Chronic Central Infusion of Ghrelin Increases Hypothalamic Neuropeptide Y and Agouti-Related Protein mRNA Levels and Body Weight in Rats

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Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), was originally purified from the rat stomach. Like the synthetic growth hormone secretagogues (GHSs), ghrelin specifically releases growth hormone (GH) after intravenous administration. Also consistent with the central actions of GHSs, ghrelin-immunoreactive cells were shown to be located in the hypothalamic arcuate nucleus as well as the stomach. Recently, we showed that a single central administration of ghrelin increased food intake and hypothalamic agouti-related protein (AGRP) gene expression in rodents, and the orexigenic effect of this peptide seems to be independent of its GH-releasing activity. However, the effect of chronic infusion of ghrelin on food consumption and body weight and their possible mechanisms have not been elucidated. In this study, we determined the effects of chronic intracerebroventricular treatment with ghrelin on metabolic factors and on neuropeptide genes that are expressed in hypothalamic neurons that have been previously shown to express the GHS-R and to regulate food consumption. Chronic central administration of rat ghrelin (1 μg/rat every 12 h for 72 h) significantly increased food intake and body weight. However, it did not affect plasma insulin, glucose, leptin, or GH concentrations. We also found that chronic central administration of ghrelin increased both neuropeptide Y (NPY) mRNA levels (151.0 ± 10.1% of saline-treated controls; P < 0.05) and AGRP mRNA levels (160.0 ± 22.5% of saline-treated controls; P < 0.05) in the arcuate nucleus. Thus, the primary hypothalamic targets of ghrelin are NPY/AGRP-containing neurons, and ghrelin is a newly discovered orexigenic peptide in the brain and stomach. Diabetes 50:2438–2443, 2001

Energy intake and expenditure are tightly regulated in mammals (1). Several neuronal populations, particularly in the hypothalamic arcuate nucleus (ARC), are involved in the regulation of energy homeostasis and have been implicated as possible targets of orexigenic peptides (1). These include neuropeptide Y (NPY)/agouti-related protein (AGRP) coexpressing neurons, pro-opiomelanocortin (POMC) neurons, and growth hormone–releasing hormone (GHRH) neurons, which are known to be stimulated (NPY and AGRP) or suppressed (POMC and GHRH) by starvation (2–10). However, central administration of NPY, AGRP, and GHRH increased food intake in rats, whereas α-melanocyte-stimulating hormone (α-MSH), an end product of POMC processing, decreased food intake when injected centrally (11–13).

The growth hormone secretagogues (GHSs) are synthetic peptide and nonpeptide compounds that stimulate the pulsatile release of growth hormone (GH) after intravenous administration (14). Central administration of GHSs also stimulates feeding behavior (15). GHS receptor (GHS-R) mRNA is expressed in the pituitary gland and in several areas of the brain, including the hypothalamus (16). In the hypothalamus, GHS-R mRNA is expressed in NPY/AGRP, POMC, and GHRH-containing neurons (17–19). Ghrelin, an endogenous ligand for the GHS-R, was purified from the rat stomach (20). Similar to the synthetic GHSs, ghrelin specifically releases GH after intravenous administration. Recently, we showed that a single central administration of ghrelin increased food intake and hypothalamic AGRP gene expression in rodents, and the orexigenic effect of this peptide seems to be independent of its GH-releasing activity (21). Thus, ghrelin has an alternative role in stimulating food intake in addition to releasing GH from the pituitary. It has been reported that previous exposure to GHS can induce desensitization of the subsequent GH responses to GHS, indicating that homologous desensitization is observed after continuous exposure to GHS with respect to GH release (22,23). However, the effect of chronic infusion of ghrelin on food consumption and body weight and their possible mechanisms has not been elucidated. In this study, we determined the effects of chronic intracerebroventricular treatment with ghrelin on metabolic factors and on the hypothalamic NPY, AGRP, POMC, and GHRH mRNA levels using radioactive in situ hybridization histochemistry.
RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley rats (250–280 g) were housed in air-conditioned animal quarters, with lights on between 06:00 and 20:00 h, and were given food and water ad libitum. The animals were anesthetized with ether, and a 23-gauge stainless-steel cannula was implanted into the right lateral ventricle using a stereotaxic apparatus, as previously described (24). The upper incisor bar was set 3.3 mm below the interauricular line, and the bregma was taken as A-P zero. The cannula tip was placed at A – 0.9, L – 1.2, and V – 3.6 mm and secured in place with dental acrylic. Only those rats whose cerebrospinal fluid overflowed through the cannula were used for the experiment. They were kept in individual cages and habituated by handling every day. Injections of the peptide were performed 14 days after placement of the cannula. Experiments were conducted according to the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocol. Rat ghrelin (1 μg/rat; provided by Dr. K. Kangawa, National Cardiovascular Center Research Institute, Osaka, Japan) or saline was injected every 12 h for 72 h. Food intake and body weights were measured daily. Animals were anesthetized with pentobarbital (70 mg/kg i.p.) ~4 h after the final injection of ghrelin. The blood was collected, and plasma was separated by centrifugation and stored at −70°C until assay. Plasma ghreline was measured by the ghreline oxidase method. Plasma insulin and leptin were measured by radioimmunoassay (RIA) using a commercially available rat insulin RIA kit (Amersham, Tokyo) and rat leptin RIA kit (Linco Research, St. Charles, MO), respectively. GH levels were measured with a double-antibody RIA using materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD), as previously described (21). Epidermal, retroperitoneal, and inguinal fat pads were dissected and weighed. Anesthetized rats were perfused with 2% paraformaldehyde, and the brain was kept overnight at 4°C before the brain was fixed in a fixative containing 20% sucrose.

