Ghrelin in neonatal rats: distribution in stomach and its possible role

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Abstract

Ghrelin, a 28 amino acid peptide, has recently been isolated from the rat stomach as an endogenous ligand for the GH secretagogue receptor. The fact that administration of ghrelin, centrally or peripherally, stimulates both food intake and GH secretion suggests that stomach ghrelin has an important role in the growth of rats. We used immunohistochemistry and radioimmunoassay to determine the age at which ghrelin-immunostained cells begin to appear in the rat stomach. Ghrelin-immunoreactive cells were found to be expressed in the fetal stomach from pregnancy day 18. The number of ghrelin-immunoreactive cells in the fetal stomach increased as the stomach grew. The amount of ghrelin in the glandular part of the rat stomach also increased, in an

age-dependent manner, from the neonatal stage to adult. Eight hours of milk restriction significantly decreased the ghrelin concentration in the stomachs of 1-week-old rats, and increased the ghrelin concentration in their plasma. Administration of ghrelin to 1- and 3-week-old rats increased plasma GH concentrations. The daily subcutaneous administration of ghrelin to pregnant rats from day 15 to day 21 of pregnancy caused an increase in body weight of newborn rats. In addition, daily subcutaneous administration of ghrelin to neonatal rats from birth advanced the day of vaginal opening from 30.7 ± 0.94 to 27.9 ± 0.05 . These results suggest that ghrelin may be involved in neonatal development.

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Introduction

Growth hormone (GH) secretagogues (GHSs), which are synthetic peptides or non-peptides, stimulate release of GH from the anterior pituitary by acting on the hypothalamus (Smith *et al.* 1999). Although an endogenous GHS receptor (GHS-R) has been isolated and cloned, the endogenous GHS ligand was difficult to identify (Howard *et al.* 1996, McKee *et al.* 1997*a,b*). Recently, the peptide ghrelin was purified from rat stomach, and was identified as an endogenous ligand for GHS-R (Kojima *et al.* 1999). Ghrelin consists of 28 amino acids, including an *O*-*n*-octanoylated Ser³ residue, which is essential for GH release. An analogue of ghrelin, des-Gln14-ghrelin, has also been purified from rat stomach, and is a second endogenous ligand for GHS-R (Hosoda *et al.* 2000).

Immunohistochemical studies, including *in situ* hybridization, have revealed that, in the gastrointestinal tract, ghrelin is produced mainly from the neck to the base of the oxyntic glands, especially in the X/A-like cells, the physiological role of which is unknown (Date *et al.* 2000*a*). In addition to playing a part in GH release (Kojima *et al.*

1999, Date *et al.* 2000*b*), ghrelin is involved in various physiological functions (Tschöp *et al.* 2000, Asakawa *et al.* 2001, Date *et al.* 2001, Nakazato *et al.* 2001). We have already demonstrated that intracerebroventricular (i.c.v.) or intravenous (i.v.) administration of ghrelin gives rise to an intense orexigenic action (Nakazato *et al.* 2001). Daily treatment with ghrelin increases food intake and body weight gain in rats (Tschöp *et al.* 2000, Nakazato *et al.* 2001). It has also been suggested that, in the rat, ghrelin participates in gastric acid secretion through the vagus system (Date *et al.* 2001). These results suggest that ghrelin may have an important role in energy balance. If this is so, there is a possibility that ghrelin may be involved in neonatal development or milk intake. We therefore examined the localization of ghrelin cells and the content of ghrelin in the glandular part of the rat fetal stomach, and the effect of milk restriction on ghrelin concentrations in the peripheral blood and stomach of neonatal rats. In addition, we examined whether a single injection of ghrelin into neonates increases GH secretion, and whether daily treatment with ghrelin advances the rate of sexual maturation, for example vaginal opening.

Material and Methods

Adult male and female Wistar rats weighing about 400 g or 250 g respectively (Charles River Japan Inc., Shiga, Japan) were kept under a lighting regimen of 12 h light:12 h darkness (lights on at 0700 h) and a temperature of $23 \pm 1^\circ\text{C}$. Animals were supplied with standard laboratory chow and water, available *ad libitum*. The day when sperm was found in vaginal smears was defined as day 0 of pregnancy.

