Regulation of food intake and body weight by recombinant proghrelin

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Zhang W, Majumder A, Wu X, Mulholland MW. Regulation of food intake and body weight by recombinant proghrelin. Am J Physiol Endocrinol Metab 297: E1269–E1275, 2009. First published September 29, 2009; doi:10.1152/ajpendo.00337.2009.—Ghrelin is a 28-amino-acid hormone derived from the endoproteolytic processing of its prehormone proghrelin. Although ghrelin has been reported to regulate food intake and body weight, it is still unknown whether proghrelin exercises any biological function. Here we show that recombinant proghrelin alters food intake and energy metabolism in mice. After intraperitoneal administration of recombinant proghrelin (100 nmol/kg body wt), cumulative food intake was significantly increased at days 1, 2, and 3 (6 ± 0.3, 13 ± 0.5, and 20 ± 0.8 g vs. 5 ± 0.2, 10 ± 0.2, and 16 ± 0.3 g of the control mice receiving normal saline, respectively, n = 6, P < 0.05). Twelve-hour cumulative food intake in the light photo period in mice treated with proghrelin increased significantly relative to the control (2.1 ± 0.04 vs. 1.3 ± 0.2 g, n = 6, P < 0.05). No change in 12-h cumulative food intake in the dark photo period was observed between mice treated with proghrelin and vehicle (4.2 ± 0.6 vs. 4.3 ± 0.6 g, n = 6, P > 0.05). This is associated with a decrease in body weight (0.42 ± 0.04 g) for mice treated with proghrelin, whereas control animals gained body weight (0.31 ± 0.04 g). Mice treated with proghrelin demonstrate a significant decrease in respiratory quotient, indicating an increase in fat consumption. Recombinant proghrelin is functionally active with effects on food intake and energy metabolism.

Ghrelin; proghrelin; food intake; body weight; respiratory quotient

growth hormone release in humans and rats, following either peripheral or central administration (18, 26, 32). Although ghrelin was originally reported to stimulate growth hormone release, studies have provided evidence that ghrelin exercises a wide range of functions, including regulation of food intake and energy metabolism (5, 20, 28), modulation of cardiovascular function (29), stimulation of the proliferation of osteoblasts and bone formation (9, 14), modulation of the function of lymphocyte and inflammation (12), and stimulation of neurogenesis (40). In the gastrointestinal system, ghrelin coordinated multiple functions, including secretion of gastric acid (8, 19), gastric motility (19, 27), and pancreatic protein output (38).

Among these functions, the effect of ghrelin on the regulation of energy metabolism has been most extensively studied (5, 20, 28, 37). In rats, ghrelin has been found to stimulate food intake (20, 32), to induce adiposity (28), and to increase body weight (28, 32). The catabolic effect of ghrelin is reported to result from the reduction of fat utilization (28). Serum ghrelin levels abruptly increase before and decrease after meals, suggesting that ghrelin is a hunger hormone potentially involved in meal initiation and satiety in an inverse pattern to that of insulin (6). Interestingly, the orexigenic effect of peripherally secreted ghrelin is proposed to be mediated by a central mechanism involving the regulation of the neuropeptide Y and proopiomelanocortin neurons at the hypothalamic level (5, 11, 15, 20, 30). Because energy metabolism is regulated by insulin, it is not surprising that ghrelin affects glucose metabolism by regulating the development of pancreatic islet β-cells and insulin secretion (10).

The ghrelin gene, which is composed of four exons and three introns, encodes a molecule designated as proghrelin (24, 25, 35). Proghrelin peptide undergoes endoproteolytic processing to yield three different end products: ghrelin; des-acyl ghrelin, which has an identical amino acid sequence except its third amino acid (serine-3) is not acylated; and obestatin. All three products of the ghrelin gene are detectable in blood with des-acyl ghrelin at highest concentration. Although both des-acyl ghrelin and obestatin have been reported to counter the anabolic effect of ghrelin (4, 17, 31, 36), conflicting effect has been reported (2, 21). Although the products of the ghrelin gene have been extensively studied, it is unknown whether the precursor, proghrelin, exercises any biological function.

