EXPERIMENTAL STUDY

Influencing the between-feeding and endocrine responses of plasma ghrelin in healthy dogs

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Abstract

Objectives: Ghrelin has recently been isolated from rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagog receptor. Using beagle dogs, we investigated the distribution of ghrelin in the stomach and its possible role.

Methods: We examined: (i) GH release in response to ghrelin injection (0.5 or 5 µg/kg, i.v.), (ii) gastric localization of ghrelin-immunostained cells, (iii) changes in daily food consumption after ghrelin injection (3, 10, and 20 µg/kg, i.v.), (iv) plasma ghrelin levels under regular, but restricted feeding conditions, and (v) variations in plasma ghrelin levels in relatively lean, normal and obese dogs.

Results: Administration of ghrelin to dogs promptly increased circulating GH concentrations, although this effect was transitory and was maintained for only 20 min. Ghrelin was localized in the stomach fundus and body, but none was detected in either the pylorus or cardia. Administration of ghrelin at a dose of 20 µg/kg increased the daily food intake of beagle dogs. Plasma ghrelin levels peaked just before meal times, and then returned to basal levels. Obese dogs had higher plasma ghrelin levels than did normal and lean dogs.

Conclusions: These results indicate that ghrelin is a potent GH secretagog in dogs. The distribution of ghrelin-immunoreactive cells in the canine stomach resembles that of both the murine and human stomach. Ghrelin participates in the control of feeding behavior and energy homeostasis in dogs and may, therefore, be involved in the development of obesity.

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Introduction

Obesity and anorexia have become serious problems in companion animals such as dogs and cats, as well as in humans. Both obesity and anorexia are characterized by abnormal feeding – in the former case, hyperphagia, and in the latter, hypophagia. Recent molecular biological approaches have led to advances in research on the mechanisms of feeding regulation, such as the discovery of new peptides that regulate feeding behavior. In the near future these peptides may be useful as clinical pharmacological substrates in the treatment of obesity and anorexia. Ghrelin, which is one of these candidate substances, was recently isolated from the rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagog receptor (1). Ghrelin consists of 28 amino acids, including an O-n-octanoylated Ser3 residue that is essential for GH release. Central and peripheral injections of ghrelin stimulate GH release in many species such as rats, humans, Shiba goats, dogs and fish (1–6).

Immunohistochemical studies, including those involving in situ hybridization, have revealed that in the gastrointestinal tract, ghrelin is produced mainly from the neck to the base of the oxyntic glands, in particular in the X/A-like cells (1, 7) whose physiological role was previously unknown. This distribution of ghrelin in the stomach has been confirmed in humans, cows, pigs and horses (4, 7). The common action and distribution of ghrelin in many species suggests that the structure of ghrelin is conserved among species.

Remarkable work carried out in recent years has shown that ghrelin has various physiological functions such as stimulation of food intake, gastric acid secretion, and gastric motor activity (8–11). It is likely that the appetite-stimulating effect of peripheral ghrelin is due to its action on the afferent vagal nerve (10). On the other hand, its central effect is thought to occur via the secretion of neuropeptide Y and agouti-related peptide from the arcuate nucleus in the hypothalamus (11). These results suggest that ghrelin plays an important role in the regulation of food intake and energy expenditure in rats (9, 11, 12) and humans (13). Ghrelin levels in rats exhibit a diurnal pattern, with bimodal peaks occurring before dark and light periods (14). These two peaks are consistent.
with maximum and minimum volumes of gastric content respectively. It has been suggested that this preprandial rise and postprandial fall in circulating ghrelin levels also occurs in humans (15). Moreover, it has been shown that a transient surge in plasma ghrelin occurs in the prefeeding period in scheduled meal-fed sheep (16). These results indicate strongly that ghrelin secretion may be a trigger for endogenous hunger signals. Since the continuous administration of ghrelin to rodents results in fat deposition and obesity, ghrelin may also be involved in the development of both lean and obese conditions (12, 17).

