octanoyl modification at Ser3, is identical to ghrelin except for deletion of one glutamine, and it is produced through alternative splicing of the rat ghrelin gene. Des-Gln14-ghrelin has the same potency of activities as that of ghrelin. In the course of purifying human ghrelin from the stomach, we also isolated several minor forms of the peptide (27). These could be classified into four groups by the type of acylation observed at Ser3: nonacylated (des-acyl ghrelin, ref. 28), 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. Synthetic octanoylated and decanoylated ghrelins stimulate the increase of intracellular Ca^{2+} levels in GHS-R-expressing cells and stimulate GH release in rats to a similar degree. The nonacylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in both stomach and blood (28). In blood, des-acyl ghrelin circulates in amounts far greater than acylated ghrelin. Because a fatty acid is attached to the Ser3 of ghrelin, acylated ghrelin is unstable (29). Thus des-acyl ghrelin may represent either a pre-form 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 humans and rats. In contrast, it has been reported that des-acyl ghrelin shares with acylated ghrelin some nonendocrine actions, such as the modulation of cell proliferation and adipogenesis (30 – 32). Further study is required to determine whether des-acyl ghrelin is biologically active and binds to an as-yet-unidentified receptor.

III. Ghrelin derivatives

Based on calcium-mobilization assay in the GHS-R-expressing cell line, 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 (33, 34). When the length of the acyl modification of ghrelin was examined, the maximum response was observed at the acyl 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-metheylpentanoyl, respectively, also retained activity. Moreover, a ghrelin derivative in which the third amino acid residue was replaced with an aromatic amino acid, tryptophan, still retained weak activity. 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 (33 – 35). However, the ability of the short ghrelin derivatives to activate the GHS-R expressing cells is not predictive of their capability to stimulate GH release in rats (36). The short ghrelin derivatives are devoid of biological activity in vivo.

IV. Tissue distribution of ghrelin and ghrelin receptor (GHS-R)

A. Ghrelin-producing cells

Ghrelin is predominantly produced by the stomach, whereas substantially lower amounts are derived from the bowel, pancreas, pituitary, kidney, and placenta (15, 28, 37 – 39). Removal of the stomach or the acid-producing part of the stomach in rats reduces circulating ghrelin by approximately eighty percent, further supporting the view that the stomach is the main source of the ghrelin peptide. These other sources of ghrelin act on ghrelin secretion in a compensatory manner after gastrectomy or might act specifically in a paracrine manner. Rat ghrelin is present from the stomach to the colon, with the greatest amount in the gastric fundus (38). Ghrelin-producing cells, which are not histamine-secreting enterochromaffin-like cells, somatostatin-secreting D cells, or serotonin-secreting enterochromaffin cells, accounted for about twenty percent of the endocrine cell population in rat and human oxyntic glands (Fig. 3: A – C) (38). Rat gastric ghrelin is present in a distinct cell type, X/A-like cells, whose hormonal product and physiological functions have not previously been clarified. The X/A-like cells, now designated ghrelin-secreting cells, are not in continuity with the stomach lumen but rather are closely associated with the capillary network of the lamina propria, supporting an endocrine role. The amount of ghrelin is very low in the fetal stomach and increases in an age-dependent manner (40). The concentrations of plasma ghrelin also increase postnatally in parallel with the amount of ghrelin produced by the stomach (41). Ghrelin-immunoreactive cells are localized in the mucous membrane in the duodenum, jejunum, ileum, and colon (28, 38, 42). In the intestine, ghrelin concentration gradually decreases from the duodenum to the colon. Ghrelin cells can be classified into opened- and closed-type cells; opened-type cells are in contact with the glandular lumen, and closed-type cells do not have a luminal connection. The ghrelin cells in the stomach are closed-type cells, whereas in the duodenum, jejunum, ileum, and colon,

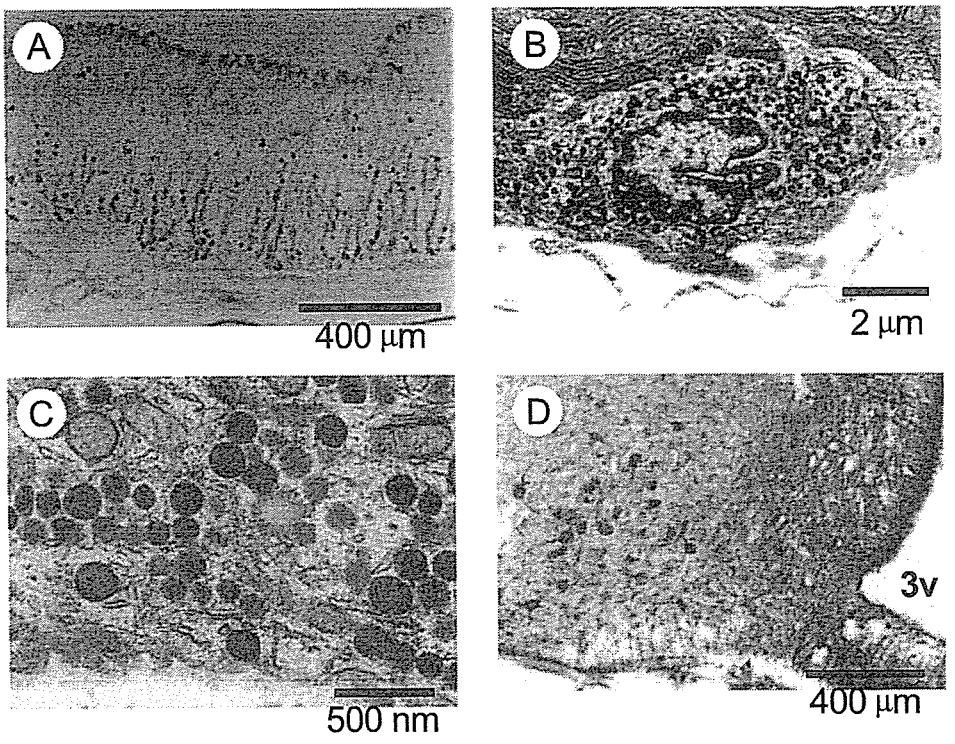


Fig. 3. Ghrelin cells in the stomach. A: ghrelin-immunoreactive cells in the stomach are found from the neck to the base of the oxyntic gland. This distribution pattern is typical for gastric endocrine cells. B and C: representative immunoelecton photograph of a ghrelin-producing cells in the oxyntic gland. C: high magnification of B. D: ghrelin-containing neurons in hypothalamic arcuate nucleus in a colchicine-treated rat. 3v, third ventricle.

opened- and closed-type of ghrelin cells are found, and the number of opened-type cells gradually increased in the direction from stomach to the lower gastrointestinal tract.

Ghrelin has been found in the hypothalamic arcuate nucleus (ARC), an important region for controlling appetite (Fig. 3D) (17, 43). 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 (PVN), and arcuate hypothalamic nuclei (44). 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, and anti-ghrelin immunoglobulin G (IgG) robustly suppresses feeding (17).

The pancreas is also a ghrelin-producing organ. In the pancreatic islets, however, the cell type of ghrelin cells remains controversial, whether it be the α cells, β cells, the newly identified islet ε cells, or a unique novel islet cell type (45 – 49). Interestingly, the pancreatic ghrelin profile changes dramatically during fetal development; 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 (50). Ghrelin mRNA expression and ghrelin concentration (mostly des-acyl ghrelin) are markedly elevated in the fetal pancreas, being several times greater than in the fetal stomach. 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 (46). The ontogenetic appearance of islet ghrelin cells precedes that of gastric ghrelin cells, which may indicate a developmental role for islet ghrelin.

B. Circulating ghrelin

To measure the plasma concentration of ghrelin, it is necessary to use EDTA and aprotinin when collecting blood samples (29). After the samples are centrifuged, the plasma fraction should be treated with 1/10 volume of 1 N hydrogen chloride. The acidified plasma should be kept in the freezer. Two major forms of ghrelin are

found in plasma: *n*-octanoyl-modified and des-acyl ghrelin. The normal plasma concentration of ghrelin in humans is 10 – 20 fmol/ml for mostly *n*-octanoyl ghrelin and 150 – 180 fmol/ml for total ghrelin, including both acyl-modified and des-acyl ghrelins. Circulating ghrelin is increased in fasting conditions and reduced after habitual feeding (51, 52), suggesting that ghrelin may be as an initiation signal for food intake or ghrelin secretion is controlled by some nutritional factors in blood.

C. Ghrelin receptor

The ghrelin receptor, or GHS-R, is a typical G-protein coupled-seven transmembrane receptor. Two distinct ghrelin receptor cDNAs have been isolated (9). The first, GHS-R type 1a, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor. Another GHS-R cDNA, type 1b, is produced by an alternative splicing mechanism (9). 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; it is thus a COOH-terminal truncated form of the type 1a receptor and is pharmacologically inactive. The ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, chicken, and pufferfish (13, 53, 54), suggesting that ghrelin and its receptor serve important physiological functions.

The mRNA of ghrelin receptor, GHS-R type 1a, is prominently expressed in the ARC and ventromedial nuclei and in the hippocampus (9, 17, 55). 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 (56). GHS-R 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. Moreover, a ghrelin-receptor transcript product is found in a mRNA sample isolated from the vagal nodose ganglion (57). Receptors in the vagus are synthesized at the cell bodies and transported to the nerve terminals through axonal transport. These results indicate that there is a close proximity between ghrelin-producing cells and vagal afferent terminals in the stomach. 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 (55, 58, 59), indicating that ghrelin has multiple functions in these tissues.

V. Physiological and pathophysiological actions of ghrelin

A. Neuroendocrine effect of ghrelin

Ghrelin is a multifaceted peptide hormone (Table 1). Because GHS is an agonist of the GHS-R, it was reasonable to expect that ghrelin possessed GH-releasing activity. In rats, large secretions of GH were observed following intravenous (i.v.), intraperitoneal, subcutaneous, and intracerebroventricular (i.c.v.) injection of ghrelin (15, 43, 60, 61), indicating that ghrelin is (directly or indirectly) a GH-releasing peptide. Intravenous ghrelin administration in healthy humans potently stimulated GH release, whereas adrenocorticotrophic hormone (ACTH), cortisol, and prolactin levels are also elevated slightly after ghrelin injection (62, 63). The coadministration of ghrelin and GHRH synergistically effect GH secretion (64).

Ghrelin stimulates GH release from primary cultured pituitary cells, which indicates that ghrelin can act directly on the pituitary (15). 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 (65). Prior administration of GHRH antagonists blocks nearly all GHS-dependent GH secretion in humans (66). Moreover, when using primary pituitary cells, the ghrelin treatment increased GH release by 2 –

Table 1. Effects of ghrelin

Hormone release	
Growth hormone release	↑
Adrenocorticotrophic hormone release	↑
Cortisol release	↑
Prolactin release	↑
Thyroid stimulating hormone release	↓ ? →
Luteinzing hormone release	↑ ? →
Follicle-stimulating hormone release	→
Insulin release	↑ ? ↓
Anabolic effects	
Appetite	↑
Adiposity	↑
Cardiovascular functions	
Cardiac output	↑
Blood pressure	↓
Apoptosis of cardiomyocytes in vitro	↓
Gastric functions	
Gastric acid secretion	↑
Gastric motility	↑

3 times above the basal level (15), which is lower than the level of induction seen when ghrelin is administered to rats in vivo. These facts suggest that other factors may be involved in vivo in order for the maximal level of GH release to be achieved by ghrelin administration. One possibility is transmission via the vagus nerve. Indeed, when the vagus nerves are cut in rats, the induction of GH release after ghrelin injection peripherally is dramatically decreased (57), indicating that the vagus nerve is needed for the maximal stimulatory effects of ghrelin. Peripheral administration of ghrelin induces c-*Fos* expression in GHRH neurons in ARC; however, this effect is canceled by chemical and surgical deafferentation of the vagus nerve. These findings imply that ghrelin elicits GH secretion from the pituitary by modulation of hypothalamic GHRH via the afferent vagus nerve system.

The effect of ghrelin on pituitary hormones is not specific to GH. Ghrelin and GHS also modulate lactotroph and corticotroph in human and animal studies (62, 67, 68). A small prolactin-releasing effect of ghrelin has been shown directly in pituitary cell cultures, so the major site of action may be either the pituitary or the hypothalamus (69). Since GHS do not stimulate ACTH release directly from pituitary cell cultures, it is probable that GHS affect the hypothalamo-pituitary-adrenal (HPA) via either one of the two major ACTH stimulators in the hypothalamus, corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP). In a hypothalamic incubation study, GHS were found to stimulate hypothalamic AVP release while no reproducible effect was observed on CRH secretion (70). When ghrelin was used in either the same system, or in hypothalamic slices, both AVP and CRH stimulation was observed (71). Interestingly, the effect of ghrelin on ACTH secretion is even more pronounced than that elicited by GHS (62, 72). These results indicate that ghrelin stimulates the HPA axis independent of the pituitary, via the hypothalamus, involving both CRH and AVP stimulation.