In situ hybridization. Frozen coronal sections, 30 μm thick, were cut with a cryostat, mounted onto Silan-coated slides (Shin-Etsu Chemical, Tokyo), and air-dried. The medial basal hypothalamus from the rostral end of the third ventricle to the caudal end of the ARC was processed for in situ hybridization using 35S-labeled riboprobes for NPY, AGRP, POMC, and GHRH mRNA, as previously described (21). The hybridization protocol has been described previously (25). In brief, sections were dried, digested by proteinase K (10 μg/ml), and acetylated with 0.25% acetic anhydride. Sections were rinsed briefly in 2× saline sodium citrate (SSC) and then air-dried. The probe was dissolved in hybridization buffer (10 mmol/l Tris [pH 8.0], containing 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 12 mmol/l EDTA, 30 mmol/l NaCl, 0.5 mg/ml yeast transfer RNA, and 10 mmol/l dithiothreitol). Thereafter, 2× 106 cpm of the probe in 100 μl buffer was applied to each slide and hybridized at 55°C overnight. The slides were rinsed in 4× SSC and digested with ribonuclease-A (20 μg/ml) for 30 min, followed by two 5-min changes of 2× SSC, 10 min in 1× SSC, 10 min in 0.5× SSC at room temperature, and 30 min in 0.1× SSC at 55°C. The slides were dehydrated in ethanol, and air-dried. These sections were dipped in Konica NR-M2 autoradiographic emulsion (Konica, Tokyo), exposed for 5 days, and developed. The sections were counterstained with cresyl violet. As a control to confirm the hybridization signal, sense NPY, AGRP, POMC, and GHRH probes were used on some adjacent sections from experimental animals, and no specific signal was detected.

Data analysis. The hybridization signal in the brain slice was determined from the autoradiograms using an MCID image analysis system (Imaging Research, St. Catherines, Canada), as previously described (25). The ARC on one side was enclosed by a circle with a 1.12-mm radius as a fixed window, which covered almost the whole area of the nucleus. The relative optical density within the window was measured. The background was estimated by measuring the relative optical density within a window placed over another area of the brain. The anatomical equivalence of hypothalamic sections among animals was obtained by selecting slides with the aid of a rat brain atlas (26) and verifying the anatomical identifications by staining sections with cresyl violet. Six sections per animal were examined. The values reported are the mean ± SE. Five rats were used for the analysis. Statistical analysis was performed with the Student’s t test to compare the means between two groups. *P < 0.05 was considered significant.