To pinpoint the location of ghrelin in the stomach, pregnant rats were decapitated at day 18 ($n=3$) or 20 ($n=4$) of pregnancy, and the fetuses and stomachs were removed. All procedures were carried out in accordance with the Japanese Physiological Society's guidelines for animal care. The tissues were immediately rinsed with saline and fixed in 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer for 2 days, then incubated in 0.1 M phosphate buffer containing 20% sucrose for 24 h at 4°C . The fetal glandular stomach was cut into sections 12 μm thick by cryostat at -20°C . The sections were then thaw-mounted on gelatin-coated glass slides and air-dried for 10 min. After pretreatment with 0.3% hydrogen peroxidase for 1 h to inactivate endogenous peroxidases, followed by incubation with normal goat serum for 1 h to block non-specific binding, all sections were incubated overnight at 4°C with anti-ghrelin antiserum. The polyclonal antibody used in this study was produced in rabbits, against the N-terminal fragment of rat ghrelin. Details of preparation and characterization of the antibody have been described by Date *et al.* (2000a). The rat anti-ghrelin antibody (antiserum no. G606) specifically recognizes ghrelin with *n*-octanoylated Ser³, and does not recognize des-acyl ghrelin. The final dilution of anti-ghrelin antiserum used in the immunohistochemistry was 1/10 000. After being washed with phosphate-buffered saline (PBS), the sections were stained by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, Vector Lab. Inc., Burlingame, CA, USA), with a diaminobenzidine (DAB) substrate kit (Vector Lab.) at room temperature. Immunostaining for ghrelin cells in the stomachs of each of four rats 1 day and 1, 3, 4 and 5 weeks old was also carried out using the methods described above. Control studies were performed with normal rabbit serum or anti-ghrelin that had been absorbed with 10 μg rat ghrelin.

To determine the content of ghrelin in the glandular part of the neonatal stomach, radioimmunoassay (RIA) of ghrelin was undertaken using a method described previously (Date *et al.* 2000a). A group of four rats was killed by decapitation every week from 1 week to 9 weeks of age. Each experiment was repeated twice. Synthetic rat [Tyr²⁹]ghrelin(1–28) was radioiodinated by the lactoperoxidase method. The iodine-125-labeled peptide was purified on a TSK ODS SIL 120A column (Tosoh Co. Ltd, Tokyo, Japan) by reverse-phase high-performance liquid chromatography. The RIA incubation buffer

was 50 mM sodium phosphate (pH 7.4) containing 0.5% bovine serum albumin (BSA) treated with *N*-ethylmaleimide, 80 mM NaCl, 25 mM EDTA, 0.05% NaN₃, and 0.5% Triton X-100. A diluted sample or the standard solution (100 μl) was incubated for 24 h with 100 μl of anti-ghrelin antiserum (final dilution 1/620 000). The tracer solution (16 000 c.p.m. in 100 μl) was added, and each mixture was incubated for 24 h. Bound and free ligands were separated by a second antibody (200 μl). All procedures were carried out at 4°C . All plasma samples were assayed in duplicate. The half-maximum inhibition of rat ghrelin(1–28) on the standard RIA curve with anti-ghrelin(1–11) antiserum (no. G606) was 3.8 fmol/tube. The dilution curves for the extracts of rat stomach paralleled both standard curves. The respective intra- and interassay coefficients of variation of RIA for the ghrelin N-terminal fragment (no. G606) were 3.5% and 3.2% at 50% binding. The recovery rates of rat ghrelin(1–28) (1 ng) and ¹²⁵I-rat ghrelin(1–28) (5000 c.p.m.) added to the tissue homogenates in the extraction performed with Sep-Pac C-18 cartridges were $92.2 \pm 0.4\%$ (mean $\pm$ S.E.M.) and $88.9 \pm 0.6\%$ (mean $\pm$ S.E.M.) respectively.