B. Ghrelin stimulates food intake

Feeding is a basic behavior that is necessary for life. 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 (73). GHS causes a short-lived increase in food intake when administered either systemically or i.c.v. (74, 75). The orexigenic or appetite-stimulating effect of GHS is not altered by pretreatment with a GHRH antagonist at a dose that completely blocked the feeding response to i.c.v. administered GHRH, indicating GHS-R mediates the effect on appetite. Both the peripheral and central administration of ghrelin also stimulated food intake in freely feeding rodents and in GH-deficient dwarf rats (17, 18, 43, 61). A GHS-R antagonist suppressed ghrelin-induced feeding and furthermore, the administration of ghrelin-specific antibodies suppressed starvation-induced feeding in a dose-dependent manner, suggesting that ghrelin is a powerful, endogenous orexigenic peptide. In humans, i.v. bolus injection or infusion of ghrelin induces hunger (76). Chronic i.c.v. administration of ghrelin strongly stimulates feeding in rats and increases body weight gain (17). Daily subcutaneous administration of ghrelin in mice induces a progressive increase in body weight, with a significant gain in fat mass but no change in lean body mass. This could result from a chronic decrease of fat oxidation as indicated by an increased respiratory quotient (18). Because ghrelin can induce adiposity that is sustained during ghrelin treatment, ghrelin might participate in the long-term regulation of body mass.

Unlike ghrelin, most other hypothalamic peptides, for example, NPY, AgRP, orexins, melanin-concentrating hormone (MCH), and galanin, that stimulate feeding when administered centrally are ineffective when administered into the periphery. Ghrelin is the first identified circulating hormone that promotes feeding following systemic administration.

C. Central actions of ghrelin

Immunohistochemical analyses indicate that ghrelin-containing neurons are found in the ARC of the hypothalamus, a region involved in appetite regulation (15, 77). 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 pro-opiomelanocortin (POMC) neurons to suppress the release of this anorexigenic peptide. The 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 γ-aminobutyric acid (GABA) release, resulting in the stimulation of CRH-expressing neurons, leading to ACTH and cortisol release.

As suggested by the distribution of ghrelin-containing neurons in the hypothalamus, i.c.v. administration of ghrelin induces c-*Fos* expression in regions of primary importance in the regulation of feeding, including ARC, PVN, and dorsomedial and ventromedial hypothalamic nuclei (17). This distribution is coincident with that of GHS-R (55). GHS-R mRNA is expressed in 94% of the neurons in the ARC that express NPY, in 8% of cells that express POMC, in 30% of those that express somatostatin, and in 20–25% of those that express

GHRH mRNA (78). Moreover, i.c.v. administration of ghrelin leads to increases in the expression of both NPY and AGRP mRNAs (17, 43). The appetite-stimulating effects of ghrelin are blocked by an antagonist of NPY-receptor 1. I.c.v. injection of an AgRP inhibitor, anti-NPY IgG or anti-AgRP IgG, inhibits ghrelin-induced feeding. These results indicate that ghrelin exerts its feeding activity by stimulating NPY/AGRP neurons in the ARC to promote the production and secretion of NPY and AgRP peptides. The ARC is a crucial target of leptin, an anorexia-mediating molecule produced from adipose tissue (73). Most NPY/AGRP-containing neurons and POMC/CART-containing neurons also express leptin receptors, and both types of neurons are regulated by leptin, albeit in an opposing manner. Leptin inhibits ghrelin-induced feeding, and ghrelin substantially reverses the anorexic effect of leptin, indicating that ghrelin may antagonize leptin action in regulating the NPY-AGRP system.

Orexin, an orexigenic hypothalamic neuropeptide, is involved in the regulation of food intake and arousal (79). Ghrelin stimulates isolated orexin neurons, whereas glucose and leptin inhibit them (80). I.c.v. administration of ghrelin induces c-*Fos* expression in orexin-producing cells (81). 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 an 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.

D. Vagus nerve and ghrelin

Peripherally injected ghrelin stimulates hypothalamic neurons and stimulates food intake (57, 76, 82). In general, peptides injected peripherally do not pass the blood-brain barrier. 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 (57, 83). Indeed, vagotomy inhibits the ability of ghrelin to stimulate food intake and GH release, and the activation of NPY-producing and GHRH-producing neurons. A similar effect was observed when capsaicin, a specific afferent neurotoxin, was applied to vagus nerve terminals to induce sensory denervation. These results indicate that the vagus afferent nerve is the major pathway conveying peripheral signals of ghrelin for starvation and GH secretion to the brain.

E. Cardiovascular functions

The gene expression of both ghrelin and its receptor has been demonstrated in heart and aorta (58, 84). In addition, a radiolabeled ghrelin was shown to bind to heart and to peripheral vascular tissue, where the density of ghrelin receptor is up-regulated with atherosclerosis (85). On the other hand, considerable specific binding of radiolabeled peptidyl GHS, such as [^{125}I]Tyr-Ala-hexarelin, is easily detectable in rat myocardium and various cardiovascular tissues (86). This binding is inhibited by unlabeled Tyr-Ala-hexarelin, hexarelin, and other peptidyl GHS, but not by the non-peptidyl GHS MK-0677 (87). Therefore, these binding sites are unlikely to be classical GHS-R because they do not bind ghrelin.

There is already evidence that ghrelin and/or GHS mediate GH-independent cardiovascular functions, both in humans and in animals. In humans an intravenous administration of ghrelin decreases mean arterial pressure without changing the heart rate (63, 84). Ghrelin also increases 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 construction compared with placebo-treated controls (88). Furthermore, ghrelin increased the diastolic thickness of the non-infarcted posterior wall, inhibited left ventricle enlargement, and increased left ventricular fractional shortening in these CHF rats. Ghrelin, thus, improves left ventricle dysfunction and attenuates the development of left ventricle remodeling and cardiac cachexia.

It has been reported that ghrelin inhibits apoptosis of primary adult and H9c2 cardiomyocytes and endothelial cells in vitro (89). These effects are regulated through activation of extracellular signal-regualted kinase-1/2 and Akt serine kinase. Interestingly, des-acyl ghrelin is similarly active, and H9c2 cardiomyocytes exhibit no gene expression of the ghrelin receptor, indicating that another unidentified receptor may be involved.

F. Gastro-entero-pancreatic functions

Intravenous administration of ghrelin dose-dependently increases gastric acid secretion and stimulates gastric motility and emptying (43, 90). These responses to ghrelin were abolished by pretreatment with either atropine or bilateral cervical vagotomy, but not by a histamine H_2-receptor antagonist. I.c.v. injection of ghrelin also increases gastric acid secretion in a dose-dependent manner and induces c-*fos* expression in the nucleus of the solitary tract and the dorsomotor nucleus of the vagus nerve (91). The gastro-prokinetic activity of ghrelin is independent of its GH-releasing effect and is likely to be mediated by the vagal-cholinergic

muscarinic pathway. Ghrelin accelerates the normal emptying process in rodents at doses compatible to those required to stimulate appetite and GH release (92). Ghrelin also accelerates the transit of the small intestine but not that of the colon. It was reported that calcitonin gene-related peptide (CGRP) [8 – 37], an antagonist of CGRP, is among the few agents with beneficial effects on the inhibition of gastric emptying after abdominal surgery (93). Ghrelin is more active than CGRP [8 – 37] and is capable of reversing the postoperative gastric ileus.

The identification of the pancreatic ghrelin-producing 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 (41, 45, 94, 95). These discrepancies may be due to experimental design and/or species differences.

VI. Control of ghrelin secretion and associated diseases

A. Regulation of ghrelin secretion

Circulating ghrelin levels are responsive to acute and chronic energy imbalance, increased by food deprivation and energy restriction, and decreased by food consumption and obesity. 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 (96, 97). Because gastric distension by water intake does not change ghrelin concentration, mechanical distention of the stomach alone clearly does not induce ghrelin release. In contrast, the ghrelin peptide contents in the stomach significantly decrease after fasting and increase after re-feeding. This inverse pattern of ghrelin levels in the stomach tissue and plasma may result from increased secretion of ghrelin from the stomach in response to fasting and subsequent decreased secretion upon resumption of feeding. Plasma ghrelin concentration showed a nocturnal increase, and this increase was blunted in obese subjects or by sleep deprivation (98, 99). Circulating ghrelin also decreases in patients with short bowel syndrome.

Obesity is characterized by a blunted GH secretion that might help to maintain the state and this decreased secretion is reversed by weight loss (100). Fasting plasma ghrelin levels in obesity are significantly lower than those in control subjects, and is negatively correlated with body mass index (BMI), percent body fat, and fasting insulin and leptin levels (101). The low levels of ghrelin might contribute to the decreased GH secretion and increased food consumption in obese subjects. Plasma ghrelin levels are also negatively correlated with plasminogen activator-1 levels that are elevated in insulin-resistant subjects (102). Gastric bypass surgery is an important treatment for morbid obesity that can produce prolonged weight reduction (103). Recent research has revealed that ghrelin may contribute to the body weight reduction that occurs following gastric bypass. In gastric bypass patients, ghrelin secretion was found to be reduced by up to 77% compared with the normal-weight control group and by up to 72% compared with the matched obese group (104). Furthermore, the normal meal-related fluctuations and diurnal rhythm of ghrelin level were absent in these patients. The mechanism for decreasing circulating ghrelin in gastric bypass patients is not known. Conversely, bypass subjects exhibited normal postprandial insulin secretion and diurnal leptin cycling. Therefore, suppression of plasma ghrelin by gastric bypass can contribute to the efficacy of this procedure as weight reduction therapy, in addition to the restriction of the stomach volume and the reduction of nutrient digestion.

Exogenous treatments with somatostatin and its analogs as well as infusion of urocortin-1, a potent anorexigenic peptide, suppress circulating ghrelin (105 – 107). However, administration of leptin does not modify ghrelin levels (108). Exogenous GH decreases stomach ghrelin mRNA expression and plasma ghrelin levels, but does not affect stomach ghrelin stores (109). These results suggest that pituitary GH exhibits a feedback regulation on stomach ghrelin production.

B. Polymorphism in the ghrelin gene and obesity

In humans, for two polymorphisms, Arg51Gln and Leu72Met, allelic frequencies are similar between obese patients and controls (110, 111). 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 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 proghrelin peptide may still be produced, although its biological activity has not been assessed.

C. Feeding disorders and cachexia

Plasma ghrelin levels in anorexia nervosa (AN) patients are high and return to control levels after weight gain by renutrition (37, 112, 113). AN patients often show markedly elevated GH levels, which may be due to

high circulating levels of ghrelin. Prader-willi syndrome (PWS) is a complex genetic disorder characterized by mild mental retardation, hyperphagia, short stature, muscular hypotonia, and distinctive behavior features. Excessive appetite in PWS causes progressive severe obesity, but the mean plasma level of ghrelin is higher by three- to four-fold in PWS than control subjects (114). It is unclear what underlies the increased ghrelin levels in these patients. Elucidation of the precise mechanism by which ghrelin gene expression is regulated may reveal the genetic cause of hyperphagia in PWS.

Recent studies suggest that ghrelin may be a clinical marker of catabolism (115). Elevated plasma ghrelin levels are observed in cachexia associated with chronic heart failure, lung cancer, and liver cirrhosis. Indeed, in patients with prolonged and severe illnesses, GHS reverses diet-induced catabolism and improves alterations in the somatotrophic axis and protein catabolism.

VII. Clinical implication

The combined administration of GHS and GHRH appears to be the most potent stimulus of GH release in humans. It may be a convenient, safe, and reliable test for the diagnosis of GH deficiency. The lack of GH is associated with alterations in body composition, increased prevalence of cardiovascular diseases, and shortened life expectancy. Most of the patients with GH deficiency respond to GHS (116). Potential targets for ghrelin and GHS compounds include children and adults with GH deficiency. Chronic treatment of elderly with MK-0677, non-peptidyl GHS, is reported to reverse age-related changes of the GH/IGF-1 axis and to improve the quality of sleep in healthy elderly subjects (117). Furthermore, ghrelin has been shown to regulate expression, of a pituitary-specific transcription factor, Pit-1, suggesting a role in somatotroph differentiation in addition to GH secretion (118).

At present, ghrelin is only a peripheral orexigenic signal that is effective upon its intravenous injection. Ghrelin may be useful as an orexigenic agent for the treatment of eating disorders such as AN. In contrast, blocking or neutralizing the orexigenic action of ghrelin 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; indeed, ghrelin-null mice as well as NPY-null mice showed no obvious abnormalities in feeding behavior (119, 120).

In humans, ghrelin and GHS possess beneficial cardiovascular effects. In fact, administration of ghrelin in normal subjects and even in patients with chronic heart failure significant reduces cardiac afterload and increases cardiac output without increasing heart rate (88); and in rats with heart failure, it improves cardiac function and attenuates the development of cardiac cachexia. In vitro, ghrelin and GHS inhibit apoptosis of cardiomyocytes and endothelial cells (89). 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.