Here we describe the cloning, production, and characterization of a recombinant rat proghrelin protein that has functional activity. Recombinant proghrelin stimulates food intake in mice. The increase in food intake occurs in the light photo period and does not cause gain in body weight. A decrease in body weight was observed, associated with a decrease in respiratory quotient and an increase in energy expenditure in mice treated with proghrelin.
Results

Expression of GST-proghrelin. As shown in Fig. 1, recombinant proghrelin was induced to be expressed by IPTG in *E. coli* as a fusion protein with the carrier protein GST. The GST-proghrelin fusion protein appeared as an ~39-kDa protein when analyzed by 10% SDS-PAGE. No expression of GST-proghrelin was observed in the absence of IPTG. Thrombin cleavage of the GST-proghrelin fusion protein resulted in a major proghrelin protein (~13 kDa) and an ~27-kDa GST intact protein.

Stimulation of food intake by recombinant proghrelin. Food intake in C57BL/6J mice was significantly increased by intraperitoneal injection of recombinant proghrelin relative to vehicle control (Fig. 2A). The extent of increase in food intake induced by recombinant proghrelin was similar to that caused by acetyl ghrelin. Further analysis revealed that the increase in food intake induced by recombinant proghrelin occurred in the light cycle (Fig. 2B), whereas no difference in food intake during the dark cycle was observed between mice treated with proghrelin and control animals (Fig. 2C). In contrast, GST-proghrelin fusion protein demonstrated no effect on the food intake compared with control mice treated with GST (P > 0.05, n = 6; Fig. 2D).

Materials and Methods

Construction of glutathione S-transferase-proghrelin and glutathione S-transferase-mutated proghrelin plasmids. Proghrelin cDNA was generated by RT-PCR as follows: total cellular RNA was isolated from adult rat gastric mucosa using TRIzol reagent according to the manufacturer’s directions. Single-strand cDNA was synthesized using reverse transcriptase SuperScript II RT (Invitrogen, Carlsbad, CA). The PCR reaction was carried out using a Perkin-Elmer Thermal Cycler (Norwalk, CT). The primers to produce the full length of proghrelin were deduced from rat published sequences with two restriction sites, EcoRI and NotI, incorporated. The nucleotide sequences of sense and antisense primers are as follows: sense: CCGAATTCAAGGCCATGGTGTCTTC and antisense: TGCCGCGCAGTGGTTACTTGT.

After the RT-PCR product was matched to the published sequence by sequencing analysis, proghrelin cDNA was excised, purified, and then cloned into a pGEX-4T vector (GE Healthcare Life Sciences, Piscataway, NJ) by using EcoRI and NotI restriction enzymes in the same reading frame as the carrier gene-glutathione S-transferase (GST). The resulting plasmid was then transferred into *Escherichia coli* BL21. The recombinant proghrelin expressed in *E. coli* has four additional amino acids (Glu-Phe-Lys-Ala) inserted in the NH2-terminal sequence when exercised and separated from GST protein by thrombin digestion.

Ghrelin mutant, ala-proghrelin, was constructed using site directed mutagenesis (Stratagene, La Jolla, CA). The third amino acid serine, where octanoylation occurs in the production of acyl ghrelin, was replaced with alanine.

Expression and purification of proghrelin. The GST-proghrelin or its mutant fusion protein was induced to be expressed from the isopropyl-β-D-thiogalactopyranoside (IPTG) tac promoter by 0.5 mM IPTG in the E. coli BL21 grown to a cell density of OD600 1.5 for 2 h at 37°C. GST-proghrelin was purified by glutathione-conjugated sepharose beads. Briefly, *E. coli* BL21 cells were incubated with 1 mg/ml lysozome, 1 mM phenylmethylsulfonyl fluoride in PBS for 20 min at 0°C, followed by treatment with 1% Triton X-100 and sonication. The cell lysate was cleared by centrifugation (10,000 g), and the GST-proghrelin protein was purified by affinity chromatography with a glutathione sepharose 4B column. Protein content was measured using a Bio-Rad protein assay kit. The GST-proghrelin fusion protein was further assessed by Western blotting using a specific GST antibody.

