Role of Endogenous Ghrelin in Pulsatile GH Secretion in Conscious Rats

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Abstract. Ghrelin is synthesized in the stomach and plays a role as a potent endogenous ligand for the GH secretagogue receptor. Although exogenously administered ghrelin stimulates GH release both in vivo and in vitro, the physiological role of endogenous ghrelin in GH secretion remains to be clarified. We generated antisera in rabbits, which were specific to the bioactive N-terminus portion of rat ghrelin (1-28). An anti-ghrelin serum (AGS, #106) was used at a final dilution of 1:12,000,000 for the ghrelin RIA, which showed negligible cross-reactivity (<0.1%) with des-acyl rat ghrelin (1-28), rat ghrelin (13-28), other gastrointestinal peptides, or pituitary hormones, but reacted 100% with human ghrelin (1-28). Tissue content of ghrelin was high in the stomach (52.8 ± 6.1 fmol/mg wwt), but negligible in the cerebral cortex, hypothalamus or anterior pituitary (<0.03 fmol/mg wwt). Ghrelin immunohistochemistry showed ghrelin-positive cells in the gastric mucosa, but not in the hypothalamus, or cerebral cortex of the animals. Quantitative measurement of ghrelin mRNA levels by the real-time PCR technique (LightCycler, Roche) showed 5 x 10^6–1 x 10^9 and 5 x 10^3–2 x 10^4 copies/μg total RNA from the stomach and pituitary, respectively, but undetectable levels (<1 x 10^3 copies/μg total RNA) in the cerebral cortex or hypothalamus. Repeated blood sampling every 20 min from 1000 h to 1800 h showed that plasma GH levels in conscious male rats given icv or iv with normal rabbit serum (NRS) were pulsatile with spontaneous GH secretory bursts, which occurred regularly every 3–4 hrs from undetectable levels (less than 2 ng/ml) to over 100 ng/ml. When animals were pre-treated iv with AGS, the plasma GH release induced by ghrelin (20 μg/rat, iv) was completely abolished. In contrast, passive immunization with AGS either iv or icv did not affect pulsatile GH secretion in conscious rats, while the 1:1,000 diluted plasma from the animals given iv with AGS showed more than 70% binding to the 125I-ghrelin throughout the experiment. These results suggest that the ghrelin gene expresses exclusively in the stomach, and that circulating ghrelin does not play any major significant roles in the regulation of pulsatile GH secretion in the rat.

Key words: immunohistochemistry of rat ghrelin, specific RIA for n-octanoylated ghrelin, real time PCR of rat ghrelin, immunoneutralization of circulating ghrelin, pulsatile GH in conscious male rat

Introduction

Ghrelin is synthesized in the stomach and plays a role as a potent endogenous ligand for the GH secretagogue receptor (1). The n-octanoylation at serine⁹ residue of ghrelin (1-28)
is essential for its bioactivity. Nevertheless, most studies including immunohistochemistry (IHC) and tissue concentrations of ghrelin have so far been conducted with polyclonal antibodies against the bioactive C-terminus portion of ghrelin. Previous studies with antibody specific to the C-terminus of ghrelin showed some ghrelin-containing neurons in the hypothalamic arcuate nucleus with nerve termini in the outer layer of the median eminence (1).

Recent advances in the real-time PCR technique provide us with an opportunity to measure gene expressions quantitatively in various tissues as well as differentiating from background noise, which may occur in qualitative PCR (2).

Passive immunization with specific and potent antisera against various peptides has previously been well utilized to demonstrate important roles for endogenous peptides in the control of GH secretion (3, 4). Although exogenously administered ghrelin stimulates GH release both in vivo and in vitro, passive immunization of circulating ghrelin with its specific antisera has not yet been conducted.

In the present study, we first generated polyclonal antisera in the rabbit against the N-terminus portion of serine\(^3\)-(n)-octanoylated ghrelin (1-28), and characterized qualitatively (by IHC) and quantitatively (by RIA) ghrelin-producing cells in the cerebral cortex, hypothalamus, pituitary, and stomach. We then measured ghrelin mRNA levels quantitatively by the real-time PCR technique in various tissues. We further examined the effect of iv or icv injection of an anti-ghrelin serum (AGS) on pulsatile GH secretion in conscious male rats.