RESULTS

Food intake, body weight, fat weight, and plasma hormones. Seven injections of rat ghrelin (1 μg) into the lateral ventricle of rats during a 72-h period of free feeding resulted in a significant increase in food intake (116.0 ± 6.2 g for ghrelin vs. 97.8 ± 4.5 g for saline; n = 5 rats per group; *P < 0.05) (Table 1) and body weight (11.8 ± 2.9 g for ghrelin vs. 2.8 ± 2.5 g for saline; n = 5 rats per group; P < 0.05 (Table 1), as compared with intracerebroventricular injection of the vehicle. However, it did not affect the weight of three fat deposits or of plasma insulin, glucose, leptin, and GH concentrations, although there was the trend toward higher levels of fat pad weights and plasma leptin and insulin levels in the ghrelin-treated group (Table 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparisons of food intake, body weight, fat weight, plasma glucose, insulin, leptin, and GH in rats treated with rat ghrelin or saline for 3 days</th>
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<tbody>
<tr>
<td>n</td>
<td>Control</td>
</tr>
<tr>
<td>Food intake (g/72 h)</td>
<td>97.8 ± 4.5</td>
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<tr>
<td>Initial body weight (g)</td>
<td>373 ± 12</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>376 ± 14</td>
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<tr>
<td>Δ Body weight (g)</td>
<td>2.8 ± 2.5</td>
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<tr>
<td>Epidydymal fat weight (g)</td>
<td>3.6 ± 0.3</td>
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<tr>
<td>Retroperitoneal fat weight (g)</td>
<td>3.2 ± 0.4</td>
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<tr>
<td>Inguinal fat weight (g)</td>
<td>3.8 ± 0.3</td>
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<tr>
<td>Plasma glucose (mg/dl)</td>
<td>144.8 ± 3.2</td>
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<td>Plasma insulin (ng/ml)</td>
<td>4.56 ± 0.43</td>
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<tr>
<td>Plasma leptin (ng/ml)</td>
<td>5.65 ± 1.39</td>
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<tr>
<td>Plasma GH (ng/ml)</td>
<td>8.61 ± 3.18</td>
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</tbody>
</table>

Data are means ± SD. *P < 0.05 compared with the control group.

DISCUSSION

In the present study, we demonstrated that chronic central infusion of ghrelin significantly increases food intake and body weight. We also found that chronic central administration of ghrelin increases hypothalamic NPY and AGRP mRNA levels, whereas central infusion of ghrelin does not alter POMC and GHRH mRNA levels. However, the infusion did not affect plasma insulin, glucose, leptin, or GH concentrations. These results indicated that the primary hypothalamic targets of ghrelin are NPY/AGRP-containing neurons, and ghrelin is a novel orexigenic peptide in the brain and stomach.

The central regulation of pituitary GH release is mediated by the opposing actions of GHRH and somatostatin (SS) (27). In this study, plasma GH levels and GHRH and SS (data not shown) mRNA levels were not altered by central infusion of ghrelin. These results are consistent with our previous report that a single central injection of 1 μg rat ghrelin did not alter GHRH and SS mRNA levels or the episodic release of GH of freely moving adult male rats (21). It also has been reported that treatment with ghrelin for 1 week increased the food intake of female dwarf rats,
which are deficient in GH (28). Taken together, these data suggest that the orexigenic action of ghrelin is unlikely to be due to its ability to stimulate GH secretion.

It has been reported that central administration of a synthetic GHS induced c-fos expression, a marker of neuronal activity, in NPY/AGRP-containing neurons (29,30). Consistent with these reports, this study showed that chronic central administration of ghrelin increased both NPY mRNA levels and AGRP mRNA levels, suggesting that ghrelin activates the NPY/AGRP neurons of the ARC. These findings are in agreement with our previous report that a single central injection of ghrelin (1 µg/rat) increased AGRP mRNA levels in the ARC (21). However, in the same report, we did not observe a significant change in NPY mRNA levels after a single injection of ghrelin, although there was a trend toward an increase. It remains to be determined whether this discrepancy is because 1) the single injection of 1 µg of ghrelin was not sufficient to increase NPY mRNA levels significantly, or 2) prolonged stimulation with ghrelin is necessary to increase NPY mRNA levels.