A recent study indicates a potent protective effect against ethanol-induced gastric lesions by central ghrelin and a partial peripheral protective effect (121). This effect of ghrelin is mediated by endogenous nitric oxide release and requires the integrity of sensory nerve fibers. Abdominal surgery inhibits gastric emptying and digestive motor activity in experimental animals and humans (92). Interestingly, the potent prokinetic activity of ghrelin has been confirmed and extended to allow for reversal of a gastric postoperative ileus in the animal model. It should be examined whether ghrelin can help prevent the ileus by abdominal surgery in humans.

The isolation of ghrelin can be considered a landmark in the GH field, allowing for new insights into understanding the regulation of the GH system, food intake, body fat composition and gastrointestinal functions. Ghrelin may prove to be an effective probe for elucidating the normal and pathological regulation of GH release and appetite in humans, and it should be explored in the clinical setting as a potential diagnostic and therapeutic tool.

Acknowledgments

Research in the authors' laboratory is supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Ministry of Health, Labor, and Welfare of Japan; and the Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

References

1 Frohman LA, Jansson JO. Growth hormone-releasing hormone. Endocr Rev. 1986;7:223–253.
2 Frohman LA, Kineman RD, Kamegai J, Park S, Teixeira LT, Coschigano KT, et al. Secretagogues and the somatotrope: signaling and proliferation. Recent Prog Horm Res. 2000;55: 269–291.
3 Muller EE, Locatelli V, Cocchi D. Neuroendocrine control of growth hormone secretion. Physiol Rev. 1999;79:511–607.
4 Bowers CY. Growth hormone-releasing peptide (GHRP). Cell Mol Life Sci. 1998;54:1316–1329.

5 Dickson SL, Bailey AR, Leng G. Growth hormone (GH) secretagogues and neuroendocrine regulation of GH secretion. Growth Horm IGF Res. 1999;9 Suppl A:89–91.
6 Ghigo E, Arvat E, Broglio F, Giordano R, Gianotti L, Muccioli G, et al. Endocrine and non-endocrine activities of growth hormone secretagogues in humans. Horm Res. 1999;51 Suppl 3:9–15.
7 Karydis I, Tolis A, Tolis G. New GH secretagogues and potential usefulness in thalassemia. J Pediatr Endocrinol Metab. 1998;11 Suppl 3:857–862.
8 Smith RG, Van der Ploeg LH, Howard AD, Feighner SD, Cheng K, Hickey GJ, et al. Peptidomimetic regulation of growth hormone secretion. Endocr Rev. 1997;18:621–645.
9 Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, et al. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 1996;273: 974–977.
10 Korbonits M, Ciccarelli E, Ghigo E, Grossman AB. The growth hormone secretagogue receptor. Growth Horm IGF Res. 1999;9 Suppl A:93–99.
11 Pong SS, Chaung LY, Dean DC, Nargund RP, Patchett AA, Smith RG. Identification of a new G-protein-linked receptor for growth hormone secretagogues. Mol Endocrinol. 1996;10:57–61.
12 Robinson IC. Hypothalamic targets for growth hormone secretagogues. Acta Paediatr Suppl. 1997;423:88–91.
13 Smith RG, Feighner S, Prendergast K, Guan X, Howard A. A new orphan receptor involved in pulsatile growth hormone release. Trends Endocrinol Metab. 1999;10:128–135.
14 Smith RG, Palyha OC, Feighner SD, Tan CP, McKee KK, Hreniuk DL, et al. Growth hormone releasing substances: types and their receptors. Horm Res. 1999;51 Suppl 3:1–8.
15 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999;402:656–660.
16 Kojima M, Hosoda H, Matsuo H, Kangawa K. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. Trends Endocrinol Metab. 2001;12:118–122.
17 Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. Nature. 2001;409:194–198.
18 Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature. 2000;407:908–913.
19 Bowers CY, Chang JK, Fong TTW. A synthetic pentapeptide which specifically release GH, in vitro. Program of Endocrine Society, 59th Annual Meeting, Chicago, p. 232.
20 Momany FA, Bowers CY, Reynolds GA, Chang D, Hong A, Newlander K. Design, synthesis, and biological activity of peptides which release growth hormone in vitro. Endocrinology. 1981;108:31–39.
21 Ghigo E, Arvat E, Giordano R, Broglio F, Gianotti L, Maccario M, et al. Biologic activities of growth hormone secretagogues in humans. Endocrine. 2001;14:87–93.
22 Cheng K, Chan WW, Barreto A Jr, Convey EM, Smith RG. The synergistic effects of His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 on growth hormone (GH)-releasing factor-stimulated GH release and intracellular adenosine 3',5'-monophosphate accumulation in rat primary pituitary cell culture. Endocrinology. 1989;124: 2791–2798.
23 Cheng K, Chan WW, Butler B, Barreto A Jr, Smith RG. Evidence for a role of protein kinase-C in His-D-Trp-Ala-Trp-D-Phe-Lys-NH2-induced growth hormone release from rat primary pituitary cells. Endocrinology. 1991;129:3337–3342.
24 Akman MS, Girard M, O'Brien LF, Ho AK, Chik CL. Mechanisms of action of a second generation growth hormone-releasing peptide (Ala-His-D-beta Nal-Ala-Trp-D-Phe-Lys-NH2) in rat anterior pituitary cells. Endocrinology. 1993;132: 1286–1291.
25 Kaiya H, Kojima M, Hosoda H, Koda A, Yamamoto K, Kitajima Y, et al. Bullfrog ghrelin is modified by n-octanoic acid at its third threonine residue. J Biol Chem. 2001;276:40441–40448.
26 Hosoda H, Kojima M, Matsuo H, Kangawa K. Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. J Biol Chem. 2000;275:21995–22000.
27 Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. J Biol Chem. 2003;278:64–70.
28 Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun. 2000;279:909–913.
29 Hosoda H, Doi K, Nagaya N, Okumura H, Nakagawa E, Enomoto M, et al. Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. Clin Chem. 2004;50:1077–1080.
30 Cassoni P, Ghe C, Marrocco T, Tarabra E, Allia E, Catapano F, et al. Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines. Eur J Endocrinol. 2004;150: 173–184.
31 Zhang W, Zhao L, Lin TR, Chai B, Fan Y, Gantz I, et al. Inhibition of adipogenesis by ghrelin. Mol Biol Cell. 2004;15: 2484–2491.
32 Kim MS, Yoon CY, Jang PG, Park YJ, Shin CS, Park HS, et al. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. Mol Endocrinol. 2004;18:2291–2301.
33 Bednarek MA, Feighner SD, Pong SS, McKee KK, Hreniuk DL, Silva MV, et al. Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem. 2000;43:4370–4376.
34 Matsumoto M, Hosoda H, Kitajima Y, Morozumi N, Minamitake Y, Tanaka S, et al. Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem Biophys Res Commun. 2001;287:142–146.
35 Matsumoto M, Matsumoto M, Kitajima Y, Iwanami T, Hayashi Y, Tanaka S, et al. Structural similarity of ghrelin derivatives to peptidyl growth hormone secretagogues. Biochem Biophys Res Commun. 2001;284:655–659.
36 Torsello A, Ghe' C, Bresciani E, Catapano F, Ghigo E, Deghenghi R, et al. Short ghrelin peptides neither displace ghrelin binding in vitro nor stimulate GH release in vivo. Endocrinology. 2002;143:1968–1971.
37 Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, et al. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity

levels in humans. J Clin Endocrinol Metab. 2001;86:4753–4758.
38 Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology. 2000;141:4255–4261.
39 Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, et al. The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. J Clin Endocrinol Metab. 2001;86:881–887.
40 Hayashida T, Nakahara K, Mondal MS, Date Y, Nakazato M, Kojima M, et al. Ghrelin in neonatal rats: distribution in stomach and its possible role. J Endocrinol. 2002;173:239–245.
41 Lee HM, Wang G, Englander EW, Kojima M, Greeley GH Jr. Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. Endocrinology. 2002;143: 185–190.
42 Sakata I, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, Kangawa K, et al. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. Peptides. 2002;23:531–536.
43 Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, et al. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology. 2001; 120:337–345.
44 Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron. 2003;37:649–661.
45 Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, et al. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. Diabetes. 2002;51:124–129.
46 Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L. Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A. 2004;101:2924–2929.
47 Wierup N, Svensson H, Mulder H, Sundler F. The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. Regul Pept. 2002;107:63–69.
48 Wierup N, Yang S, McEvilly RJ, Mulder H, Sundler F. Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. J Histrochem Cytochem. 2004;52:301–310.
49 Volante M, Allia E, Gugliotta P, Funaro A, Broglio F, Deghenghi R, et al. Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors. J Clin Endocrinol Metab. 2002;87:1300–1308.
50 Chanoine JP, Wong AC. Ghrelin gene expression is markedly higher in fetal pancreas compared with fetal stomach: effect of maternal fasting. Endocrinology. 2004;145:3813–3820.
51 Tschöp M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, et al. Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest. 2001;24:RC19–RC21.
52 Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes. 2001;50:1714–1719.
53 Palyha OC, Feighner SD, Tan CP, McKee KK, Hreniuk DL, Gao YD, et al. Ligand activation domain of human orphan growth hormone (GH) secretagogue receptor (GHS-R) conserved from Pufferfish to humans. Mol Endocrinol. 2000;14:160–169.
54 Smith RG, Leonard R, Bailey AR, Palyha O, Feighner S, Tan C, et al. Growth hormone secretagogue receptor family members and ligands. Endocrine. 2001;14:9–14.
55 Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, et al. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res. 1997;48:23–29.
56 Bennett PA, Thomas GB, Howard AD, Feighner SD, van der Ploeg LH, Smith RG, et al. Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth hormone in the rat. Endocrinology. 1997;138:4552–4557.
57 Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology. 2002;123:1120–1128.
58 Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, et al. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab. 2002;87:2988–2991.
59 Hattori N, Saito T, Yagyu T, Jiang BH, Kitagawa K, Inagaki C. GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. J Clin Endocrinol Metab. 2001;86:4284–4291.
60 Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K, et al. Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. Biochem Biophys Res Commun. 2000;275:477–480.
61 Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, et al. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology. 2000; 141:4325–4328.
62 Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, et al. Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab. 2000;85:4908–4911.
63 Nagaya N, Miyatake K, Uematsu M, Oya H, Shimizu W, Hosoda H, et al. Hemodynamic, renal, and hormonal effects of ghrelin infusion in patients with chronic heart failure. J Clin Endocrinol Metab. 2001;86:5854–5859.
64 Hataya Y, Akamizu T, Takaya K, Kanamoto N, Ariyasu H, Saijo M, et al. A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. J Clin Endocrinol Metab. 2001;86:4552–4555.
65 Popovic V, Miljic D, Micic D, Damjanovic S, Arvat E, Ghigo E, et al. Ghrelin main action on the regulation of growth hormone release is exerted at hypothalamic level. J Clin Endocrinol Metab. 2003;88:3450–3453.
66 Pandya N, DeMott-Friberg R, Bowers CY, Barkan AL, Jaffe CA. Growth hormone (GH)-releasing peptide-6 requires endogenous hypothalamic GH-releasing hormone for maximal GH stimulation. J Clin Endocrinol Metab. 1998;83:1186–1189.
67 Arvat E, di Vito L, Maccagno B, Broglio F, Boghen MF, Deghenghi R, et al. Effects of GHRP-2 and hexarelin, two

