis. It is not clear how ghrelin is regulated in the stomach and what molecules, such as receptors and transporters, are involved in regulation of its production and secretion. More detailed mechanisms must be elucidated in the future.

CONCLUSIONS AND PERSPECTIVES

Ghrelin is produced in the brain and stomach, and plays a role in the regulation of energy balance (Fig. 5). Ghrelin is the first bioactive peptide known to be modified by *n*-octanoyl acid. This structure is unprecedented in the fields of bioactive peptides and endocrinology. Historically, the discovery of endogenous receptor ligands usually precedes the development of functional analogues of these novel putative peptides and factors. In the case of the GHS system, however, the synthetic analogues GHRP-6 and hexarelin were discovered first and were followed by the discovery of the ghrelin/GHS receptor and then, finally, the natural ligand, ghrelin. Motilin-related peptide (MTLRP)/m46 was identified as a motilin-related gene by conventional molecular biological techniques [32]. However, no biological function could be attributed to it when generated in transfected COS cells, because the peptide was not modified by an *n*-octanoyl moiety. This result suggests that even in the post-genome era, classical purification methods, when paired with monitoring of biological activity, remains an important technique for identifying novel post-translationally-modified bioactive peptides.

Feeding is regulated not only in the central nervous system but also in peripheral tissues [180]. Ghrelin is the first orexigenic signal to be derived from the stomach, and is thought to counter the satiety signal of leptin secreted from adipose tissue. The discovery of ghrelin has raised the novel idea that the stomach plays an important role in growth hormone secretion and feeding regulation, providing a multitude of directions for clinical and basic GH and feeding research.

Clinical application of ghrelin in humans has already been initiated. A ghrelin antagonist for the treatment of obesity will be studied in the future. On the other hand, ghrelin-resistant conditions (hyperghrelinemia) may be amenable to treatment with ghrelin or GHS, as insulin treatment is used for insulin resistance syndrome. Further research on ghrelin will contribute to our understanding of physiological and pathophysiological feeding mechanisms

and provide a novel therapeutic tool for patients with altered nutritional homeostasis.

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Eating pattern and the effect of oral glucose on ghrelin and insulin secretion in patients with anorexia nervosa

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pmol/l) and AN-R (180 min, 418·9 ± 68·4 pmol/l) were also delayed as compared to controls (60 min, 509·2 ± 88·8 pmol/l). The glucose level at 180 min in AN-R was significantly ($P < 0·05$) higher than in controls.

CONCLUSIONS These findings suggest that differences in eating behaviour in AN may induce alterations in both ghrelin and insulin metabolism in the acute feeding state. Furthermore, metabolic changes in the restrictive eating pattern may be related to the pathophysiology of small quantitative meal intake in AN-R patients.

Summary

OBJECTIVE Ghrelin is thought to be involved in the regulation of eating behaviour and energy metabolism in acute and chronic feeding states. Circulating plasma ghrelin levels in healthy humans have been found to decrease significantly after oral glucose administration. Because it is suggested that eating behaviour may influence the secretion of ghrelin and insulin in anorexia nervosa (AN), we examined the effect of oral glucose on ghrelin and insulin secretion in subtypes of AN patients.

DESIGN AND PATIENTS Twenty female AN patients and 10 age-matched female controls were subjects. The patients were subdivided into two subtypes based on eating behaviour as follows: 11 restricting type (AN-R), nine binge-eating and purging type (AN-BP). Subjects underwent an oral glucose tolerance test at 08·00 h. Blood was collected 0, 30, 60, 120 and 180 min after the glucose load.

RESULTS Both AN-R and AN-BP had a significant increased basal ghrelin level ($P < 0·01$) and a significantly decreased basal insulin level ($P < 0·05$) as compared to controls. The time of the nadir of mean ghrelin in AN-BP (120 min, 58·1% of basal level, 204·9 ± 34·3 pmol/l, mean ± SEM) was delayed compared to controls (60 min, 60·2%, 74·3 ± 7·9 pmol/l), and in the AN-R group it kept decreasing for 180 min (80·0%, 182·4 ± 31·5 pmol/l). The peaks insulin levels in AN-BP (120 min, 319·3 ± 88·8

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Ghrelin is involved in the regulation of GH release (Kojima *et al.*, 1999; Takaya *et al.*, 2000) and recently has been found to have other actions, including effects on appetite (Tschöp *et al.*, 2000; Cummings *et al.*, 2001; Wren *et al.*, 2001; Lawrence *et al.*, 2002), one heart (Nagaya *et al.*, 2001), pancreas (Wierup *et al.*, 2002) and carbohydrate metabolism (Shiiya *et al.*, 2002). This peptide is an orexigenic peptide that has effects similar to hypothalamic neuropeptides such as neuropeptide Y (NPY; Shintani *et al.*, 2001) and agouti-related protein (AGRP; Nakazato *et al.*, 2001). Plasma ghrelin concentrations in the gastric and truncal veins of normal rats increase in response to fasting and decrease upon refeeding (Tschöp *et al.*, 2000; Dornonville *et al.*, 2001; Toshinai *et al.*, 2001). Circulating plasma ghrelin in healthy humans is found to decrease significantly after oral and intravenous (i.v.) glucose administration (Shiiya *et al.*, 2002). On the other hand, intravenous administration of ghrelin stimulates circulating gastrin and insulin levels in rats (Lee *et al.*, 2002). These findings suggest the involvement of ghrelin in the regulation of eating behaviour and energy homeostasis in both acute and chronic feeding states.

The eating disorder anorexia nervosa (AN) is characterized by chronic food restriction and has been found to cause alterations in the release of gastrointestinal peptides (Baranowska *et al.*, 2000), including increased circulating ghrelin (Ariyasu *et al.*, 2001; Otto *et al.*, 2001), and causes various metabolic changes such as glucose metabolism (Drossman *et al.*, 1979), insulin sensitivity (Zuniga-Guajardo *et al.*, 1986; Kiriike *et al.*, 1990; Fukushima *et al.*, 1993) and insulin resistance (Scheen *et al.*, 1988). However, we previously found that after subdividing AN into two subtypes based on guidelines for the differences in eating behaviour as published in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV; American Psychiatric Association, 1994) there were increased plasma