Measurement of food intake and body weight. Adult male C57 mice were maintained in individual cages under controlled conditions of temperature (21–23°C) and light (lights on 0700; lights off 1900) with ad libitum access to standard mouse chow and water unless stated otherwise. All procedures were approved by UCUC at the University of Michigan. Mice were weighed and randomly divided into control and treatment groups. Mice were separated and individually housed in a standard cage and acclimated for 2 days before intraperitoneal injections using a 1-ml syringe and a 25-gauge needle. Care was taken to avoid stressing the animals. The maximum volume of injection was 200 μl and was adjusted according to the weight of the individual animal. Recombinant proghrelin was dissolved in saline. During the early light phase (0900–1200) mice were given a daily intraperitoneal injection of saline, proghrelin, or ala-proghrelin (100 nmol/kg body wt), acetyl ghrelin (3 nmol/mouse), or des-acyl ghrelin (3 nmol/mouse) (*n* = 6/group). After injection, the mice were returned to their home cages and provided with a preweighed amount of Chow. Food intake was measured 1, 2, and 3 days postinjection. At the end of the experiment, mice were weighed again to calculate the body weight gain.

Analysis of energy expenditure. Measurement of oxygen consumption (*V*O₂) and carbon dioxide production (*V*CO₂) with indirect calorimetry was performed on 8- to 12-wk-old mice over 3 days with the metabolic platform at the University of Michigan Center for Integrative Genomics. Animals were fed standard laboratory chow and nectar fluid and maintained on 12:12-h light-dark cycles beginning at 0700 and 1900, respectively. Animals were acclimated in measuring chambers for 2 days before recording. Measurements of *V*O₂ and *V*CO₂ were made every 24 min for each animal over a period of 3 days. Respiratory quotient was calculated by dividing *V*CO₂ by *V*O₂. Energy expenditure was calculated by the following equation: energy expenditure = (3.815 + 1.232 × respiratory quotient) × *V*O₂.

Establishment of HEK 293 cells stably expressing growth hormone secretagogue receptor 1a. HEK 293 cells were maintained in DMEM containing 10% FCS, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% glutamine. Cells (1.5 × 10⁶) were plated, and vectors (pcDNA3.1) and growth hormone secretagogue receptor 1a (GHSR1a) constructs were transfected in cells by Lipofectamine (Invitrogen, Carlsbad, CA). Cells expressing GHSR1a constructs were selected with 800 μg/ml neomycin. Stable cell lines were maintained with 400 μg/ml neomycin.

Measurement of intracellular calcium. Fura 2-AM (4 μmol/ml) was added to cultured HEK 293 cells in fresh warm serum-free growth media and incubated at 37°C for 30 min before experimentation. Cells were washed and maintained in Hanks’ balanced salt solution. Cover slips were then placed in a perfusion chamber mounted on the stage of a Nikon inverted fluorescence microscope. The rate of superfusion of buffer and reagents was kept constant at 1 ml/min. A Nikon inverted microscope with a ×40 oil immersion objective and TILLvisION digital imaging system (TILL Photonics) were used. Single-cell cytoplasmic calcium was determined from the ratio of fluorescence intensity of fura 2-AM at 340 and 380 nm, monitored by an intensified charge-couple device camera, and subsequently digitized. Background intensity at each emission wavelength was corrected. The ratio of fluorescence intensities at 340 and 380 nm was plotted as the change in fluorescence (ΔF/ΔF₀), expressed as a percentage of the basal fluorescence ratio observed in the absence of stimulus. Cells were considered responsive if maximal ΔF/ΔF₀ increment was ≥15% above the baseline for each experimental condition.

Data analysis. The software used was GraphPad Prism from GraphPad Software (San Diego, CA). Results are expressed as means ± SE. Data were analyzed using ANOVA and a Student’s *t*-test as appropriate. Significance was accepted as *P* < 0.05.
Alteration of energy metabolism. To further confirm the observation that recombinant proghrelin is functionally active, we investigated the ability of proghrelin to induce an alteration of respiratory quotient in C57BL/6J mice. As shown in Fig. 3, recombinant proghrelin significantly reduced the respiratory quotient relative to control animals (n = 6, P < 0.05), suggesting an increase in consumption of fatty acid. On the other hand, energy expenditure was significantly increased in mice administered proghrelin relative to control animals (n = 6, P < 0.05).