synthetic GH-releasing peptides, on GH, prolactin, ACTH and cortisol levels in man. Comparison with the effects of GHRH, TRH and hCRH. Peptides. 1997;18:885–891.
68 Riley LG, Hirano T, Grau EG. Rat ghrelin stimulates growth hormone and prolactin release in the tilapia, Oreochromis mossambicus. Zoolog Sci. 2002;19:797–800.
69 Rubinfeld H, Hadani M, Taylor JE, Dong JZ, Comstock J, Shen Y, et al. Novel ghrelin analogs with improved affinity for the GH secretagogue receptor stimulate GH and prolactin release from human pituitary cells. Eur J Endocrinol. 2004;151:787–795.
70 Korbonits M, Little JA, Forsling ML, Tringali G, Costa A, Navarra P, et al. The effect of growth hormone secretagogues and neuropeptide Y on hypothalamic hormone release from acute rat hypothalamic explants. J Neuroendocrinol. 1999;11: 521–528.
71 Mozid AM, Tringali G, Forsling ML, Hendricks MS, Ajodha S, Edwards R, et al. Ghrelin is released from rat hypothalamic explants and stimulates corticotrophin-releasing hormone and arginine-vasopressin. Horm Metab Res. 2003;35:455–459.
72 Arvat E, Ramunni J, Bellone J, Di Vito L, Baffoni C, Broglio F, et al. The GH, prolactin, ACTH and cortisol responses to Hexarelin, a synthetic hexapeptide, undergo different age-related variations. Eur J Endocrinol. 1997;137:635–642.
73 Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature. 2000; 404:661–671.
74 Torsello A, Luoni M, Schweiger F, Grilli R, Guidi M, Bresciani E, et al. Novel hexarelin analogs stimulate feeding in the rat through a mechanism not involving growth hormone release. Eur J Pharmacol. 1998;360:123–129.
75 Locke W, Kirgis HD, Bowers CY, Abdoh AA. Intracerebroventricular growth-hormone-releasing peptide-6 stimulates eating without affecting plasma growth hormone responses in rats. Life Sci. 1995;56:1347–1352.
76 Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab. 2001;86:5992–5995.
77 Lu S, Guan JL, Wang QP, Uehara K, Yamada S, Goto N, et al. Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. Neurosci Lett. 2002;321:157–160.
78 Willesen MG, Kristensen P, Romer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology. 1999;70:306–316.
79 Sakurai T. Orexin: a link between energy homeostasis and adaptive behaviour. Curr Opin Clin Nutr Metab Care. 2003;6: 353–360.
80 Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, et al. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron. 2003;38:701–713.
81 Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, et al. Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology. 2003;144:1506–1512.
82 Wang L, Saint-Pierre DH, Tache Y. Peripheral ghrelin selectively increases Fos expression in neuropeptide Y-synthesizing neurons in mouse hypothalamic arcuate nucleus. Neurosci Lett. 2002;325:47–51.
83 Sakata I, Yamazaki M, Inoue K, Hayashi Y, Kangawa K, Sakai T. Growth hormone secretagogue receptor expression in the cells of the stomach-projected afferent nerve in the rat nodose ganglion. Neurosci Lett. 2003;342:183–186.
84 Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, et al. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol. 2001;280:R1483–R1487.
85 Katugampola SD, Pallikaros Z, Davenport AP. [125I-His(9)]-ghrelin, a novel radioligand for localizing GHS orphan receptors in human and rat tissue: up-regulation of receptors with atherosclerosis. Br J Pharmacol. 2001;134:143–149.
86 Bodart V, Bouchard JF, McNicoll N, Escher E, Carriere P, Ghigo E, et al. Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. Circ Res. 1999;85:796–802.
87 Papotti M, Ghe C, Cassoni P, Catapano F, Deghenghi R, Ghigo E, et al. Growth hormone secretagogue binding sites in peripheral human tissues. J Clin Endocrinol Metab. 2000;85: 3803–3807.
88 Nagaya N, Kangawa K. Ghrelin, a novel growth hormone-releasing peptide, in the treatment of chronic heart failure. Regul Pept. 2003;114:71–77.
89 Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, et al. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol. 2002;159:1029–1037.
90 Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, et al. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun. 2000;276:905–908.
91 Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun. 2001;280:904–907.
92 Trudel L, Tomasetto C, Rio MC, Bouin M, Plourde V, Eberling P, et al. Ghrelin/motilin-related peptide is a potent prokinetic to reverse gastric postoperative ileus in rat. Am J Physiol Gastrointest Liver Physiol. 2002;282:G948–G952.
93 Plourde V, Wong HC, Walsh JH, Raybould HE, Tache Y. CGRP antagonists and capsaicin on celiac ganglia partly prevent postoperative gastric ileus. Peptides. 1993;14:1225-1229.
94 Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, et al. Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. J Clin Endocrinol Metab. 2001;86:5083–5086.
95 Reimer MK, Pacini G, Ahren B. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. Endocrinology. 2003;144:916–921.
96 McCowen KC, Maykel JA, Bistrian BR, Ling PR. Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents. J Endocrinol. 2002;175:R7–R11.
97 Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, et al. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab. 2002;87:240–244.
98 Dzaja A, Dalal MA, Himmerich H, Uhr M, Pollmacher T, Schuld A. Sleep enhances nocturnal plasma ghrelin levels in healthy subjects. Am J Physiol Endocrinol Metab. 2004;286: E963–E967.
99 Yildiz BO, Suchard MA, Wong ML, McCann SM, Licinio J. Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. Proc Natl Acad Sci USA. 2004;101:10434–10439.

100 Casanueva FF, Dieguez C. Growth hormone secretagogues: physiological role and clinical utility. Trends Endocrinol Metab. 1999;10:30–38.
101 Tschöp M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. Diabetes. 2001;50:707–709.
102 Ikezaki A, Hosoda H, Ito K, Iwama S, Miura N, Matsuoka H, et al. Fasting plasma ghrelin levels are negatively correlated with insulin resistance and PAI-1, but not with leptin, in obese children and adolescents. Diabetes. 2002;51:3408–3411.
103 Fobi MA. Surgical treatment of obesity: a review. J Natl Med Assoc. 2004;96:61–75.
104 Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med. 2002; 346:1623–1630.
105 Barkan AL, Dimaraki EV, Jessup SK, Symons KV, Ermolenko M, Jaffe CA. Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels. J Clin Endocrinol Metab. 2003;88: 2180–2184.
106 Davis ME, Pemberton CJ, Yandle TG, Lainchbury JG, Rademaker MT, Nicholls MG, et al. Urocortin-1 infusion in normal humans. J Clin Endocrinol Metab. 2004;89:1402–1409.
107 Norrelund H, Hansen TK, Orskov H, Hosoda H, Kojima M, Kangawa K, et al. Ghrelin immunoreactivity in human plasma is suppressed by somatostatin. Clin Endocrinol (Oxf). 2002;57: 539–546.
108 Chan JL, Bullen J, Lee JH, Yiannakouris N, Mantzoros CS. Ghrelin levels are not regulated by recombinant leptin administration and/or three days of fasting in healthy subjects. J Clin Endocrinol Metab. 2004;89:335–343.
109 Qi X, Reed J, Englander EW, Chandrashekar V, Bartke A, Greeley GH Jr. Evidence that growth hormone exerts a feedback effect on stomach ghrelin production and secretion. Exp Biol Med (Maywood). 2003;228:1028–1032.
110 Miraglia del Giudice E, Santoro N, Cirillo G, Raimondo P, Grandone A, D'Aniello A, et al. Molecular screening of the ghrelin gene in Italian obese children: the Leu72Met variant is associated with an earlier onset of obesity. Int J Obes Relat Metab Disord. 2004;28:447–450.
111 Poykko S, Ukkola O, Kauma H, Savolainen MJ, Kesaniemi YA. Ghrelin Arg51Gln mutation is a risk factor for Type 2 diabetes and hypertension in a random sample of middle-aged subjects. Diabetologia. 2003;46:455–458.
112 Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, et al. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. Eur J Endocrinol. 2001;145:669–673.
113 Soriano-Guillen L, Barrios V, Campos-Barros A, Argente J. Ghrelin levels in obesity and anorexia nervosa: effect of weight reduction or recuperation. J Pediatr. 2004;144:36–42.
114 Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS, et al. Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med. 2002;8:643–644.
115 Inui A. Ghrelin: an orexigenic and somatotrophic signal from the stomach. Nat Rev Neurosci. 2001;2:551–560.
116 Dieguez C, Casanueva FF. Ghrelin: a step forward in the understanding of somatotroph cell function and growth regulation. Eur J Endocrinol. 2000;142:413–417.
117 Chapman IM, Bach MA, Van Cauter E, Farmer M, Krupa D, Taylor AM, et al. Stimulation of the growth hormone (GH)-insulin-like growth factor I axis by daily oral administration of a GH secretogogue (MK-677) in healthy elderly subjects. J Clin Endocrinol Metab. 1996;81:4249–4257.
118 Garcia A, Alvarez CV, Smith RG, Dieguez C. Regulation of Pit-1 expression by ghrelin and GHRP-6 through the GH secretagogue receptor. Mol Endocrinol. 2001;15:1484–1495.
119 Sun Y, Wang P, Zheng H, Smith RG. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci U S A. 2004;101:4679–4684.
120 Palmiter RD, Erickson JC, Hollopeter G, Baraban SC, Schwartz MW. Life without neuropeptide Y. Recent Prog Horm Res. 1998;53:163–199.
121 Sibilia V, Rindi G, Pagani F, Rapetti D, Locatelli V, Torsello A, et al. Ghrelin protects against ethanol-induced gastric ulcers in rats: studies on the mechanisms of action. Endocrinology. 2003;144:353–359.

0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(5):2306–2314

doi: 10.1210/en.2005-1357

Des-Acyl Ghrelin Induces Food Intake by a Mechanism Independent of the Growth Hormone Secretagogue Receptor

Koji Toshinai, Hideki Yamaguchi, Yuxiang Sun, Roy G. Smith, Akihiro Yamanaka, Takeshi Sakurai, Yukari Date, Muhtashan S. Mondal, Takuya Shimbara, Takashi Kawagoe, Noboru Murakami, Mikiya Miyazato, Kenji Kangawa, and Masamitsu Nakazato

Department of Internal Medicine (K.T., H.Y., Y.D., M.S.M., T.SH., T.K., M.N.), Miyazaki Medical College, University of Miyazaki, Miyazaki 889-1692, Japan; Department of Veterinary Physiology (N.M.), University of Miyazaki, Miyazaki 889-2151, Japan; Huffington Center on Aging (Y.S., R.G.S.), Baylor College of Medicine, Texas 77030; Department of Pharmacology (A.Y., T.SA.), University of Tsukuba, Ibaraki 305-8575, Japan; and National Cardiovascular Center Research Institute (M.M., K.K.), Osaka 565-8565, Japan

Ghrelin, an acylated peptide produced predominantly in the stomach, stimulates feeding and GH secretion via interactions with the GH secretagogue type 1a receptor (GHS-R1a), the functionally active form of the GHS-R. Ghrelin molecules exist in the stomach and hypothalamus as two major endogenous forms, a form acylated at serine 3 (ghrelin) and a des-acylated form (des-acyl ghrelin). Acylation is indispensable for the binding of ghrelin to the GHS-R1a. Ghrelin enhances feeding via the neuronal pathways of neuropeptide Y and orexin, which act as orexigenic peptides in the hypothalamus. We here studied the effect of des-acyl ghrelin on feeding behavior. Intracerebroventricular (icv) administration of rat des-acyl ghrelin to rats or mice fed *ad libitum* stimulated feeding during the light phase; neither ip nor icv administration of des-acyl ghrelin to fasting mice suppressed feeding. The icv administration of des-acyl ghrelin induced the expression of Fos, a marker of neuronal activation, in orexin-expressing neurons of the lateral hypothalamic area, but not neuropeptide Y-expressing neurons of the arcuate nucleus. Peripheral administration of des-acyl ghrelin to rats or mice did not affect feeding. Although icv administration of ghrelin did not induce food intake in GHS-R-deficient mice, it did in orexin-deficient mice. In contrast, icv administration of des-acyl ghrelin stimulated feeding in GHS-R-deficient mice, but not orexin-deficient mice. Des-acyl ghrelin increased the intracellular calcium concentrations in isolated orexin neurons. Central des-acyl ghrelin may activate orexin-expressing neurons, perhaps functioning in feeding regulation through interactions with a target protein distinct from the GHS-R. (*Endocrinology* 147: 2306–2314, 2006)

GHRELIN IS A 28-amino-acid peptide isolated from human and rat stomach as an endogenous ligand for the GH secretagogue receptor (GHS-R) (1). The GHS-R, a G protein-coupled seven-transmembrane domain receptor, was initially identified as a receptor for small synthetic molecules termed GH secretagogues (GHSs), such as L-692,429, GHRP-6, and MK-0677, all of which act on the pituitary to stimulate GH secretion (2, 3). Two GHS-R subtypes are generated by alternative splicing of a single gene: the full-length type 1a receptor (GHS-R1a) and a carboxyl-terminally truncated form, the GHS-R type 1b (GHS-R1b), that encodes a protein containing transmembrane domain one to five (2, 3). The GHS-R1a is the functionally active, signal transducing form of the GHS-R, whereas the GHS-R1b is devoid of high-affinity ligand binding and signal transduction activity. Ghrelin molecules, predominantly produced by endocrine cells of the gastric oxyntic glands (4, 5), exist in two major molecular forms, ghrelin and des-*n*-octanoyl ghrelin (des-acyl ghrelin) (6). These two ghrelin molecules are also produced in the rat hypothalamus, as demonstrated by the combination of reverse-phase HPLC (RP-HPLC) with two separate RIAs recognizing ghrelin and des-acyl ghrelin (7, 8). All ghrelin species identified in fish, amphibians, birds, and many mammals possess a unique structural modification of the hydroxyl group of their third residue, which is either serine or threonine, by *n*-octanoic acid (9). This acylation is essential for the binding of ghrelin to the GHS-R1a (1, 10, 11); thus, the acylated form has been designated as ghrelin in our original description (1). Administration of ghrelin stimulates food intake in humans and rats (12–16) but does not change feeding behavior in GHS-R-deficient mice (17), suggesting that ghrelin enhances food intake via GHS-R-mediated signaling.