We have, therefore, examined the relationship between ghrelin and food intake in the beagle dog. Ghrelin secretion should be affected by feeding behavior, but little is known about ghrelin-induced appetite stimulation in dogs. First, we examined whether ghrelin stimulates GH release, and whether ghrelin-immunostained cells are localized in the dog stomach. Secondly, we investigated whether intravenous (i.v.) administration of ghrelin increases food intake in dogs, and determined peripheral ghrelin levels before and after feeding. Finally, we measured and then compared the plasma ghrelin levels in lean, normal and obese dogs.

Materials and methods

Animals and experimental design

All procedures were performed in accordance with the Japanese Physiological Society’s guidelines for animal care. Healthy adult male and female beagle dogs were used for this study. The animals were housed individually in a roofed enclosure in cages (1.0 m × 0.8 m × 0.6 m) at ambient temperature and under natural photoperiod conditions.

In the first experiment, we used 12 healthy male beagle dogs (Kitayama Labs, Yamaguchi, Japan) weighing approximately 6.0–8.3 kg (age range 7–9 months; median 7.9 months), in which we evaluated the effect of ghrelin administration on plasma GH levels. The dogs were divided randomly into three experimental groups: a saline-treated (0.5 ml/kg) control group, and two groups that were administered rat synthetic ghrelin (Peptide Ins. Inc. Osaka, Japan) by i.v. injection, one group at a dose of 0.5 µg/kg and the other at 5 µg/kg. Samples of blood (2 ml) were drawn from conscious animals into tubes containing disodium ethylenediaminetetraacetic acid (EDTA; 1 mg/ml blood) via puncture of the jugular vein using sterile needles and syringes, before injection and 10, 20, 40, 80, and 160 min after injection. Plasma was separated by centrifugation and was kept at −80°C until determination of ghrelin levels.

In the second experiment, the immunohistochemical localization of ghrelin in the dog stomach was examined. The Animal Hospital of Miyazaki University supplied the stomach, esophagus, and duodenum from three adult beagle dogs (age range 8–9.3 months, mean 8.6 months) after euthanasia induced by an overdose injection of pentobarbital.

In the third experiment, we studied the regulation of food intake by ghrelin in six male beagle dogs (6.9–10.2 kg; age range 6–9 months; median 7.4 months). The animals were fed a sufficient quantity of commercial canine laboratory diet (DS-A; Oriental Yeast, Chiba, Japan), and water was available ad libitum. Food consumption was measured at 0900 h every 24 h for 10 consecutive days – the pre-experiment period. Each animal then received an i.v. injection of saline and then ghrelin in the order of dosage of 3, 10, and 20 µg/kg ghrelin at 3-day intervals. Food consumption was measured in the 24 h after each injection in each dog. The 3-day interdose interval and the increasing order of dose were imposed to avoid any potential carry-over effects of individual doses.

In the fourth experiment, 12 male beagle dogs (8.6–11.2 kg; age range 7–10 months; median 8.2 months) were randomly subdivided into 2 mealtime groups that were fed a restricted diet at 1000 or 1700 h, regularly for 10 days. On the 10th day, we collected blood samples from each dog at 0730, 0930, 1100, 1430, 1630 and 1930 h, into chilled tubes containing disodium EDTA (1 mg/ml blood) and aprotinin (500 U/ml blood). Plasma was separated by centrifugation, and we added 10% plasma volume of 0.1 mol/l HCl. Samples were stored at −80°C until determination of ghrelin levels.