Materials and Methods

Chemicals and peptides

All synthetic brain-gut peptides, motilin, glucagon, secretin, NPY, GHRH (1-43)OH and somatostatin, were purchased from Bachem (Torrance, CA), with the exception of ghrelin. Pituitary hormone, including rat and human GH, prolactin, TSH, ACTH, LH, FSH and AVP, were provided by NIDDK, NIH (Washington, DC). Synthetic [serine\(^3\)]-(n)-octanoylated human ghrelin (1-28)-[Cys\(^{28}\)] was purchased from Peptide Institute, Inc. (Osaka, Japan). Synthetic human ghrelin (1-28), rat ghrelin (1-28), (n)-octanoylated ghrelin (1-11)-Cys\(^{12}\) and ghrelin (13-28) were kindly provided by Dr. M. Kojima (Research Institute, National Cardiovascular Center, Osaka, Japan).

Tissue collection and extraction

Fresh individual cerebral frontal cortexes, hypothalami, anterior pituitaries and gastric bodies from 40-day-old male SD rats were collected at the time of decapitation. Tissues were rapidly dissected, frozen on dry ice, and stored at -80°C until subsequent extraction (5). Individual frozen tissues, ca. 100 mg, were weighed, diced and immediately added to test tubes containing ten-fold volume of 1 N acetic acid, and the tubes were boiled for 20 min. The tissues were individually homogenized with a glass-Teflon homogenizer. The homogenates were centrifuged at 10,000 \(\times\) g for 20 min at 4°C and the supernatants were lyophilized and stored at -20°C for subsequent rat ghrelin measurement. Recovery of rat ghrelin added prior to extraction was 96%.

RIA for rat ghrelin

Synthetic (n)-octanoylated ghrelin (1-11)-[Cys\(^{12}\)] or [serine\(^3\)]-(n)-octanoylated human ghrelin (1-28)-[Cys\(^{28}\)] was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mKLH, Pierce, Rockford, IL) in conjugation buffer (Pierce). The mixture was dialyzed against distilled water for 24 h at 4°C. Female Japanese albino rabbits (2.5–3.0 kg) were injected subcutaneously at multiple sites with 1.0 ml of an equal mixture (vol/vol) of ghrelin conjugate and Freund's complete
adjuvant with an initial immunizing dose of 100 μg ghrelin. The animals received booster injections of 50 μg ghrelin in Freund’s incomplete adjuvant at monthly intervals. Antibodies were detected in all rabbits within 8 weeks after the initial immunization and maximal titers were achieved by 5 months. RIA of rat ghrelin was performed according to a method previously described (5). Iodinated human ghrelin, 125I-[Ile5]-human ghrelin(1-28) (IM347, Amersham Biosciences AB, Uppsala, Sweden), was used for both RIA and assessment of binding potency of the injected antiserum into animals.

Animals
Adult three-month old male Sprague Dawley rats were purchased from Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan, and were kept for at least one week in an environmentally controlled room (lights on 0600–1800 h, temperature, 24 ± 1°C) with tap water and food ad libitum. Both icv and iv cannulae were then implanted into the lateral cerebral ventricle and right atrium, respectively, as previously described (6). After cannulation, animals were caged individually, handled and weighed daily by the same investigator, and adapted to specific blood sampling procedures. Since previous studies by us and others have shown that plasma GH response to ghrelin is GH-burst dependent in conscious male rats (data not shown), two groups of animals were anesthetized with pentobarbital, and pretreated with NRS or anti-ghrelin serum (AGS, #106) (0.8 ml/rat, iv) 20 min prior to the iv injection of rat ghrelin (20 μg/rat). Plasma GH levels before and 10 min after ghrelin injection between normal rabbit serum (NRS) and AGS treated rats were compared for assessment of the biological potency of the antiserum. All animal experiments were conducted in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committee, Miyazaki Medical college.

