Physiological role of ghrelin in dogs

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General Introduction

Obesity, a condition already at epidemic proportions (the prevalent population across the USA, Europe and Japanese markets reached 114 million in 2003) is largely attributable to an indulgent lifestyle and is forecast to grow approximately 20% by 2013 (reaching 140 million). In most adults, adiposity and body weight are remarkably constant despite huge variations in daily food intake and energy expended. A powerful and complex physiological system exists to balance energy intake and expenditure, composed of both afferent signals and efferent effectors. This system consists of multiple pathways which incorporate significant redundancy in order to maintain the drive to eat. In the circulation, there are both hormones which act acutely to initiate or terminate a meal and hormones which reflect body adiposity and energy balance. These signals are integrated by peripheral nerves and brain centres, such as the hypothalamus and brain stem. The integrated signals regulate central neuropeptides, which modulate feeding and energy expenditure. This energy homeostasis, in most cases, regulates body weight tightly. However, it has been argued that evolutionary pressure has resulted in a drive to eat without limit when food is readily available. The disparity between the environment in which these systems evolved and the current availability of food may contribute to over-eating and the increasing prevalence of obesity. Naturally occurring mutations, as well as ablative lesions, have shown that the brain regulates both aspects of energy balance and that abnormalities in energy expenditure contributes to obesity. Indeed, early evidence pointing towards a model for hypothalamic control of energy balance came from specific brain lesion experiments, either through physical or chemical destruction of neurones. In particular lesions of the ventromedial hypothalamic nucleus (VMH), resulted in obesity, whilst lesions of the lateral hypothalamic area (LHA) lead to anorexia and weight loss(27). From this first conception, it is now understood that sensory

information from the upper gastrointestinal tract, abdominal viscera and taste information from the oral cavity (85) are all integrated in the nucleus of the tractus solitarius (NTS), an area in the caudal brainstem. Satiety-inducing signals, initiated by mechanical or chemical stimulation of the stomach and small intestine, neural-inputs related to energy metabolism in the liver (30) and humoral signals also converge on the NTS via ascending vagal fibres from the spinal cord (64). Afferent fibres then carry the signals to the hypothalamus and other forebrain regions. Needless to say, the pharmacological potential of several endogenous peripheral peptides released prior to, during and/or after feeding are being explored.

The physiological regulation of energy homeostasis is critical to an animal's long-term survival and is relevant to the companion animals in relation to animal production and welfare. In various farm animals, the regulation of food intake and energy balance has been a focus of research interest and has been the subject of several recent reviews. Energy balance is a metabolic state that exists when total body energy expenditure equals dietary energy intake. The regulation of food intake can be divided into short-term and long-term components. In mammals, rapid developments have been made over the past 10 years in identifying key neurochemicals and neural pathways involved in energy homeostasis.

Even though this energy balance is usually fine-tuned, in some individuals there is an imbalance between energy intake and expenditure, resulting in obesity. In fact, obesity and excess body weight are the most common nutritional disorders encountered in small animal medicine. They are estimated to affect approximately 25% to 44% of dogs receiving veterinary care in Western countries (46,54). Obesity develops when energy intake consistently exceeds daily energy expenditure. Numerous environmental and social factors contribute to the formation of obesity. These include lack of exercise, overfeeding, or unbalanced diet. Ad libitum

feeding of a high-fat diet, for example, is a well-known factor of obesity development (40). Genetic factors such as breed or physiological factors such as neutering have been associated with an increase in the risk of obesity. Medicines (eg. Corticosteroids and progesterons) or endocrine abnormalities have also been implicated as causes of obesity.

Recent molecular biological approaches have advanced our knowledge of the mechanisms involved in the regulation of feeding. An interdisciplinary approach in discovery research has led to the identification and characterization of many peptidyl endocrine factors in the last decade. Leptin (100), agouti related protein; AgRP (57), orexin (66) and galanin like peptide; GALP (55) are examples of such peptidyl factors, each of which is known to be involved in the regulation of diverse physiological functions in mammals. In December 1999, information about the isolation, characterization and some in vitro and in vivo biological actions of ghrelin were first reported (42). The rapidly growing literature on ghrelin indicates that it is a multifunctional hormone (44,90).

Ghrelin is a 28 amino-acid acylated peptide which was first isolated from the rat stomach, where it was localized mainly from the neck to the base of the oxyntic gland of rat and human stomachs and characterized to the neuroendcrine X/A-like cells of the gastric mucosa (20,42). This peptide has been identified as an endogenous ligand for the growth hormone (GH) secretagogue receptor, and has been shown to regulate GH release from the pituitary gland in vivo and in vitro (21,42,69,97). However, there are a few reports on the expression in ghrelin in the stomach and GH-releasing action of ghrelin by using healthy dogs (8,63). In addition, evidence from many species indicates that ghrelin exerts a variety of actions, affecting energy balance (38), gastrointestinal motility and secretion (48) and feeding behavior (94). The central orexigenic effects of ghrelin are independent on growth hormone stimulation and appear to be

mediated, at least in part, through activation of neuropeptide Y /agouti-related peptide hypothalamic neurons (38). Administration of exogenous ghrelin increases neuropeptide Y gene expression and blocks leptin-induced feeding reduction, thus implying a possible competitive interaction between ghrelin and leptin in feeding regulation (52).