Effect of recombinant proghrelin on body weight. Despite the effect of recombinant proghrelin to stimulate food intake, significant decreases in body weight were observed in mice receiving intraperitoneal injection of recombinant proghrelin relative to control animals (Fig. 4A). Administration of GST-proghrelin fusion protein did not alter body weight in C57 BL/6J mice (P > 0.05, n = 6; Fig. 4B).

Fig. 1. Expression of recombinant proghrelin. Proghrelin cDNA was generated by RT-PCR using the ghrelin sense and antisense primers as indicated in text. The recombinant glutathione S-transferase (GST)-proghrelin fusion protein was induced to be expressed 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the Escherichia coli BL21 strain of bacteria, purified by glutathione-conjugated sepharose beads, separated by 10% SDS-PAGE gel electrophoresis (A) and assessed by Western blotting using a specific GST antibody (B). Ext, unpurified protein extract; Pro-ghr, purified proghrelin; ala-proghrelin, purified ala-proghrelin.

Fig. 2. Stimulation of food intake. Male adult C57BL/6J mice were administered normal saline (control), proghrelin at a dose of 100 nmol·kg body wt⁻¹·day⁻¹, acyl ghrelin (3 nmol/mouse), or des-acyl ghrelin (3 nmol/mouse) by ip injection. Food intake was measured daily unless described otherwise. A: cumulative food intake on days 1, 2, and 3. B: food intake during the 12-h light cycle postinjection. C: food intake during the 12-h dark cycle. D: cumulative food intake after administration of GST (control) or GST-proghrelin. *P < 0.05.
Structural requirement for proghrelin activity in food intake. To determine the structural requirements for proghrelin in the modulation of food intake, we generated a proghrelin mutant (ala-proghrelin). As shown in Fig. 5, replacement of the third amino acid serine residue of ghrelin by alanine did not alter the effect of proghrelin on food intake. Food intake over 24 h in mice treated with recombinant ala-proghrelin was similar to that of animals receiving recombinant proghrelin (5.7 \pm 0.5 vs. 5.8 \pm 0.3 g respectively). Both of these groups showed a significant increase in food intake relative to control mice (4.7 \pm 0.2 g, \( P < 0.05 \)).

Effect of recombinant proghrelin on intracellular calcium signaling. Ghrelin has been demonstrated to activate its receptor (GHSR1a) with an increase in intracellular calcium signaling. To examine whether recombinant proghrelin is capable of activating the GHSR1a receptor, we established a HEK 293 cell line in which GHSR1a constructs are stably expressed and used these cells as a model to test the effect of recombinant proghrelin on the intracellular calcium signaling. As shown in Fig. 6, cells expressing GHSR1a demonstrated a significant increase in intracellular calcium concentration upon stimulation by synthetic ghrelin (10^{-8} M), indicating activation of GHSR1a. Recombinant proghrelin (10^{-7} M) demonstrated no effect on intracellular calcium signaling in cells responding to synthetic ghrelin with an increase in the intracellular calcium concentration. This result suggests that recombinant proghrelin is unlikely to activate the classical ghrelin receptor GHSR1a.

