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

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Ghrelin, an acylated peptide produced predominantly in the stomach, stimulates feeding and GH secretion via interactions with the GH secretagogue type 1α receptor (GHS-R1α), 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-R1α. 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 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, GRHRP-6, and MK-0677, 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 protein containing transmembrane domain one to five (2, 3). Ghrelin molecules exist in the stomach and hypothalamus as two major endogenous forms, ghrelin and des-acyl 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 cardiomyocytes 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 cytotoxic effect on cultured cardiomyocytes

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

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(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 (Abbott 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 (CH₃CN) 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 with the avidin-biotin complex method (Vectorstain 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% CH₃CN 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.](https://academic.oup.com/endo/article-abstract/147/5/2306/2501041)
Alexa 488-conjugated donkey anti-goat IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400). After washing with PBS (pH 7.4), samples were incubated with a rabbit anti-orexin-A antiseraum 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 to 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 icv 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). Sev- enth, 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 random- ized to receive either test substance with a washout period of 3 d between each administration.

Measurement of cytosolic Ca2+ concentration ([Ca2+])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 NaHCO3, 4.7 mM KCl, 1.2 mM KH2PO4, 1.8 mM CaCl2, 1.2 mM MgSO4, and 10 mM HEPES (pH 7.4)] containing 10 mM glucose. The LHA was incubated in HKRB supplemented with 1 ng/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 [Ca2+]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 MgCl2, 2.0 mM CaCl2, 10 mM glucose, and 2 μM Fluo-3/acetoxymethylether (Dojindo Labs, Kumamoto, Japan) (pH 7.4)). 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. [Ca2+]i was determined by measuring fluorescence signal from the Ca2+ indicator Fluo-3/acetoxymethylether, 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 [Ca2+]i in a single neuron was recorded for 6 min after the administration of des-acyl ghrelin. After [Ca2+]i measurement, the neurons were fixed with 4% paraformaldehyde overnight. They were incubated with rabbit anti-orexin-A antiseraum (dilution 1:5000) 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 [Ca2+]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 gland 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.
(Amersham, Buckinghamshire, UK). The experiment was concluded by treatment with 60 mM KCl to induce depolarization.

**Statistical analysis**

Data (mean ± SEM) were analyzed by ANOVA and the post hoc Scheffe’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 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 ghrelin increased food intake in rats given ghrelin, whereas pretreatment with anti-NPY IgG did not affect des-acyl ghrelin-induced feeding in comparison to rats given control IgG and des-acyl ghrelin (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).
ghrelin to rats significantly increased locomotor activity compared with saline administration (Fig. 8). Orexin-A also significantly increased locomotor activity in these rats.

**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 palmityl-coenzyme A, functions in the stomach (32–34). In contrast, the enzyme that catalyzes the acyl modification of ghrelin has not been identified.
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 electrophysiological 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 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.

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