Several recent *in vitro* studies have demonstrated that radiolabeled ghrelin and des-acyl ghrelin bound to the membranes of PC-3 prostate tumor cells, H9c2 cadiomyocytes and isolated adipocytes, none of which expressed the GHS-R (18–20). This binding could be displaced by ghrelin, des-acyl ghrelin, and synthetic GHSs. Ghrelin and des-acyl ghrelin exhibit similar GHS-R-independent biological activities, including a cytoprotective effect on cultured cardiomyocytes

First Published Online February 16, 2006

Abbreviations: AgRP, Agouti gene-related protein; CRF, corticotropin-releasing factor; 2-DG, 2-deoxy-D-glucose; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; HKRB, Krebs-Ringer bicarbonate buffer; icv, intracerebroventricular(ly); LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; PVN, paraventricular nucleus; RP-HPLC, reverse-phase HPLC.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

(18), the inhibition of cell proliferation of breast carcinoma cell lines (19), the reduction of glycerol release from rat epididymal adipocytes (20), an ionotropic effect on guinea pig papillary muscle (21), and the promotion of bone marrow adipogenesis (22). Although the signaling molecules downstream of des-acyl ghrelin remain undefined, des-acyl ghrelin appears to share a subset of biological activities with ghrelin in peripheral tissues through an unidentified receptor or a target protein unique from the GHS-R.

The coordination of the regulation of food intake and energy expenditure occurs in the hypothalamus. Glucoprivic states induced by fasting or treatment with 2-deoxy-D-glucose (2-DG), a selective inhibitor of carbohydrate metabolism, increase feeding through the activation of orexigenic peptides, neuropeptide Y (NPY) and agouti gene-related protein (AgRP) in the arcuate nucleus (23). Secretion of des-acyl ghrelin from the rat hypothalamus increased in glucoprivic states induced by fasting or treatment with 2-DG (7). The axonal terminals of neurons that produce ghrelin and des-acyl ghrelin make direct synaptic contacts with NPY- and orexin-expressing neurons, which participate in hypothalamic feeding regulation (16, 24). Both ghrelin and des-acyl ghrelin may have a direct central action on the control of feeding.

We here investigated the effect of central or peripheral administration of des-acyl ghrelin on food intake in rats and mice and Fos expression, a marker of neuronal activation (25), in neurons that produce the orexigenic hypothalamic peptides, NPY/AgRP, orexin, or melanin-concentrating hormone (MCH). We studied the functional signaling downstream of des-acyl ghrelin using orexin-deficient mice and rats pretreated with antiorexin-A and -B IgGs. We confirmed that des-acyl ghrelin increased the intracellular calcium concentrations in orexin neurons dispersed from the lateral hypothalamic area (LHA) by the calcium-imaging analysis. We demonstrated that des-acyl ghrelin increased feeding by activation of orexin neurons in the LHA. We examined whether des-acyl ghrelin-induced food intake was mediated by the GHS-R pathway using GHS-R-deficient mice. Des-acyl ghrelin appears to regulate feeding via a receptor or target protein independent of the GHS-R.

Materials and Methods

Animals

We used male Wistar rats (Charles River Japan, Inc., Shiga, Japan), weighing 300–350 g, male C57BL/6 (Charles River Japan, Inc.), weighing 24–28 g, and male ddy mice (Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan), weighing 35–39 g. Orexin-deficient mice (12-wk-old, male) and GHS-R-deficient mice (12-wk-old, male) were generated by targeted mutation of embryonic stem cells as reported (17, 26). All animals were individually housed in plastic cages at a constant room temperature in a 12-h light (0800–2000 h)/12-h dark cycle and given standard laboratory chow and water *ad libitum*. All procedures were approved by University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care. Anesthesia was given as an ip injection of sodium pentobarbital (Abbot Labs., Chicago, IL). Intracerebroventricular (icv) cannulae were implanted into the lateral cerebral ventricles of rats and mice. Proper placement of the cannulae was verified at the end of the experiment by dye administration. Intravenous cannulae were implanted into the rat right jugular vein. Only animals that exhibited progressive weight gain after surgery were used.

Peptide synthesis

Rat ghrelin and des-acyl ghrelin were purchased from Peptide Institute, Inc. (Osaka, Japan). Adequate purification of synthesized peptides was ascertained by RP-HPLC, ion-exchange-HPLC, capillary zone electrophoresis, amino acid sequencing, and mass spectrometry (MALDI-MS). Ghrelin and des-acyl ghrelin were separately eluted, each as single peaks by RP-HPLC performed using a TSK ODS SIL 120A column (4.6 × 150 mm) (Tosho Co., Tokyo, Japan) with a linear gradient of 10–60% acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (Fig. 1).

Fos expression

Ghrelin (200 pmol/10 μl saline), des-acyl ghrelin (200 pmol/10 μl saline), or saline was injected icv into Wistar rats or GHS-R-deficient mice 90 min before transcardial perfusion with fixative containing 4% paraformaldehyde. The brain was sectioned into 20- or 40-μm-thick samples. Fos-specific immunohistochemistry was performed as described (27). Hypothalamic sections from rats and GHS-R-deficient mice were incubated for 2 d with goat anti-Fos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:1500), then stained using the avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA). These sections were also stained with either rabbit antiorexin-A (dilution 1:3000) (16, 27) or rabbit anti-MCH (Phoenix Pharmaceuticals, Inc., Belmont, CA; dilution 1:200) antisera. We observed orexin- and MCH-expressing neurons by light microscopy. For immunofluorescence microscopy, we incubated hypothalamic sections of GHS-R-deficient mice with goat anti-Fos antiserum (dilution 1:1500) for 2 d at 4 C, then performed an additional 2 h incubation with

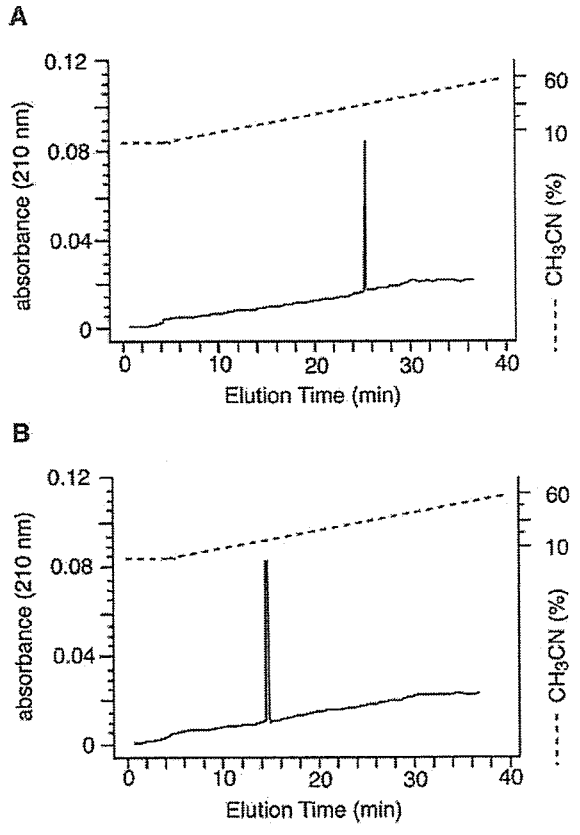


FIG. 1. RP-HPLC analysis of synthetic rat ghrelin (A) and des-acyl ghrelin (B) used for experimentation. Each peptide (0.3 nmol) was loaded onto a TSK ODS SIL 120A column using a linear gradient of 10–60% CH_3CN containing 0.1% trifluoroacetic acid at a rate of 1.0 ml/min for 40 min. Each peptide is eluted as a single peak whose elution position was identical with that of the corresponding synthetic peptide.

Alexa 488-conjugated donkey antigoat IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400). After washing with PBS (pH 7.4), samples were incubated with a rabbit antiorexin-A antiserum for 2 d at 4 C and Alexa 546-labeled goat antirabbit IgG antibody (Molecular Probes; dilution 1:400) for a final 2 h. Slides were observed on a fluorescence microscope (BH2-RFC; Olympus, Tokyo, Japan).

Food intake

Experiments were performed 1 wk after the implantation of icv or iv cannulae. First, ghrelin or des-acyl ghrelin (each at 200 pmol/10 μl saline) was administered icv at 1000 h to rats fed *ad libitum* (n = 10 per group). The 1-, 2-, and 4-h food intake amounts were then measured. Second, des-acyl ghrelin (1 nmol/10 μl saline) was administered icv to rats (n = 6 per group) 10 min before the beginning of the dark phase, after which the 30-min food intake was measured. Third, ghrelin (1.5 nmol) or des-acyl ghrelin (1.5 or 5 nmol/100 μl saline) was administered iv to rats at 1000 h through an iv cannula. Fourth, des-acyl ghrelin (1 or 5 nmol/2 μl saline) was administered ip at 1000 h to C57BL/6 mice fed *ad libitum* (n = 8 per group). Fifth, 3 h after an icv administration of antiorexin-A and -B (each at 0.25 μg/2.5 μl saline), anti-NPY (0.5 μg/5 μl saline), or normal rabbit serum (0.5 μg/5 μl saline) IgGs, ghrelin or des-acyl ghrelin (each at 200 pmol/5 μl saline) was administered at 1200 h to rats (n = 10–12 per group). Sixth, ghrelin or des-acyl ghrelin (each at 200 pmol/2 μl saline) was administered icv at 1000 h to orexin-deficient mice or their wild-type littermates (n = 6–8 per group). Seventh, ghrelin (200 pmol/2 μl saline), des-acyl ghrelin (200 pmol/2 μl saline) or NPY (1 nmol/2 μl saline; Peptide Institute, Inc.) was administered icv at 1000 h to GHS-R-deficient mice or their wild-type littermates (n = 6–8 per group). With the exception of the first and second experiments, 2-h food intake was measured in all tests. Eighth, ghrelin or des-acyl ghrelin (each at 1 nmol/2 μl saline) was administered icv at 1000 h to ddy mice fed *ad libitum*. Ninth, des-acyl ghrelin (1 nmol/2 μl saline) was administered icv at 1000 h to ddy mice that had fasted for the previous 16 h (n = 8 per group). Tenth, des-acyl ghrelin (1 nmol/50 μl saline) was administered ip at 1000 h to ddy mice that had fasted for the previous 16 h (n = 8 per group). After the injections of ddy mice in the eighth, ninth, and tenth experiments outlined above, we measured 20-min, 1-h, and 2-h food intake. These feeding tests were performed using a cross-over design experiments in which animals were randomized to receive either test substance with a washout period of 3 d between each administration.

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$)

The LHA of rat brain was punched out according to the *Atlas of the Rat Brain* (28). The tissue was washed twice with HEPES and Krebs-Ringer bicarbonate buffer (HKRB) [129 mM NaCl, 5.0 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4)] containing 10 mM glucose. The LHA was incubated in HKRB supplemented with 1 mg/ml papain (Sigma-Aldrich, St. Louis, MO), 5 mg/ml deoxyribonuclease, and 0.025% BSA for 20 min at 36 C in a shaking water bath, then LHA cells were dispersed by mechanical desegregation for 4 min. The cell suspension was diluted with HKRB and centrifuged at 100 × *g* for 5 min. The pellet was resuspended in HKRB and distributed onto the glass well (Nunc 96 Microwell Optical Bottom Plate; Nalge Nunc International, Rochester, NY). Measurement of $[Ca^{2+}]i$ was carried out 2–4 h after the preparation of cells. The cells were loaded with Fluo-3 for 20 min in HEPES buffer solution [10 mM HEPES, 140 mM NaCl, 5.0 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM glucose, and 2 μm Fluo-3/acetoxymethylester (Dojindo Labs, Kumamoto, Japan) (pH 7.2)]. They were washed twice with HEPES buffer, then filled with 100 μl HEPES buffer. One min after, 50 μl of 3 μM rat des-acyl ghrelin were added into the well. $[Ca^{2+}]i$ was determined by measuring fluorescence signal from the Ca^{2+} indicator Fluo-3/acetoxymethylester, with 480 nm excitation and 530 nm emission using a cooled charge-coupled device camera, and the ratio image was produced in Functional Imaging Cell-Sorting System (IMACS; Hamamatsu Photonics, Hamamatsu, Japan). The level of $[Ca^{2+}]i$ in a single neuron was recorded for 6 min after the administration of des-acyl ghrelin. After $[Ca^{2+}]i$ measurement, the neurons were fixed with 4% paraformaldehyde overnight. They were incubated with rabbit antiorexin-A antiserum (dilution 1:1500) for 2 d at 4 C, then Alexa 350-conjugated goat antirabbit IgG antibody (dilution 1:400) for 2 h (16, 27). The picture of calcium imaging was collated with the immunohistochemical picture. Fluorescence signals from Fluo-3 were converted automatically to pseudo colors in IMACS. The levels of $[Ca^{2+}]i$ were assigned pseudo colors ranging from blue of the lowest value through yellow to red of the highest value. Fluorescence signals from Alexa-350 were shown in white.