In the fifth experiment, 28 adult female beagle dogs (age range 8–13 months; median 9.6 months) were chosen from 290 dogs by 8 keepers, and divided into 4 groups under the randomized block design: relatively lean ‘lean’, normal ‘normal’, relatively light obese ‘obese L’, and relatively heavy obese ‘obese H’, and each group had mean ± S.E.M. body weights of 7.2±0.2 kg (P < 0.05 vs normal), 10.1±0.2 kg, 12.4±0.4 kg (P < 0.05 vs normal) and 15.9±1.2 kg (P < 0.05 vs obese L) respectively. In addition, to compare the gender difference of plasma ghrelin levels, we measured the plasma ghrelin levels in each of four male and female beagle dogs at about 7 weeks of age. We collected blood samples from all of these dogs after they were fasted overnight. Sampling was carried out as described earlier and the plasma was stored at −80°C until determination of ghrelin concentrations.

RIA of GH and ghrelin

We developed an RIA system for canine GH measurement by using a canine GH RIA kit supplied by NIDDK (National Hormone and Peptide Program, Harbor-UCLA, CA, USA). Iodination was performed by the chloramine-T method. The second antibody was goat anti-monkey IgG serum (HAC-MKA2 – 02GTP88), which was supplied by the Biosignal Research Center.
Institute for Molecular and Cellular Regulation, Gunma University, Japan. After completion of the kit protocol, 25 μl plasma were diluted with 175 μl assay buffer for use in the assay. All samples were analyzed in duplicate within one RIA, and the minimum detectable mass was 0.25 ng/ml. The assay procedure was performed according to the method described by Hayashida et al. (4). The intra- and interassay coefficients of variation were 6.4% and 3.9% respectively.

Plasma ghrelin levels were measured with a ghrelin (human) RIA kit (Linco Research, St Louis, MO, USA). Without exception, all assay procedures were carried out in accordance with the protocol supplied by the manufacturer. Since this RIA was for use with rat and human plasma, we verified that dog plasma contained suitable matrices. The RIA technique detected rat and human ghrelin with equal accuracy in dog. The intra- and interassay coefficients of variation were 5.1% and 2.4% respectively. The sensitivity of the assay was 10 pg/ml.

**Immunohistochemical staining**

Tissue blocks of the canine stomach, esophagus and duodenum, taken from a total of three dogs, were rinsed with ice-cold saline and fixed in 4% paraformaldehyde plus 0.2% picric acid in 0.1 mol/l phosphate buffer for 2 days, then incubated for 24 h at 4°C in 0.1 mol/l phosphate buffer containing 20% sucrose. Samples were then frozen and stored at −80°C until immunohistochemical staining. Immunostaining for ghrelin in cells in the cardia, fundus, body, and pylorus of the stomach, and in the esophagus and duodenum of each dog was performed as follows. Ten-micrometer-thick sections were prepared with the aid of a cryostat at −20°C and were then thaw-mounted onto gelatin-coated glass slides and air-dried for 10 min. After pretreatment with 0.3% hydrogen peroxidase for 1 h to inactivate endogenous peroxidases and then incubation with normal goat serum for 1 h to block nonspecific 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 the preparation and characterization of the antibody have been described by Date et al. (7). The rat anti-ghrelin antibody specifically recognizes ghrelin with n-octanoylated Ser3, and does not recognize des-acyl ghrelin. The final dilution of the anti-ghrelin antiserum used in the immunohistochemistry was 1:10000. After being washed with phosphate-buffered saline, the sections were stained by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA), with a diaminobenzidine substrate kit (Vector Laboratories) at room temperature.

Data are expressed as the mean ± S.E.M. Statistical analyses were carried out by Student’s t-test, or one-way analysis of variance and Tukey’s post hoc test, as required.

**Results**

**GH release in response to ghrelin injection**

Plasma GH levels increased in a monophasic manner after a single ghrelin injection (Fig. 1). An i.v. administration of 5 μg/kg ghrelin led to a peak GH value of 15.45 ng/ml 10 min later, a value that is eight times higher than the preinjection level. The GH release response to ghrelin was significantly increased both 10 and 20 min after injection (P < 0.05). GH levels had returned to basal values by 40 min after injection. Administration of a dose of 0.5 μg/kg had no significant effect on plasma GH concentrations.