DISCUSSION

The major finding of the present study is that recombinant proghrelin stimulates food intake, likely by an action on an unidentified receptor distinct from the classical ghrelin receptor GHSR1a. This conclusion is supported by five distinct observations: 1) systemic administration of recombinant proghrelin significantly increases food intake in the light cycle; 2) the stimulatory effect of recombinant proghrelin on food intake is accompanied by a decrease in body weight; 3) respiratory quotient is decreased, while energy expenditure is significantly increased by recombinant proghrelin; 4) replacement of the third amino acid serine with alanine does not alter the functional activity of proghrelin on food intake; and 5) unlike synthetic acyl ghrelin, recombinant proghrelin does not induce intracellular calcium signaling. Gastric X/A-like cells are the major source
of circulating ghrelin. Proghrelin is synthesized and subsequently undergoes endopeptidolytic processing and post-translational modification to produce acyl-ghrelin and des-acyl ghrelin in these cells. Des-acyl ghrelin has the same amino acid sequence as acyl ghrelin, but the third amino acid (serine-3) is not acylated (18). A third putative proghrelin peptide, termed “obestatin,” has been identified (36), but biochemical and functional evidence supporting its existence has not been forthcoming. All three products of the ghrelin gene are detectable in blood, with des-acyl ghrelin at highest concentration (25, 35). Despite the presence of three different products of the ghrelin gene, acyl ghrelin is the only ligand capable of binding and activating the ghrelin receptor to exercise orexigenic effects (20, 28). It is currently unknown whether proghrelin is present in the circulation, nor whether it has biological effects. Our study provides the first evidence that proghrelin may be functionally active.

Similar to acyl ghrelin, proghrelin increases food intake in mice. The stimulatory effect of proghrelin in food intake does not lead to a gain in body weight observed for acyl ghrelin. Instead, loss of body weight occurs after systemic administration of proghrelin. Because both studies in animals and in humans have demonstrated that systemic administration of acyl ghrelin increases food intake and body weight, likely through the activation of ghrelin receptor GHSR1a on hypothalamic and brain stem neurons (5, 13, 20), it is unlikely that proghrelin acts on the GHSR1a to exercise its orexigenic effect. This notion is further supported by our study in which replacement of the ghrelin active core by alanine does not alter the function of proghrelin on food intake. This observation suggests that acylation of the third amino acid serine is not required for the activity of proghrelin on the regulation of food intake. Furthermore, proghrelin had no effect on the intracellular calcium signaling in HEK 293 cells expressing GHSR1a, indicating that proghrelin may not be the ligand for the classical ghrelin receptor GHSR1a. It seems unlikely that proghrelin exercises its effect through a mechanism similar to those of either des-acyl ghrelin or obestatin, because the effects of these two peptides are still quite controversial (2, 4, 17, 21, 31, 36) and we did not observe any effect of des-acyl ghrelin on food intake in the present study.

The classical ghrelin receptor GHSR1a is mainly present in the central nervous system and accounts for the neuroendocrine effect of ghrelin (5, 16). Activation of the GHSR1a requires the ghrelin active core composed of a motif of amino acid sequence surrounding the third amino acid serine and octanoyl modification of this serine (3). Functional activities of ghrelin have been reported in cardiac myocytes, tumor cells, and adipocytes in which no conventional ghrelin receptor GHSR1a has been observed (1, 23, 41). Radioisotope labeled ligand has been used to confirm ghrelin-binding activities in these cells that are distinct from that of the conventional ghrelin receptor GHSR1a (23, 41). Activation of this unidentified receptor leads to a distinct intracellular signaling pathway: phosphatidylinositol 3-kinase/mitogen-activated protein kinase signaling (23). Taken together, these studies indicate that there exits at least one other novel subtype of ghrelin receptor that is functionally active. The findings that proghrelin is functionally active and that its effect on food intake is not mediated by the classical ghrelin receptor suggest that proghrelin may provide a tool for the identification of the unidentified subtype of ghrelin receptor. However, the functional domain of proghrelin critical for binding and activating its receptor is unknown. The observation showing that GST-proghrelin fusion protein does not possess the functional activity of proghrelin on food intake suggests that the GST tag may block the binding of proghrelin with its receptor. It is possible that the functional domain of proghrelin may be within the NH2-terminal region, which is subject to spatial occupation by the adjacent GST molecule. It is generally agreed that proghrelin is synthesized and cleaved by an intracellular enzyme prohormone convertase 1/3 before being secreted in the circulation within the ghrelin-producing cells (25, 35). Currently, there is no reported mechanism by which proghrelin is secreted and cleaved outside the cells. It is therefore unlikely that the recombinant proghrelin is cleaved into peptide fragments, which then affect food intake.

In summary, this study suggests that proghrelin is functionally active in regulation of food intake and body weight in a manner distinct from the action of acyl ghrelin.
REFERENCES