Measurement of locomotor activity

Locomotor activity of rats was measured using a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface and a computer. The infrared sensors were placed above the cages and measured all locomotor activity. A cage with the infrared sensor was placed in an isolated chamber with a controlled light/dark cycle. Rats were given icv des-acyl ghrelin (1 nmol/10 μl saline), human orexin-A (Peptide Institute, Inc.; 1 nmol/10 μl saline) or saline administration icv at 0900 h (n = 5 per group), then these rats were immediately returned to their individual cages. Locomotor activity counts were made 30 min and analyzed by Compact ACTAM Software (Muromachi Co. Ltd.).

GH response

Ghrelin or des-acyl ghrelin (each at 1.5 nmol/100 μl) was administered iv to rats (n = 6 per group) at 1000 h. Blood samples (80 μl) were obtained from the tail veins at 0, 15, 30, and 60 min after administration. After removal, six anterior pituitary glands of rats were immersed in Hanks' balanced salt solution, then incubated at 37 C for 30 min. Each one pituitary grand was placed in a polystyrene well (16 mm in diameter; Iwaki Glassware Co., Tokyo, Japan) filled with oxygenated medium (DMEM containing 2.5% fetal calf serum and 2.5% bovine serum). After rinsing twice in 500 μl medium for 1 min each, 750 μl medium was added into each well for 5 min. The medium was then collected into plastic microtubes to evaluate basal GH secretion. The pituitary glands were then stimulated for 5 min with medium containing either ghrelin or des-acyl ghrelin (1 μM). The medium was collected into plastic microtubes to quantify GH concentration with a Biotrak Rat GH RIA kit

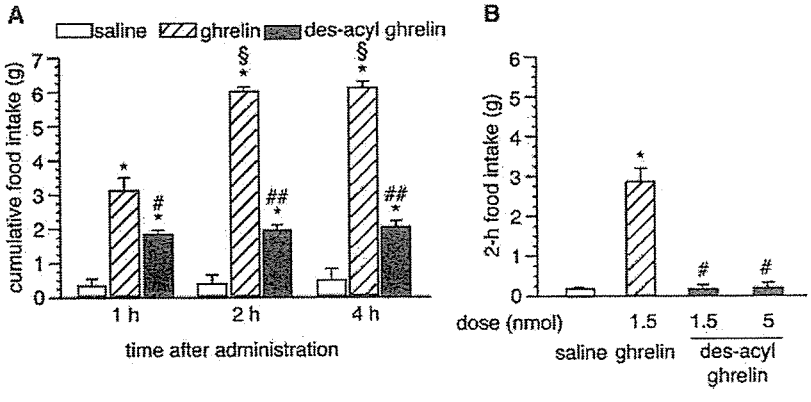


FIG. 2. Effect of ghrelin or des-acyl ghrelin on food intake in rats. A, Intracerebroventricular administration of des-acyl ghrelin or ghrelin (each at 200 pmol) at 1000 h. *, $P < 0.001$ *vs.* saline; #, $P < 0.05$; ##, $P < 0.001$ *vs.* ghrelin; §, $P < 0.001$ *vs.* 1-h food intake. B, Intravenous administration of ghrelin (1.5 nmol) or des-acyl ghrelin (1.5 or 5 nmol) at 1000 h. *, $P < 0.001$ *vs.* saline; #, $P < 0.001$ *vs.* ghrelin.

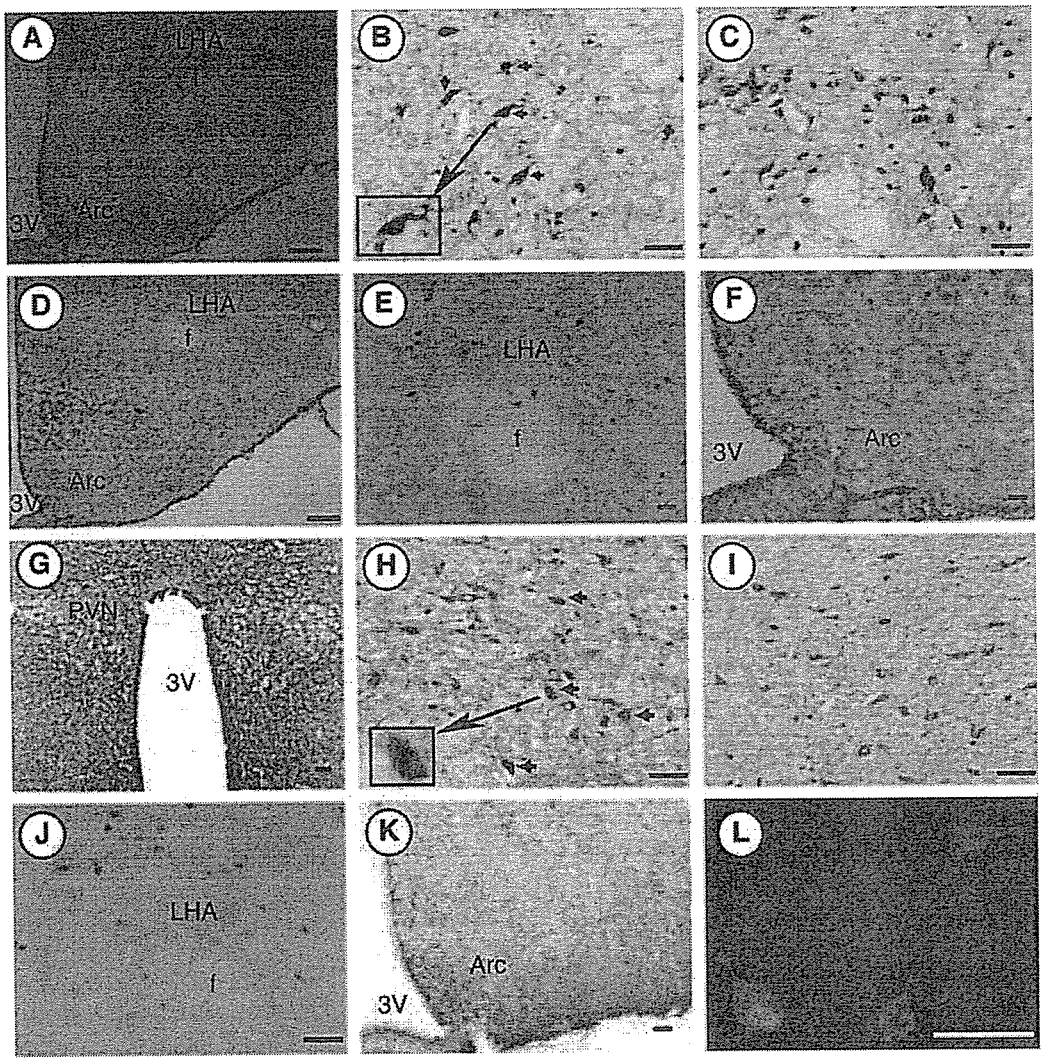


FIG. 3. Fos expression in the hypothalamus after icv administration of ghrelin or des-acyl ghrelin (each at 200 pmol). A, Fos expression (*dark blue-black*) in the LHA and arcuate nucleus of rats given ghrelin. B, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in rats given ghrelin. The *inset* is a higher magnification of Fos expression in an orexin-expressing neuron. C, No Fos (*dark blue-black*) expression in MCH-expressing neurons (*brown*) after ghrelin administration. D, Fos expression (*dark blue-black*) in the hypothalamus of rats given des-acyl ghrelin. E, Fos expression (*dark blue-black*) in the LHA of rats given des-acyl ghrelin. No Fos (*dark blue-black*) expression in the arcuate nucleus (F) or the PVN (G) after des-acyl ghrelin administration. H, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in rats given des-acyl ghrelin. The *inset* is a higher magnification of Fos expression in an orexin-expressing neuron. I, No expression of Fos (*dark blue-black*) is observed in MCH-expressing neurons (*brown*) after des-acyl ghrelin administration. J, Fos expression (*dark blue-black*) in the LHA of GHS-R-deficient mice given des-acyl ghrelin. K, No Fos expression (*brown*) in the arcuate nucleus of GHS-R-deficient mice given des-acyl ghrelin. L, Costaining of Fos (*green*) and orexin (*red*) in the LHA neurons of GHS-R-deficient mice given des-acyl ghrelin. 3V, Third ventricle; Arc, arcuate nucleus; f, fornix. *Scale bars*, A, D, 200 μm; B, C, E, F, H, G, H, I, J, K, L, 50 μm.

(Amersham, Buckinghamshire, UK). The experiment was concluded by treatment with 60 mM KCl to induce depolarization.

Statistic analysis

Data (mean ± SEM) were analyzed by ANOVA and the *post hoc* Scheffé's F test. Differences were considered to be significant when the P values were less than 0.05.

Results

Des-acyl ghrelin-induced food intake

The icv administration of either ghrelin or des-acyl ghrelin to rats stimulated food intake (Fig. 2A). Although ghrelin increased food intake for at least 2 h after administration, the effect of des-acyl ghrelin lasted for only 1 h. Des-acyl ghrelin also significantly increased the early dark-phase food intake in rats in comparison to saline administration (des-acyl ghrelin, 2.04 ± 0.37 g/30 min; saline, 0.96 ± 0.23 g/30 min, $P <$ 0.05). Next, we studied the effect of peripherally administered des-acyl ghrelin on feeding. A single iv administration of ghrelin significantly increased feeding, whereas des-acyl ghrelin did not (Fig. 2B). An ip administration of des-acyl ghrelin to C57BL/6 mice fed *ad libitum* did not increase food intake (des-acyl ghrelin 1 nmol, 0.05 ± 0.03 g/2 h; 5 nmol, 0.04 ± 0.02 g/2 h; saline, 0.05 ± 0.02 g/2 h).

Fos expression

Intracerebroventricular administration of ghrelin induced Fos expression in the LHA and arcuate nucleus (Fig. 3A). In contrast, icv administration of des-acyl ghrelin induced Fos in the LHA, but not the arcuate nucleus or the paraventricular nucleus (PVN) (Fig. 3, D–G). By double immunohistochemistry, ghrelin induced Fos in 32 ± 7% of orexin-immunoreactive neurons (Fig. 3B). Des-acyl ghrelin induced Fos in 22 ± 5% of orexin-immunoreactive neurons (Fig. 3H). In the LHA, neither ghrelin nor des-acyl ghrelin induced Fos in MCH-immunoreactive neurons (Fig. 3, C and I). Intracerebroventricular administration of des-acyl ghrelin to GHS-R-deficient mice induced Fos in the LHA, but not the arcuate nucleus (Fig. 3, J and K). In GHS-R-deficient mice, des-acyl ghrelin induced Fos expression in 28 ± 2% of orexin-immunoreactive neurons, whereas ghrelin did not induce the expression of Fos in any hypothalamic neurons (Fig. 3L).

Functional relationship between des-acyl ghrelin and orexin in feeding

We examined the effects of orexin and NPY blockades on des-acyl ghrelin-induced food intake. Both ghrelin and des-acyl ghrelin increased food intake in rats given an icv administration of control IgG (Fig. 4). Pretreatment with anti-orexin-A and -B IgGs, however, reduced ghrelin-induced food intake by 29% from the amounts seen in rats given control IgG and ghrelin, whereas pretreatment with anti-orexin-A and -B IgGs completely abolished des-acyl ghrelin-induced food intake. Whereas pretreatment with anti-NPY IgG reduced ghrelin-induced feeding in rats in comparison to rats given control IgG and ghrelin, anti-NPY IgG did not affect des-acyl ghrelin-induced feeding in comparison to rats given control IgG and des-acyl ghrelin (Fig. 4).

Orexin-deficient mice were used to verify the functional relationship between des-acyl ghrelin and orexin in feeding regulation. Although ghrelin induced food intake in orexin-deficient mice, the potency of this induction in these mice was significantly reduced from that seen in wild-type littermates (Fig. 5A). Des-acyl ghrelin stimulated feeding in wild-type mice, but not in orexin-deficient mice (Fig. 5A). To investigate whether des-acyl ghrelin regulates feeding through the GHS-R, we gave an icv administration of des-acyl ghrelin to GHS-R-deficient mice. Des-acyl ghrelin, but

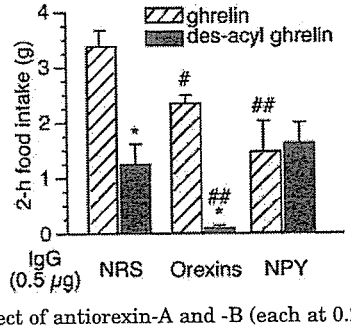


FIG. 4. The effect of antiorexin-A and -B (each at 0.25 μg) and anti-NPY (0.5 μg) IgGs on ghrelin- or des-acyl ghrelin-induced feeding. Ghrelin or des-acyl ghrelin (each at 200 pmol) was given to rats 3 h after icv administration of IgG. The 2-h food intake was then measured. NRS, Normal rabbit serum. *, $P < 0.01$ *vs.* ghrelin; #, $P < 0.05$; ##, $P < 0.01$ *vs.* NRS IgG.