**Distribution of ghrelin-immunostained cells**

We detected ghrelin-immunoreactive cells in the oxyntic glands of the fundus and body of the stomach (Fig. 2). In accordance with the physiological role and the conformation changes of acid secretion that occur in the dog stomach, the scatter of ghrelin-positive cells was higher in the fundus than in the body of the stomach. Ghrelin-positive cells were restricted to the gastric mucosa and were scattered from the glandular base to the glandular neck. A comparison with hematoxylin and eosin stained sections of the same tissue revealed that these cells resemble endocrine cells, with unstained nuclei and dense granules in the cytoplasm. No immunostained cells were detected in the esophagus, cardia, pylorus, or duodenum (data not shown). All of these findings were confirmed in tissues taken from all three animals.

**Change of daily food consumption after ghrelin injection**

Figure 3 shows the effects of three different doses of ghrelin (3, 10, and 20 μg/kg) on food intake measured...
24 h after the injections. The average daily food intake was increased significantly by injection of 20\( \mu \)g/kg ghrelin compared with saline treatment (Fig. 3). Food consumption gradually increased from 75 g/day up to 125 g/day after injections of 3, 10 and 20\( \mu \)g/kg ghrelin, but only the highest dose resulted in a statistically significant change.

**Ghrelin response to feeding**

Restricting feeding times to 1000 or 1700 h resulted in the same peak patterns of plasma ghrelin levels. The peaks were observed just before feeding, at 0930 h and at 1630 h (Fig. 4). After feeding, plasma ghrelin levels immediately fell and then remained almost constantly low throughout the day.

**Variations in plasma ghrelin levels in lean, normal and obese dogs**

Relatively higher and lower plasma concentrations of ghrelin were observed in relatively light obese and lean dogs respectively (Fig. 5). Although no significant difference was observed between lean and normal or between relatively light obese and normal, relatively heavy obese dogs showed a significant increase in plasma ghrelin levels in comparison with normal and lean dogs. There was no significant difference in plasma ghrelin levels between normal female and male dogs (female 476\( \pm \)59.8 vs male 497\( \pm \)98.3, mean\( \pm \)S.E.M. \( n = 4 \)).

**Discussion**

Injection of ghrelin at a dose of 5\( \mu \)g/kg body weight caused GH secretion to rise to eight times higher than basal levels by 10 min after injection. The time course of changes in GH secretion was close to that recorded in previous reports (5). The cDNA encoding the dog ghrelin precursor has been sequenced (18), and it has been shown that there is only one amino acid sequence difference between rat and canine ghrelin. Our study demonstrates that rat synthetic ghrelin is able adequately to stimulate GH secretion in the dog, and that endogenous ghrelin may play an important role in GH secretion in this animal. The GH response to stimulation with ghrelin

*Figure 3* Effect of a single i.v. injection of ghrelin on daily food intake in dogs. Daily food intake increased after treatment with ghrelin. Bars and vertical lines represent the mean values\( \pm \)S.E.M. \( n = 6 \). \( \ast P < 0.05 \), significantly different from the saline-treated group. BW, body weight.

*Figure 4* Influence of daily feeding time on plasma ghrelin levels in dogs. The white and black bar represents the light and dark periods. Areas a and b represent the meal time in group a and group b respectively. Plasma ghrelin peaked just before the restricted daily feeding time at 1000 h (group a; closed circles) and 1700 h (group b; open circles) and then decreased immediately after the end of feeding. Symbols and vertical lines represent the mean value\( \pm \)S.E.M. \( n = 6 \).
recorded in this study was considerably lower than that reported previously in healthy dogs in response to a dose of 2 μg/kg (5). Indeed, it has been shown that ghrelin strongly stimulates GH release in humans, whereas in dogs, either ghrelin is not a very potent stimulator of GH release or else the results reflect methodological differences in the assays used to determine GH levels.