Immunocytochemistry
Forty-eight hours after colchicine treatment (100 μg, icv), animals were anesthetized with sodium pentobarbital and perfused cephalad through the thoracic aorta with 100 ml ice-cold 50 mM PBS, pH 7.4, followed by 70 ml PBS containing either 4% acrolein (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) or 4% paraformaldehyde. The brain was removed, rinsed overnight in PBS, and immersed in PBS containing 30% sucrose for 24–48 h until tissues sank to bottom of the container. The tissue was sectioned at 40-μm intervals on a cryostat, stored for up to 1 week in PBS at 4°C to clear fixative and sucrose, and then subjected to immunohistochemistry using the ABC method previously described (4). The fresh stomachs from another group of 3-month-old SD male rats were obtained after decapitation, fixed with 4% paraformaldehyde, and embedded in paraffin wax (7). Sections were mounted on glass slides at 2 μm thickness. Antibodies against rat GHRH (#51) (8) were used at a final dilution of 1:5,000 for the hypothalamus, while AGS at 1:500–1:100,000 and 1:50,000 was used for the hypothalamus and stomach, respectively.

Real-time PCR for ghrelin gene expression in the cerebral cortex, hypothalamus, pituitary, and stomach
Serial dilutions of a single stranded vector, which contained the PCR-generated full length rat ghrelin cDNA, were carried out in duplicate from 1 × 10⁶ molecules to 10 molecules and used in triplicate RT-PCR reactions. Standard curves were obtained by plotting the log [calculated copy number] against the threshold cycle. The log copy numbers (N) of unknown samples were calculated from the regression line according to the formula: logN = (CT – b)/m, where CT is the threshold cycle, b is the y-intercept, and m is the slope of the standard curve line. As normalization to the
GAPDH housekeeping gene is inaccurate, mRNA expression levels are presented as the mRNA copy number per μg total RNA. Any copy number under 1,000 copies/μg total RNA was assumed to be due to illegitimate transcription. The threshold cycle number is plotted against the amount of standard added to the reaction. Total RNA from the cerebral cortex, hypothalamus, anterior pituitary and gastric antrum (body) of individual rats (n=3) was prepared using Isogen (Nippon Gene, Co., Ltd, Toyama, Japan). PCR primers for rat ghrelin cDNA (forward: GAGGCAGAGGAGGAGCTGGAATCAGGT, reverse: TCCGCCAGGGCGCGCCATGCTGCTGGA) were designed to amplify the size of a 100 bps target segment. A plasmid vector, which contained a PCR-generated full length segment of rat ghrelin cDNA, was used as a standard for quantitative PCR. In brief, 20 μL of reaction mixture including 1 μg of total RNA template, dNTP mixture, and oligo (dT) 20 primer were heated for 20 min at 94°C in the presence of reverse transcriptase (ReverTra Ace, Toyobo Co., Ltd., Osaka, Japan). The reaction mixture was then denatured at 99°C for 5 min, followed by 4°C for 5 min. The PCR reactions were performed, recorded, and analyzed using the LightCycler Quick System 330® (Roche Biosystems, Mannheim, Germany). We used an initial denaturation step of 95°C for 10 min followed by 55 cycles of 95°C for 10 sec, 56°C for 5 sec, and 72°C for 5 sec in the presence of Sybergreen I and oligoprobes. To check the specificity of the PCR products obtained, a conventional electrophoresis of amplified samples was routinely performed.

Evaluation of spontaneous GH secretion and anti-ghrelin serum responsiveness in vivo

On the day of experiments, blood samples (0.2 ml) were withdrawn from the intratrial cannula at 20 min intervals from 1000 h to 1800 h to assess the pattern of spontaneous GH secretion according to a previously described method (6, 9). NRS or AGS was injected iv (0.8 ml) or icv (10 μl) over 2 min or 1 min, respectively, at 1040 h into conscious male rats. All blood samples were immediately centrifuged and the plasma was separated and stored at -20°C for subsequent GH radioimmunoassay (RIA). After blood sampling, erythrocytes were resuspended in saline and returned to the same animal after collecting the subsequent blood sample. In immunoneutralization experiments in conscious rats, binding potency of diluted plasma samples (1:1000) obtained for 7 hr, 1040 h–1800 h, from animals given iv with AGS was assessed by RIA.