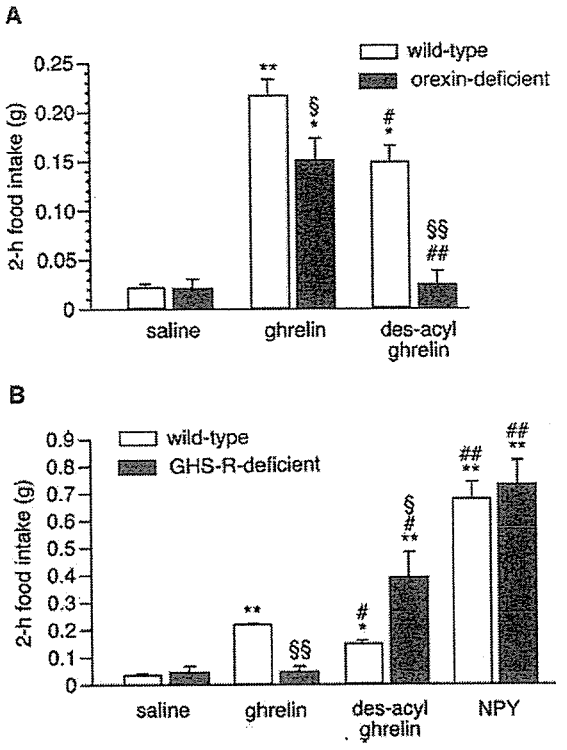


FIG. 5. A, Effect of icv administration of ghrelin or des-acyl ghrelin (each at 200 pmol) on 2-h food intake in orexin-deficient mice. *, $P < 0.05$, **; $P < 0.01$ *vs.* saline; #, $P < 0.05$; ##, $P < 0.01$ *vs.* ghrelin, §, $P < 0.05$, §§, $P < 0.01$ *vs.* wild-type mice. B, Effect of icv administration of ghrelin (200 pmol), des-acyl ghrelin (200 pmol), or NPY (1 nmol) on 2-h food intake in GHS-R-deficient mice. *, $P < 0.05$; **, $P < 0.001$ *vs.* saline; #, $P < 0.001$ *vs.* ghrelin; §, $P < 0.05$; §§, $P < 0.01$ *vs.* wild-type mice.

not ghrelin, stimulated feeding in GHS-R-deficient mice (Fig. 5B). Des-acyl ghrelin-induced feeding in GHS-R-deficient mice was more potent than that induced in wild-type littermates. NPY was used as a positive control to evaluate the orexigenic effects on GHS-R-deficient mice. NPY-induced food intake was similar in both GHS-R-deficient mice and their wild-type littermates (Fig. 5B).

Intracerebroventricular administration of des-acyl ghrelin increased food intake in ddy mice fed *ad libitum* (Fig. 6A). Neither icv nor ip administration of des-acyl ghrelin suppressed food intake in ddy mice that had fasted for 16 h (Fig. 6, B and C).

Cytosolic $[Ca^{2+}]i$ response in orexin neurons

We studied the cytosolic $[Ca^{2+}]i$ response of orexin-expressing neurons to des-acyl ghrelin. Some cells dispersed from the rat LHA showed increased cytosolic $[Ca^{2+}]i$ in response to des-acyl ghrelin administration (Fig. 7, A and B). These cells showed orexin immunoreactivity by immunohistochemistry (Fig. 7C).

Locomotor activity

We examined the effect of des-acyl ghrelin on locomotor activity. Intracerebroventricular administration of des-acyl

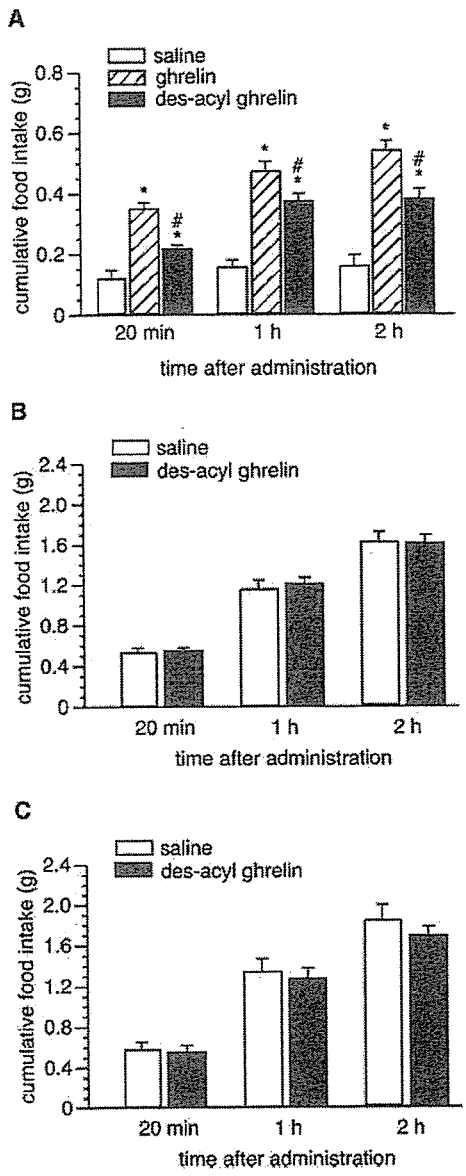


FIG. 6. Effect of des-acyl ghrelin on food intake in ddy mice. A, Intracerebroventricular administration of ghrelin or des-acyl ghrelin (each at 1 nmol) at 1000 h to mice *ad libitum*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* saline; #, $P < 0.01$ *vs.* ghrelin. Neither icv (B) nor ip (C) administration of des-acyl ghrelin (1 nmol) suppressed feeding in mice that had fasted for 16 h.

ghrelin to rats significantly increased locomotor activity compared with saline administration (Fig. 8). Orexin-A also significantly increased locomotor activity in these rats.

FIG. 8. Effects of icv administration of des-acyl ghrelin or orexin-A (each at 1 nmol) on locomotor activity in rats. *, $P < 0.05$ *vs.* saline.

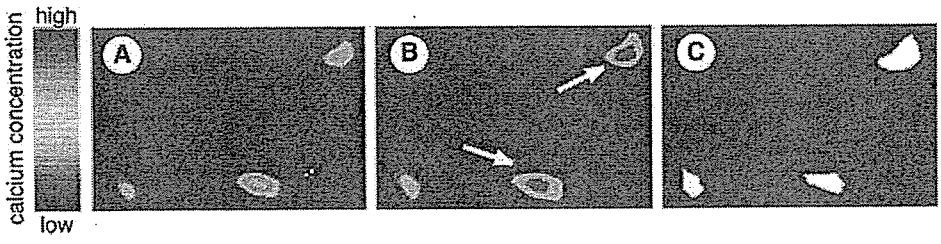
GH response

We studied the release of GH in response to peripheral des-acyl ghrelin administration. Intravenous administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration (Fig. 9A). Intravenous administration of des-acyl ghrelin did not stimulate GH release. We examined the effect of des-acyl ghrelin on GH release from isolated samples of the rat anterior pituitary. GH concentrations in the culture medium of the anterior pituitary cultures increased in response to ghrelin administration, but not to des-acyl ghrelin administration (Fig. 9B).

Discussion

At present, the amino acid sequences of ghrelin peptides in 21 species of fish, amphibians, birds, and mammals have been determined (9). All of the ghrelin molecules identified possess a serine or threonine as the third amino acid residue. A hydroxyl group of this amino acid forms an ester with a monocarboxylic acid of medium-chain fatty acid (6). Ghrelin peptide is present in the stomach of humans, rats, and mice as two major molecular forms: ghrelin and des-acyl ghrelin (6). In the plasma, ghrelin accounts for only 2–20% of total ghrelin immunoreactivity (6, 7, 28–30). This is likely due to the shorter half-life of ghrelin than that of des-acyl ghrelin because plasma ghrelin rapidly disappears from the circulation because of binding to the GHS-R in the systemic tissues (31). Deacylation of ghrelin to des-acyl ghrelin, which rapidly occurs in the plasma, is also responsible for the reduced half-life of ghrelin. Two enzymes involved in the deacylation of ghrelin have been identified: high-density lipoprotein-associated paraoxonase functions in the plasma, whereas lysophospholipase I, a thioesterase active against palmitoyl-Gsα and plamitoyl-coenzyme A, functions in the stomach (32–34). In contrast, the enzyme that catalyzes the acyl modification of ghrelin has not been identified.

FIG. 7. Effect of des-acyl ghrelin on $[Ca^{2+}]i$ response in isolated orexin-expressing neurons. A, A picture shows the basal level of $[Ca^{2+}]i$ in neurons (*blue*) before administration of des-acyl ghrelin. B, Des-acyl ghrelin increased cytosolic $[Ca^{2+}]i$ in two neurons (*arrows*). C, Immunostaining of orexin-expressing neurons (*white*) after the measurement of $[Ca^{2+}]i$ response.



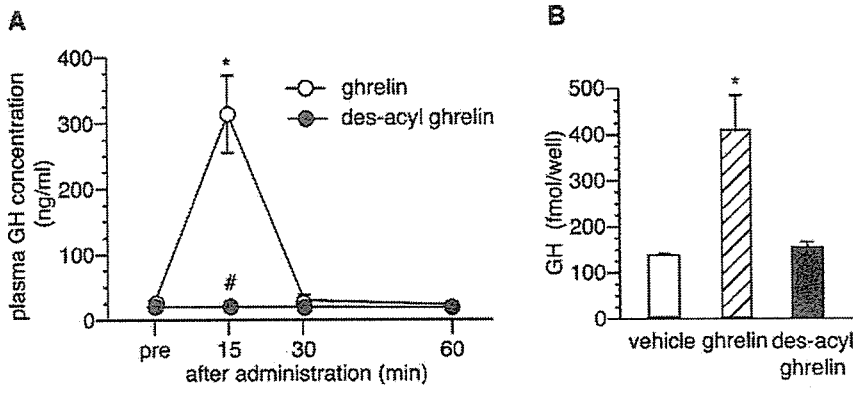


FIG. 9. A, Effect of iv administration of ghrelin or des-acyl ghrelin (each at 1.5 nmol) on the plasma GH concentration in rats. *, $P < 0.001$ *vs.* preadministration; #, $P < 0.001$ *vs.* ghrelin at the same time point. B, Effect of ghrelin or des-acyl ghrelin administration (each at 200 pmol) on GH release from rat pituitary gland (n = 6 per group). *, $P < 0.001$ *vs.* control vehicle.

Acylation of ghrelin is essential for ghrelin's GH-releasing activity (1, 9, 10); several recent *in vitro* studies have shown that des-acyl ghrelin exhibits biological activities on the cell proliferation and metabolism of cardiomyocytes, adipocytes, myocytes, and myelocytes (18–22). Although many of these cells did not express the GHS-R, des-acyl ghrelin bound to their cell membranes (18–20). We here examined the orexigenic activity of des-acyl ghrelin. We confirmed the purity of ghrelin and des-acyl ghrelin by several biochemical methods before using these substances in feeding experiments. Both ghrelin and des-acyl ghrelin were completely pure by RP-HPLC, ion-exchange-HPLC, capillary zone electrophoresis, and mass spectrometry. Des-acyl ghrelin did not stimulate GH release when either peripherally administered to rats or applied directly to the rat pituitary *in vitro*. Intracerebroventricular administration of des-acyl ghrelin significantly induced feeding during both the light and dark phases in rats. Intracerebroventricular administration of des-acyl ghrelin also increased food intake in GHS-R-deficient mice and their wild-type littermates. Two recent studies reported the anorexic activity of des-acyl ghrelin in rats and mice (35, 36). In these studies, ip administration of des-acyl ghrelin suppressed feeding in rats had fasted for 16 h (35). Both ip and icv administrations of des-acyl ghrelin suppressed feeding in ddy mice that had been fasting for 16 h; icv administration of des-acyl ghrelin did not significantly change the light phase food intake in ddy mice fed *ad libitum* (36). These studies described that icv and ip administrations of des-acyl ghrelin expressed Fos in the PVN neurons, presumably corticotropin-releasing factor (CRF) neurons (35, 36). We also examined the effect of des-acyl ghrelin on feeding in ddy mice because the anorexic effect noted above contrasted the orexigenic effect observed in C57BL/6 mice. An icv administration of des-acyl ghrelin significantly increased the light phase food intake of ddy mice fed *ad libitum*. The icv administration of des-acyl ghrelin did not suppress food intake in ddy mice that had fasted for 16 h. In addition, an icv administration of des-acyl ghrelin did not express Fos in any neurons of the PVN where CRF-producing neurons are present. Because the effects of peptides in feeding experiments are hampered by unsatisfactory habituation (37, 38), all of the rats and mice used in these experiments were satisfactorily acclimated to handling before ip and icv injections. We do not know why our findings conflicted with previous results; des-acyl ghrelin, however, reproducibly stimulated feeding in rats, C57BL/6 mice and ddy mice. Des-acyl ghrelin, which was synthesized in the rat hypothalamus, was released in response to fasting (7). The ratio of des-acyl ghrelin to ghrelin in the rat hypothalamus was 2:1 under *ad libitum* conditions, and this ratio did not change in upon fasting. Des-acyl ghrelin, as well as ghrelin, may serve as orexigenic peptides in the hypothalamus.