The immunohistochemical studies carried out in the present investigation revealed that ghrelin-immuno- stained cells were detected in the oxyntic glands of the canine stomach. They are particularly abundant in the fundus and body of the stomach, and are entirely lacking in the pylorus, cardia, esophagus and duodenum. This pattern of distribution correlates well with that of the expression of the ghrelin precursor, as determined by Northern blotting analysis of dog tissues (18). The dog gastric mucosa has been investigated at the electron microscope level by Rindi et al. (19), who found that murine and canine ghrelin-immunoreactive cells closely resemble those of the human stomach in their general ultrastructure, including the structural patterns of their compact granules. It seems, therefore, that there is some structural homogeneity of these cells among species, and we can therefore probably expect them to have a functional homogeneity similar to that of endocrine cells among various animals, including the dog.

Daily treatment with ghrelin or a single injection increases food intake and body weight gain in both rats (9, 11, 12) and humans (13). However, little is known about ghrelin-induced appetite stimulation in dogs. We have found that administration of ghrelin induces an increase in food intake, suggesting that endogenously released ghrelin is involved in the control of daily food intake. We observed that plasma ghrelin levels increase just before a meal time, and then rapidly return to the basal level after the end of feeding. In free-feeding rats, ghrelin secretion follows a diurnal pattern, with bimodal peaks occurring before the dark and light periods (14). Both peaks are consistent with the periods just before feeding, in accordance with the circadian rhythms of rats. Moreover, it has been shown that a transient surge of plasma ghrelin occurs in the prefeeding period in scheduled meal-fed sheep (16). When sheep are fed two or four times a day, the ghrelin levels rise just before each feeding (20). Plasma ghrelin levels showed a nocturnal rise that exceeded the meal-associated increase in lean human (21). These results indicate that the ghrelin secretory response to feeding in dogs is similar to that of sheep, rodents and human. A transient surge of ghrelin secretion has also been observed just before pseudofeeding in sheep (16). The regulation of this secretion seems to be complicated by the influences of the gastric contents, gastric acid secretion, and the central nervous system via the vagus nerve (22), since ghrelin signals may be involved centrally and/or peripherally via the gut-brain axis. It would be worthwhile examining further the stimulation of food intake by ghrelin and how ghrelin levels are regulated under conditions of negative and positive energy balance, such as during feeding.

From our results and those of these other studies it seems that ghrelin is involved in the regulation of eating behavior and energy metabolism in both the acute and chronic feeding states (13, 15, 23, 24). Circulating plasma ghrelin levels in healthy dogs decreased significantly after eating. Since this suggests that eating behavior influences the secretion of ghrelin, we examined ghrelin secretion in relatively lean, normal, and obese dogs to determine the ghrelin status in obesity. The obese dogs had higher plasma concentrations of ghrelin than did the lean dogs. These results contrast with those published previously for obese humans, in whom significantly lower ghrelin levels have been detected (25). Several studies have demonstrated that plasma ghrelin levels are inversely correlated with the body mass index (26 –29), suggesting that ghrelin levels are downregulated in obesity. On the other hand, our results in dogs are in agreement with the ghrelin secretion patterns recorded in lean and obese Zucker rats, which show, respectively, low and high ghrelin levels in plasma (30). The Zucker fa/fa rat is a widely used model of obesity that is characterized by massive obesity, overeating, and alterations of growth hormone metabolism. Whether or not obesity can be linked to plasma ghrelin levels needs to be clarified by further characterization of the pathophysiology of obesity in dogs. Recently, abnormal circulating ghrelin levels have been reported in patients with anorexia nervosa (31) and Prader–Willi syndrome (26). Studies such as these will help us to understand whether ghrelin plays a role in the pathogenesis of simple or secondary obesity in humans, and the use of dogs with obesity-associated disease could prove an interesting approach.
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References

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