For the assessment of ghrelin concentration in peripheral blood and stomach after 8 h of milk restriction, four male pups were selected from each litter on postpartum day 7 and separated from their mothers for 8 h (from 0900 h). The remaining three nursing male pups in each group were used as controls. Each experiment was repeated twice. Immediately after peripheral blood was collected by heart puncture, the stomachs were removed. Blood was drawn into chilled polypropylene tubes containing EDTA.2Na (1 mg/ml blood), aprotinin (500 U/ml blood) and Pefabloc SC (2.4 mg/ml blood; Roche, Nutley, NJ, USA), and then centrifuged. After the plasma had been diluted twofold with 0.9% saline, it was applied to a Sep-Pak C-18 cartridge and washed with saline and 10% CH₃CN solution containing 0.1% trifluoroacetic acid (TFA). The absorbed peptides were eluted with 60% CH₃CN solution containing 0.1% TFA, lyophilized, then subjected to RIA. The stomach tissues were immediately heated at 95–100 $^\circ\text{C}$ for 5 min in a 10-fold volume of water, to inactivate the intrinsic proteases, then cooled to 4°C . CH₃COOH and HCl were then added to respective final concentrations of 1 M and 20 mM, after which the tissues were homogenized in a Polytron for 10 min. The homogenate was centrifuged at 11 500 *g* for 30 min. The supernatants were applied to a Sep-Pac C-18 cartridge, and the peptides eluted with 60% acetonitrile solution containing 0.1% TFA and lyophilized, then subjected to RIA.

To examine the effect of a single injection of ghrelin on GH secretion in neonates, six male pups at 1 and 3 weeks old were selected from each litter and were injected with 1 nmol ghrelin subcutaneously (s.c.) at 0800 h. Remaining male pups in the same litters were injected with saline. The same experiment was repeated three times. Twenty

minutes after injection, blood samples were collected after decapitation of the animal. GH was measured by radioimmunoassay as described previously (Date *et al.* 2000b).

To examine the effect of daily injections of ghrelin into pregnant rats on the body weight of newborn rats, eight pregnant rats were each injected s.c. with saline or 3 nmol ghrelin three times per day at 0800, 1300 and 1700 h. On the day of delivery, body weights of newborn rats were measured. To examine the effect of daily injections of ghrelin on the time of vaginal opening, six female pups were selected from each litter and were injected with ghrelin at 0800 h every day. Remaining female pups from the same litters were injected with saline. Two groups were used in this experiment. In one group, 0.5, 1.0 and 1.5 nmol ghrelin was injected s.c. from the day of birth to day 15, from days 16 to day 30, and from day 31 to the day of vaginal opening respectively. In the other group, 1.0 and 1.5 nmol ghrelin was injected s.c. from the day of birth to day 15 and from day 16 to the day of vaginal opening respectively. The same experiment was repeated twice. The day of vaginal opening was determined by visual inspection.

The statistical analysis was carried out by analysis of variance with the *post hoc* Fisher test.

Results

Stomach concentrations of ghrelin measured by RIA using antibody specific for the N-terminal fragment of ghrelin increased linearly from 1 week until 5 weeks of age, when they reached a plateau (Fig. 1). Immunohistochemistry revealed that cells immunostained for ghrelin were already present, albeit in very low numbers, in the gastric mucosa of the fetus on pregnancy days 18, 20 and 22. The numbers of these cells increased gradually from postnatal day 1. Ghrelin-immunoreactive cells were abundant in the bases of the oxyntic glands in 1-week-old rats, and then became widely distributed from the necks to the bases of the gastric glands at 3–5 weeks of age (Fig. 2). No immunoreactive cells were detected in the gastric mucosa of the rat stomach when normal rabbit serum (not shown) or the antisera were absorbed with excessive ghrelin (Fig. 2F). Eight-hour milk restriction significantly reduced ghrelin concentrations in the stomach ($P < 0.05$) but, in contrast, increased those in plasma ($P < 0.05$) (Fig. 3). A single injection of 1 nmol ghrelin into 1- or 3-week-old neonates caused a significant increase in GH secretion (Fig. 4A, B). The daily administration of a high dose of ghrelin (1.0 nmol ghrelin injected s.c. from the day of birth to day 15, followed by treatment with 1.5 nmol ghrelin until the day of vaginal opening) advanced the day of vaginal opening in immature female rats from day 30.7 ± 0.94 to day 27.9 ± 0.05 ($P < 0.05$) (Fig. 5A),