Statistical analysis

The peak GH response to ghrelin was compared between NRS- and AGS-injected rats. The integrated spontaneous GH secretion from 1100 h to 1800 h was determined by measuring the area subscribed by the plasma GH values during the specified time interval. The significance of differences between groups was determined with Students' t-test as performed on a using a computer program, StatView 4.5J for Macintosh computer. A probability of p<0.05 was considered significant.

Results and Discussion

RIA for rat ghrelin and tissue concentration

Dilution curves of an antiserum (#106) with [125I]-[Ile9]-human ghrelin(1-28), as shown in Fig. 1a, indicated high binding capacity for ghrelin. At 1:12,000,000 dilution, the antiserum showed 30–35% binding to iodinated human ghrelin. As shown in Fig. 1b, AGS (#106) used in the present study at a final dilution of 1:12,000,000 exhibited no significant cross-reactivity (<1 pmole/tube) with des-acyl rat ghrelin (1-28), rat ghrelin (13-28), motilin, rat growth hormone-releasing hormone (1-43)OH, rat corticotropin-releasing factor, peptide histidyl isoleucine, vasoactive intestinal polypeptide, glucagon, secretin,
gonadotropin-releasing hormone, thyrotropin-releasing hormone, gastric inhibitory polypeptide, gastrin releasing peptide, cholecystokinin 8, neurotensin, substance P, somatostatin 28, SRIF, angiotensin 1, and rat GH, TSH, or prolactin. The cross-reactivity of the antiserum with human (n)-octanoylated ghrelin (1-28) was 100%. The intra- and inter-assay coefficients of variation, as determined by a pooled rat stomach extract, were 7.2 and 10.4%, respectively, at a concentration of 5 fmol/tube rat ghrelin (1-28). The dilution curves of rat stomach extract were parallel to those of the synthetic rat ghrelin standards. The sensitivity of the rat ghrelin assay was between 0.2 and 0.4 fmol/tube, with half-maximal displacement of 3 fmol/tube (IC50). Another antiserum (#104) against human ghrelin (1-28) at 1:150,000 showed binding and standard curves similar to those of AGS against ghrelin (1-11) (#106).

The highest concentration of ghrelin was found in the stomach (body 52.8 ± 6.1 fmol/mg wwt), but it was negligible in the cerebral cortex, hypothalamus or anterior pituitary (<0.03 fmol/mg wwt). These results confirm the findings of Hosoda et al. (10).

**Ghrelin immunohistochemistry in the hypothalamus and stomach**

Dense GHRH immunoreactivity was observed both within the external zone of the median eminence and neuronal perikarya in the arcuate nucleus of animals treated with colchicines (Fig. 2a). In contrast, no immunoreactivity of ghrelin was found either in the median eminence, arcuate nucleus (Fig. 2b) or cerebral cortex (data are not shown) of the same animals. This negative ghrelin immunostaining in the rat brain was confirmed with various dilutions of AGS, at 1:1,000, 1:5,000, 1:25,000 and 1:100,000 and with different fixatives, i.e. acrolein and paraformaldehyde. On the other hand,
abolished the positive staining of ghrelin in the gastric mucosa.

The present immunohistochemical findings in the rat hypothalamus with AGS specific to the bioactive N-terminus of ghrelin are different from those of a previous report by Kojima et al. (1), which utilized ghrelin antiserum specific to the bioinactive C-terminus portion of ghrelin (13-28). The differences may be explained by antisera which recognize different portions of the molecule, since there are two major forms of ghrelin, (n)-octanoylated ghrelin and bioinactive des-acyl ghrelin. The C-terminus specific antisera recognize both forms, whereas our AGS only recognizes the bioactive form of ghrelin, which is a minor form in the hypothalamus, but not in the stomach. Another explanation may be that the C-terminus specific antisera used by Kojima et al. can bind to another, yet unidentified structure(s) of the hypothalamus because an immunoneuralization study with excess amount of ghrelin was not conducted in their report.