During starvation, when circulating leptin, an adipose tissue–derived hormone, levels fall (31,32), NPY and AGRP mRNA levels increase in the ARC. Because the hypothalamic NPY/AGRP-containing neurons also express the leptin receptor (33), these neurons may be direct targets for leptin. Because leptin inhibits NPY and AGRP mRNA levels (5,34,35), one mechanism by which fasting leads to
an increase in NPY and AGRP mRNA may be through the reduction of circulating leptin. However, the reduction of leptin is unlikely to be the sole mechanism by which NPY and AGRP mRNA levels are increased during fasting, because NPY and AGRP mRNA levels also are elevated by fasting in the leptin-insensitive \textit{db/db} mice, which, as a result of a mutation in the leptin receptor, are resistant to the effects of leptin (8,35). It also has been reported that fasting increases plasma ghrelin levels (28), and we showed that central infusion of ghrelin increased NPY and AGRP mRNA levels. Therefore, it has been hypothesized that part of the mechanism by which fasting increases NPY and AGRP mRNA levels is through an elevation in plasma ghrelin that accompanies fasting. So far, the source of the ghrelin that activates the NPY/AGRP neurons is unknown, but the principal site of ghrelin synthesis is the stomach and not the hypothalamus (20). In addition, it has been reported that intraperitoneal administration of ghrelin in freely feeding rats stimulated food intake (36). These results led to the suggestion that ghrelin is the peripheral signal from the stomach that regulates food intake by acting on the hypothalamic neuropeptides in addition to leptin. However, additional studies are required to determine how ghrelin enters the brain. We cannot, however, exclude the possibility that the ghrelin that originates in the hypothalamus acts within the brain to increase food intake, because ghrelin-immunoreactive cells were shown to be located in the hypothalamic ARC as well as the stomach (20). In the latter case, it remains to be determined whether the hypothalamic levels of ghrelin are affected by nutritional status.

AGRP and NPY are potent stimulators of feeding

FIG. 3. Effect of intracerebroventricular administration of ghrelin on POMC mRNA in the ARC of adult male rats. Rat ghrelin (1 μg) or its vehicle was administered into the lateral ventricle every 12 h for 72 h. POMC mRNA levels in the ARC were determined by in situ hybridization. \textit{A}: Representative in situ hybridization histochemistry for POMC mRNA, as revealed by emulsion autoradiography in the ARC after central administration of ghrelin or the vehicle. The scale bar indicates 500 μm. \textit{B}: A summary of the data, which are expressed as a percentage of the vehicle-treated control group. The data represent the mean ± SE (n = 5 animals/group).

FIG. 4. Effect of intracerebroventricular administration of ghrelin on GHRH mRNA in the ARC of adult male rats. Rat ghrelin (1 μg) or its vehicle was administered into the lateral ventricle every 12 h for 72 h. GHRH mRNA levels in the ARC were determined by in situ hybridization. \textit{A}: Representative in situ hybridization histochemistry for GHRH mRNA, as revealed by emulsion autoradiography in the ARC after central administration of ghrelin or the vehicle. The scale bar indicates 500 μm. \textit{B}: A summary of the data, which are expressed as a percentage of the vehicle-treated control group. The data represent the mean ± SE (n = 5 animals/group).
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(11,12). During fasting, NPY released from NPY/AGRP neuron binds to and activates NPY-Y1 and -Y5 receptors in the hypothalamus and results in the stimulation of feeding behavior (1). The simultaneous release of AGRP antagonizes the melanocortin-4 receptor, which suppresses the anorectic effects of α-MSH (12). Therefore, NPY and AGRP increase food intake via different pathway. It has been reported that mice with a deletion in the NPY gene also increase food intake after central administration of ghrelin (28), raising the possibility that redundant mechanisms exist to ensure that this drive is maintained, suggesting that ghrelin has a physiological relevance in the regulation of food intake. The present studies, demonstrating that chronic central infusion of ghrelin increase body weight in rats, support the hypothesis that ghrelin plays a role in the physiological regulation of body weight. Orexigenic factors such as NPY or AGRP may contribute to the obesity syndrome and diabetes. In fact, diabetic hyperphagia induced by streptozotocin is associated with elevated expression of the NPY and AGRP genes in the ARC (37). Additional work will be necessary to determine whether ghrelin contributes to the pathophysiological mechanisms in diabetes. We are currently investigating these possibilities.

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REFERENCES