Ghrelin-producing neurons localize to the hypothalamic arcuate nucleus and adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei of rats and mice (1, 24). Ghrelin fibers synapse on NPY/AgRP neurons in the arcuate nucleus and orexin neurons in the LHA (16, 24, 39). We investigated the signaling events downstream of des-acyl ghrelin that stimulates feeding. Fos expression, induced by icv administration of des-acyl ghrelin, was restricted to orexin-expressing neurons in the LHA. Des-acyl ghrelin-induced food intake was completely abolished in rats by pretreatment with antiorexin IgG, but not anti-NPY IgG or control serum IgG. Des-acyl ghrelin did not stimulate feeding in orexin-deficient mice. These results indicate that des-acyl ghrelin-induced feeding is mediated by the activation of the orexin pathway. Orexin-A and -B are hypothalamic peptides functioning in the regulation of feeding, energy homeostasis, and arousal (40). Approximately 3000 orexin-expressing neurons are present in the LHA of rats and mice. Orexin-positive nerve fibers have wide projections onto a variety of brain regions, such as the arousal centers in the forebrain and brain stem and the feeding center within the hypothalamus (40). Orexin-expressing neurons are heterogeneous in their anatomical projections and physiological functions, playing multifaceted roles in the brain. Ghrelin fibers project to orexin-positive neurons (16) and ghrelin stimulated elecrophysiological activity of isolated orexin neurons in the whole-cell patch-clamp study (41); we demonstrate here that ghrelin and des-acyl ghrelin acted on orexin-expressing neurons and that des-acyl ghrelin increased intracellular calcium concentration in isolated orexin neurons. There are three possible subtypes of orexin neurons: those that express the GHS-R as a receptor for ghrelin, those expressing an as-yet unknown target protein of des-acyl ghrelin, neurons possessing both proteins. Orexin also functions to maintain wakefulness (40). We examined the effect of des-acyl ghrelin on locomotor activity. As expected, icv administration of des-acyl ghrelin increased locomotor activity, suggesting that des-acyl ghrelin may increase wake-

fulness and locomotor activity for food seeking by stimulating orexin neurons.

We next investigated the functional relationship between des-acyl ghrelin and the GHS-R using GHS-R-deficient mice. The icv administration of des-acyl ghrelin to GHS-R-deficient mice induced food intake and Fos expression in orexin-expressing neurons. The icv administration of ghrelin to GHS-R-deficient mice did not stimulate food intake. Des-acyl ghrelin did not bind to GHS-R-expressing Chinese hamster ovary cells and did not inhibit the binding of ghrelin to rat pituitary culture cells expressing the GHS-R (1, 10, 42), implying that des-acyl ghrelin does not compete with ghrelin for the binding to the GHS-R. Thus, des-acyl ghrelin is thought to stimulate feeding via a mechanism independent of the GHS-R.

A number of gastrointestinal peptides transmit satiety or starvation signals to the nucleus of the solitary tract via the vagal afferents and/or to the hypothalamus via the bloodstream (43). Although iv administration of ghrelin stimulated both vagal afferents and feeding, iv administration of des-acyl ghrelin affected neither (44). Peripheral administration of des-acyl ghrelin to rats and mice did not affect feeding. Receptors on vagal afferents are generated by nodose ganglion neurons, transported to the nerve terminals through axonal transport (45). These results indicate that a receptor or a target protein binding to des-acyl ghrelin is not expressed in nodose ganglion neurons. The plasma concentration of des-acyl ghrelin increased upon fasting (7). The peripheral des-acyl ghrelin does not act to suppress feeding.

In summary, centrally administered des-acyl ghrelin increased feeding through activation of the orexin pathway. In addition to its peripheral actions, which include cell proliferation, inhibition of apoptosis, and fat metabolism (18–22), des-acyl ghrelin may function in hypothalamic feeding regulation. Central administration of des-acyl ghrelin to GHS-R-deficient mice stimulated feeding, suggesting that des-acyl ghrelin acts on a target protein that is specific for des-acyl ghrelin and independent of the GHS-R. Ghrelin and des-acyl ghrelin act in the regulations of peripheral cell functions through a common putative target protein (18–22). Ghrelin and des-acyl ghrelin function as orexigenic peptides in the hypothalamus. Des-acyl ghrelin may have basal effects of ghrelin-related peptides. Further studies examining the physiological and neuroanatomical interactions between des-acyl ghrelin and its target will establish roles of ghrelin peptides in the regulation of feeding and energy homeostasis.

Acknowledgments

We thank Tomoko Tsuruta, Etsuko Masuda, and Ryusuke Koshida for assistance.

Received October 25, 2005. Accepted February 9, 2006.

Address all correspondence and requests for reprints to: Masamitsu Nakazato, M.D., Ph.D., Third Department of Internal Medicine, Miyazaki Medical College, Miyazaki University, Kiyotake, Miyazaki 889-1692, Japan. E-mail: nakazato@med.miyazaki-u.ac.jp.

This study was supported in part by grants from 21st Century Center of Excellence Program and grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology; the Ministry of Health, Labor, and Welfare; Novartis Foundation for Gerontological Research; Society of Molecular Mechanism of Digestive Tract; The Fujisawa Foundation; and Takeda Medical Research Foundation.

Disclosure summary: all authors have nothing to declare.

References

1. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a novel growth hormone releasing acylated peptide from stomach. Nature 402:656–660
2. **Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJS, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, Demartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LHT** 1996 A receptor in pituitary and hypothalamus that functions in growth hormone rrelease. Science 273:974–977
3. **MaKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van der Ploeg LHT, Howard AD** 1997 Molecular analysis of rat pituitary and hypothalamus growth hormone secretagogue receptors. Mol Endocrinol 11:415–423
4. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M** 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141: 4255–4261
5. **Dornonville de la Cour C, Björkqvist M, Sandvik AK, Bakke I, Zhao CM, Chen D, Håkanson R** 2001 A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. Regul Pept 99:141–150
6. **Hosoda H, Kojima M, Matsuo H, Kangawa K** 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 281:1220–1225
7. **Sato T, Fukue Y, Teranishi H, Yoshida Y, Kojima M** 2005 Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-D-glucose administration. Endocrinology 146:2510–2516
8. **Mondal MS, Date Y, Yamaguchi H, Toshinai K, Tsuruta T, Kangawa K, Nakazato M** 2005 Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. Regul Pept 126:55–59
9. **Kojima M, Kangawa K** 2005 Ghrelin: Structure and function. Physiol Rev 85:495–522
10. **Muccioli G, Tschöp M, Papotti M, Deghenghi R, Heiman M, Ghigo E** 2002 Neuroendocrine and peripheral activities of ghrelin: implication in metabolism and obesity. Eur J Pharmacol 440:235–254
11. **Matsumoto M, Hosoda H, Kitajima Y, Morozumi N. Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y, Kangawa K** 2001 Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem Biophys Res Commun 287:142–146
12. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S** 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198
13. **Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi, T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K** 2001 Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227–232
14. **Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I** 2001 Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. Diabetes 50: 2438–2443
15. **Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE** 2003 Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. Regul Pept 111:161–167
16. **Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, Guan LL, Wand QP, Funahashi H, Sakurai T, Shioda S, Matsukura S, Kanagawa K, Nakazato M** 2003 Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506–1512
17. **Sun Y, Wang P, Zheng H, Smith RG** 2003 Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci USA 101:4679–4684
18. **Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A** 2004 Ghrelin and de-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 159:1029–1037
19. **Cassoni P, Ghe C, Marrocco T, Tarabra E, Allia E, Catapano F, Deghenghi R, Ghigo E, Papotti M, Muccioli G** 2004 Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell line. Eur J Endocrinol 150:173–184
20. **Muccioli G, Pons N, Ghe C, Catapano F, Granata R, Ghigo E** 2004 Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. Eur J Pharmacol 498:27–35

21. **Bedendi I, Allatti G, Marcantoni A, Malan D, Catapano F, Ghe C, Deghenghi R, Ghigo E, and Muccioli G** 2003 Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. Eur J Pharmacol 476: 87–95
22. **Thompson NM, Gill DAS, Davies R, Loveridge N, Houston PA, Robinson ICAF, Wells T** 2004 Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. Endocrinology 145:234–242
23. **Fraley GS, Ritter S** 2003 Immunolesion of norepinephrine and epinephrine afferents to medial hypothalamus alters basal and 2-deoxy-D-glucose-induced neuropeptide Y and agouti gene-related protein messenger ribonucleic acid expression in the arcuate nucleus. Endocrinology 144:75–83
24. **Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL** 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661
25. **Sagar SM, Sharp FR, Curran T** 1988 Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240:1328–1331
26. **Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M** 1999 Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98:437–451
27. **Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M** 1999 Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci USA 96:748–753
28. **Paxinos G, Watson C** 1998 The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press
29. **Nishi Y, Hiejima H, Hirohara M, Sato T, Kangawa K, Kojima M** 2005 Developmental changes in the pattern of ghrelin's acyl modification and the levels of acyl-modified ghrelin in murine stomach. Endocrinology 146:2709–2715
30. **Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Nakai Y, Kangawa K** 2005 Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. J Clin Endocrinol Metab 90:6–9
31. **Akamizu T, Takaya K, Irako T, Hosoda H, Teramukai S, Matsuyama A, Tada H, Miura K, Shimizu A, Fukushima M, Yokode M, Tanaka K, Kangawa K** 2004 Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. Eur J Endocrinol 150:447–455
32. **Beaumont NJ, Skinner VO, Tan TM, Ramesh BS, Byrne DJ, MacColl GS, Keen JN, Bouloux PM, Mikhailidis DP, Bruck-dorfer KR, Vanderpump MP, Srai KS** 2003 Ghrelin can bind to a species of high density lipoprotein associated with paraxonase. J Biol Chem 278:8877–8880
33. **Shanado Y, Kometani M, Uchiyama H, Koizumi S, Teno N** 2004 Lysophospholipase I identified as a ghrelin deacylation enzyme in rat stomach. Biochem Biophys Res Commun 325:1487–1494
34. **Duncan JA, Gilman AG** 1998 A cytoplasmic acyl-protein thioesterase that removes plamitate from G protein α subunits and p21(RAS). J Biol Cehm 273:15830–15837
35. **Chen CY, Imui A, Asakawa A, Fujino K, Kato I, Chen CC, Ueno N, Fujimiya M** 2005 Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. Gastroenterology 129:8–25
36. **Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M** 2005 Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54:18–24
37. **Tschöp M, Castaneda TR, Joost HG, Thone-Reineke C, Ortmann S, Klaus S, Hagan MM, Chandler PC, Oswald KD, Benoit SC, Seeley RJ, Kinzig KP, Moran TH, Beck-sickinger AG, Koglin N, Rodgers RJ, Blundell JE, Ishii Y, Beattie AH, Holch P, Allison DB, Raun K, Madsen K, Wulff BS, Stidsen CE, Birringer M, Kreuzer OJ, Schindler M, Arndt K, Rudolf K, Mark M, Deng XY, Whitcomb DC, Halem H, Taylor J, Dong J, Datta R, Culler M, Craney S, Flora D, Smiley D, Heiman ML** 2004 Physiology: does gut hormone PYY3–36 decrease food intake in rodents? Nature 430:1–4
38. **Halatchev IG, Ellacott KL, Fan W, Cone RD** 2004 Peptide YY3–36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology 145:2585–2590
39. **Jobst EE, Enriori PJ, Cowley MA** 2004 The electrophysiology of feeding circuits. Trends Endocrinol Metab 15:488–499
40. **Sakurai T** 2005 Reverse pharmacology of orexin: from an orphan GPCR to integrative physiology. Regul Pept 126:3–10
41. **Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, Tominaga M, Yagami K, Sugiyama F, Goto K, Yanagisawa M, Sakurai T** 2003 Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron 38:701–713
42. **Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R** 2000 Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest 24:RC7–RC9
43. **Konturek SJ, Konturek JW, Pawlik T, Brzozowski T** 2004 Brain-gut axis and its role in the control of food intake. J Physiol Pharmacol 55:137–154
44. **Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M** 2002 The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion. Gastroenterology 123:1120–1128
45. **Date Y, Toshinai K, Koda S, Miyazato M, Shimbara T, Tsuruta T, Niijima A, Kangawa K, Nakazato M** 2005 Peripheral interaction of ghrelin and cholecystokinin on feeding regulation. Endocrinology 146:3518–3525

Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**