Quantitative measurement of ghrelin mRNA in the cerebral cortex, hypothalamus, pituitary and stomach

To clarify the ghrelin gene expression in various tissues in a quantitative and more sensitive manner than conventional RIAs or immunohistochemistries, we carried out a quantitative PCR by relating the PCR threshold cycle obtained from tissue samples to amplicon-specific standard curves (Fig. 3a). As shown in Fig. 3b, ghrelin mRNA levels by the real-time PCR technique in the stomach (body, SB) and anterior pituitary (AP) were \(5 \times 10^6-1 \times 10^8\) copies and \(5 \times 10^3-2 \times 10^4\) copies/\(\mu g\) total RNA, respectively, but were undetectable (<1 \(\times 10^3\) copies/\(\mu g\) total RNA) in the cerebral cortex (CC) and hypothalamus (HT). Nass et al. reported similar mRNA quantification results by the competitive PCR technique with a different set of primers (12). Although translation of ghrelin mRNA to its peptide may vary among different immunostaining of sections of the rat stomach with AGS showed scattered cells that tended to be in the basal portion of the gastric glands, as shown in Fig. 2c. The staining was cytoplasmic and intensified in the side of the cells that faced the blind end of the gland, as reported previously (11). Excess amount of rat ghrelin (10 \(\mu g/ml\))
tissues, it seems worthy to discuss the amount of ghrelin in the hypothalamus and stomach. Granted that 1 mg wwt yields 1 μg total RNA in these tissues, numbers of ghrelin peptide molecules and mRNA copy numbers in the stomach are $3.2 \times 10^{10}$ molecules and $4.5 \times 10^7$ copies/mg wwt. The amount of ghrelin peptide in the hypothalamus and cerebral cortex would be less than $7.1 \times 10^9$ molecules/mg wwt, which is far less than the least detectable values by the most sensitive EIA ($6 \times 10^8$ molecules/mg wwt, S. Hashida and H. Katakami et al., submitted for publication). All of these results suggest that ghrelin is a gastric peptide and there is no significant gene expression of ghrelin in the hypothalamus and cerebral cortex.

**Immunoneutralization with AGS of circulating ghrelin in anesthetized and conscious rats**

Since pretreatment with AGS (0.8 ml/rat, iv, ~20 min) completely blocked the GH response to iv injection of rat ghrelin (20 μg/rat, iv), as shown in Fig. 4, the amount of antiserum used is potent enough to immunoneutralize the circulating ghrelin.

GH secretion in conscious male rats given iv or icv with NRS was pulsatile with secretory bursts, which occurred regularly every 3–4 hr, from undetectable low GH levels (<2 ng/ml) to peaks of over 100 ng/ml. The same amount of potent and specific AGS given iv or icv, however, failed to affect spontaneous occurrence of GH secretion, as shown in Fig. 5. The integrated GH secretion (AUC 1100–1800 h, ng·min/ml, mean ± SE, n=6) was not different between NRS- and AGS-treated rats (NRS iv; 7084.0 ± 1010.0,
Fig. 4  Effect of AGS on the GH release induced by ghrelin in pentobarbital-anesthetized male rats. NRS or AGS (0.8 ml) was injected iv 20 min before the iv injection of rat ghrelin. The peak GH response was obtained 10 min after ghrelin injection in NRS-pretreated rats. ** represents p<0.01 v.s. NRS. The number of animals is shown in parentheses.

v.s. AGS iv; 7539.6 ± 525.5, p>0.1, NRS icv; 7325.5 ± 753.4, v.s. AGS icv; 7417.8 ± 416.1, p>0.1), while the 1:1,000 diluted plasma from animals given iv with AGS bound to more than 70% of $^{125}$I-ghrelin in vitro throughout the experiment.

These results suggest that the ghrelin gene expresses exclusively in the stomach, and that circulating ghrelin does not play any major significant roles in the regulation of pulsatile GH secretion in the rat.

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Fig. 5  Representative spontaneous rat GH secretory profiles obtained from conscious male rats given iv (a) or icv (b) with either NRS (open circles) or AGS (closed circles). Plasma GH levels were measured every 20 min from 1000 h to 1800 h. NRS or AGS was injected iv or icv at 1040 h.
References