Figure 1 Changes in stomach ghrelin content during neonatal development. Ghrelin concentrations increased linearly until 5 weeks of age. Bars and T-markers represent mean values $\pm$ S.E.M. ($n = 8$). *Significant differences ($P < 0.05$).

without a significant increase in body weight in comparison with that in saline-treated rats ($P < 0.1$ for ghrelin compared with control; data not shown). The daily administration of ghrelin into pregnant rats during late pregnancy caused a significant increase in body weight in newborn rats (Fig. 5B).

Discussion

Ghrelin-producing cells have been detected in the rat hypothalamic arcuate nucleus and the oxyntic glands of rat and human stomach (Kojima *et al.* 1999). From their morphology and distribution, the immunostained cells in the stomach were considered to be X/A-like cells, the hormonal product of which was previously unclear (Date *et al.* 2000a). In our study, the cells that were immunoreactive for ghrelin in the fetal and neonatal stomach were similarly round and compact, with electron-dense granules in the cytoplasm, suggesting that secretion of ghrelin from the fetal stomach might already have started during late pregnancy. Recently, Lee *et al.* (2002) reported that ghrelin was not detectable in the fetal rat stomach in northern blotting analysis; however, ghrelin mRNA expression and peptide concentrations increased dramatically during the second and third postnatal weeks. The discrepancy in detection of ghrelin in fetal stomach between our data and the findings of Lee *et al.* (2002) may be due to differences of analytical method.

Figure 2 Localization of ghrelin-immunoreactive cells in the neonatal and fetal stomach. Antiserum against rat ghrelin (1–11) was used for detection. Few ghrelin cells were expressed in the gastric mucosa on pregnancy days 18 (A), 20 (B), or 22 (C). The ghrelin-positive cells (black cells) were distributed mainly in the bases of the oxyntic glands in 1-week-old pups (D), but by 5 weeks of age (E) they were more widespread, appearing from the necks to the bases of the oxyntic glands. (Photographs for rats at 3 and 4 weeks of age have been omitted, as they were almost identical to those for rats of 5 weeks of age). Scale bars: A–F, 0.1 mm; A'–E' are 2 × magnifications of A–E respectively. No immunoreactive cells were detected in the gastric mucosa of the rat stomach when the antisera were absorbed with excessive ghrelin (F' compared with F).

These observations prompted us to ask why ghrelin is synthesized in the fetal and neonatal stomach. Ghrelin has so far been shown to have several physiological roles. Firstly, it is likely that the most typical role of ghrelin is stimulation of GH secretion (Kojima *et al.* 1999, Date *et al.* 2000b, Arvat *et al.* 2000). Administration of ghrelin either centrally or peripherally increases GH secretion in a dose-dependent manner in rats and humans. Secondly, ghrelin increases food intake. I.c.v. injection of antiserum to ghrelin before exposure to a 12-h period of darkness decreases food intake for the full 12 h of darkness, suggesting that endogenous ghrelin is involved in the regulation of

daily food intake (Nakazato *et al.* 2001). It has also been reported that the stimulatory effect of ghrelin on food intake is due to the stimulation of neuropeptide Y and agouti-related peptide (Nakazato *et al.* 2001). Thirdly, ghrelin stimulates gastric acid secretion. I.c.v. injection of ghrelin increases gastric acid output in a dose-dependent manner in urethane-anesthetized rats, and this stimulation is blocked by vagotomy or administration of atropine (Date *et al.* 2001). As the fetus has no need to take food orally, it is possible that ghrelin acts directly on the fetal pituitary gland and stimulates GH secretion. In the present study, it was confirmed that ghrelin also stimulates GH secretion

Figure 3 Effect of 8-h milk restriction on stomach and plasma ghrelin concentrations in 1-week-old pups. White and black bars and T-markers represent mean $\pm$ S.E.M. stomach and plasma concentrations of ghrelin respectively ($n=6-8$). *Significant differences ($P<0.05$).

in 1- and 3-week-old neonates. Recently, it has been reported that the effect of peripheral administration of ghrelin on GH secretion is blocked by vagotomy, and that the neuronal activity of the vagus efferent is affected by i.v. injection of very small amounts (fmol) of ghrelin (Asakawa *et al.* 2001). It has been hypothesized that peripheral ghrelin may act on peripheral sensors, so that the afferent signal then stimulates GH secretion. The existence of peripheral sensors for ghrelin may be indicated by the fact that GHS-R mRNA is distributed in various peripheral organs, such as the stomach and intestine (Guan *et al.* 1997, McKee *et al.* 1997b, Date *et al.* 2000a). If this hypothesis is correct, then the presence of a small amount of ghrelin in the fetal and neonatal stomach may stimulate GH secretion. In our study, stomach ghrelin content increased with growth postpartum. This observation is consistent with the distribution of cells that are immunoreactive for ghrelin, because the distribution of these cells spreads from the neck to the base of the gastric gland as the neonate ages. Increased concentrations of ghrelin in the stomach during infancy may be related to increased gastric acid output, GH secretion and milk intake. An 8-h restriction of milk caused an increase and decrease in ghrelin concentrations in the blood and stomach respectively. Probably, this reciprocal pattern of ghrelin

Figure 4 Effect of a single s.c. injection of ghrelin on GH secretion in (A) 1-week-old and (B) 3-week-old pups. Bars and T-markers represent mean values $\pm$ S.E.M. ($n=18$). Note that the scale on the Y axis differs between (A) and (B). *Significant differences ($P<0.05$).

concentrations between blood and stomach indicates that ghrelin is secreted from stomach to blood in neonatal rats. This finding also suggests that ghrelin in the blood may be involved in stimulating the appetite in order to increase milk intake.

The fact that daily injection of ghrelin advanced the day of vaginal opening by about 2 days without an associated significant increase in body weight suggests that ghrelin may also be involved in sexual maturation. We previously reported that continuous treatment with ghrelin increased body weight, in addition to food intake, in adult rats (Nakazato *et al.* 2001), and we do not know why daily injection of ghrelin did not increase the body weight in neonatal rats. However, neonatal rats would never be able to take an excess of milk, even if their appetite was stimulated by ghrelin. Further studies are required to elucidate the mechanism of ghrelin in sexual maturation and the relationship between ghrelin and the reproductive axis. The daily administration of ghrelin into pregnant rats

Figure 5 Effect of daily injection of ghrelin on the day of vaginal opening in immature rats (A), and effect of daily injection of ghrelin into pregnant rats on the body weight of newborn rats (B). (A) Ghrelin (L): 0.5, 1.0 and 1.5 nmol ghrelin were injected s.c. from the day of birth to day 15, from day 16 to day 30, and from day 31 to the day of vaginal opening respectively. Ghrelin (H): 1.0 and 1.5 nmol ghrelin were injected s.c. from the day of birth to day 15 and from day 16 to the day of vaginal opening respectively. The Y axis represents the day of vaginal opening. (B) Ghrelin 3 nmol was injected s.c. three times per day from day 15 to day 22 of pregnancy. Bars and T-markers represent mean values $\pm$ S.E.M. ($n=12$). *Significant differences ($P<0.05$).

caused an increase in body weight of newborn rats, suggesting that, if blood concentrations of ghrelin in pregnant rats increase during late pregnancy, this may influence fetal body weight. In this study, it was unclear whether ghrelin injected into the mother directly affected the fetus through the placenta, or if ghrelin-induced GH in the mother indirectly affected fetal body weights. Nevertheless, the present study demonstrates that ghrelin may have an important role in neonatal development, including sexual maturation, in rats.

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