In mammals, ghrelin homologs have been identified in human (42), rhesus monkey (1), rat (42), mouse (79), mongolian gerbil (GenBank accession no. AF442491), cow (GenBank accession no. AB035702), pig (GenBank accession no. AB035703), sheep (GenBank accession no. AB060699), and dog (84). The amino acid sequences of mammalian ghrelins are well conserved; in particular, the 10 amino acids in their NH2 termini are identical. This structural conservation and the universal requirement for acyl-modification of the third residue indicate that this NH2-terminal region is of central importance to the activity of the peptide. Bovine and ovine ghrelins are 27-amino acid peptides that, like rat des-Gln14 ghrelin, lack the Gln14 residue. In the genes encoding these ghrelins, there is only one AG splice acceptor site between exons 2 and 3, resulting in the production of only one mRNA that gives rise the 27-residue ghrelin. Ghrelin immuno-positive cells were abundant from the neck to the base of the oxyntic glands of cows, sheep, pig and horse stomachs as well as rats (35). The plasma ghrelin concentration in cows decreased significantly after feeding and then recovered to pre-feeding levels such as rats and humans. Moreover, it has been shown that a transient surge of plasma ghrelin occurs in the prefeeding period in scheduled-meal-fed sheep (75).

GH produced by somatotrophs of the adenohypophysis is the anabolic hormone crucial for long bone growth, muscle accretion, energy homeostasis and the metabolism of proteins, sugars, fats, and minerals in mammals. A series of stimulatory and inhibitory releasing hormones of hypothalamic and peripheral origins controls the pulsatile release of GH from somatotrophs.

Until recently, the consensus was that two antagonistic hypothalamic peptides: a stimulatory GH-releasing hormone (GHRH) and an inhibitory somatostatin-14 (sst or SRIF) controlled the pulsatile pattern of GH secretion. GH participates in its own rhythmic secretion through feedback action on GHRH and SRIF neurons (14). GHRH and SRIF receptors belong to the family of seven transmembrane receptors coupled to a heterotrimeric GTP-binding protein. SRIF receptor and subtypes are coupled to a Gi protein and its activation inhibits adenylate cyclase. The GHRH receptor is coupled to a Gs protein and its activation stimulates adenylate cylase activity that results in increased intracellular cyclic AMP and protein kinase A levels. GHRH, a 1–44 amino-acid peptide, and its analogs (i.e., human pancreatic GRF [hp GRF(1, 40)OH; Nle27 rGRF(1, 29)NH2; rhGRF(1–32)OH] are potent releasers of GH in vivo in cattle (59,60) and pigs (36). Somatic growth in vertebrates is thought to be dependent on pituitary GH; without pituitary GH production or peripheral GH action, postnatal growth is severely stunted (31,89). The hypothalamus regulates episodic GH secretion from the pituitary partly by its endogenous release of GHRH, SRIF, ghrelin, GH and other hypothalamic neuropeptide hormones affecting feeding behavior and satiety (12,73,82). The neurohypophyseal link between the hypothalamus and the pituitary is essential for connecting these releasing and inhibiting hormones affecting endogenous GH secretion (31,36). In the young animal, episodic GH secretion occurs during stages of rapid growth and wanes during maturity and senescence. Although aging animals and humans lack robust episodic GH secretion, the pituitary is fully capable of responding to GHRH or GHS challenge with supraphysiological GH release. In vivo approaches such as stalk sectioning, hypothalamic deafferentation, and hypophysectomy provide strong evidence for the neuroendocrine control of GH release. Less clear is the role of episodic GH release in long-term growth.

On the basis of such findings, I propose that ghrelin alters appetite and energy metabolism in dogs, and the extent to which the physiological mechanisms involved have been conserved between dogs and human will be considered. I examined that, including the effects of ghrelin administration, distribution of ghrelin in the stomach and its possible role in beagle dogs. I also examined a possible interactive role for ghrelin and GH in juvenile beagle dogs.

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

Abstract

Ghrelin has recently been isolated from rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagog receptor. Using beagle dogs, I investigated the distribution of ghrelin in the stomach and its possible role. RIA for plasma canine ghrelin was validated. 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. Obesity resulted in a significant increase in plasma free fat acid, cholesterol and high density lipoprotein cholesterol. 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.

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 (42). 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 (8,21,35,41,42,69).

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 (20,42) whose physiological role was previously unknown. This distribution of ghrelin in the stomach has been confirmed in humans, cows, pigs and horses (20,35). The common action and distribution of ghrelin in many species suggests that the structure of ghrelin is conserved among species.

Although the structure of canine ghrelin is very close to rat ghrelin except one amino acid, assay system of canine ghrelin has not been established. It is likely that anti-rat ghrelin antiserum recognize the canine ghrelin, because anti-rat ghrelin antiserum used in radioimmunoassay is polyclonal antibody. Therefore, at first, I examined whether the dilution carve for canine serum is parallel to dilution curve for rat serum in standard curve for rat ghrelin. If it is parallel, I will be able to measure the canine ghrelin using the assay system of rat ghrelin.

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 (23,48,52,94). It is likely that the appetite-stimulating effect of peripheral ghrelin is due to its action on the afferent vagal nerve (23). 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 (52). These results suggest that ghrelin plays an important role in the regulation of food intake and energy expenditure in rats (52,86,94) and humans (92). Ghrelin levels in rats exhibit a diurnal pattern, with bimodal peaks occurring before dark and light periods (51). These two peaks are consistent with maximum and minimum volumes of gastric content respectively. It has been suggested that this pre-prandial rise and postprandial fall in circulating ghrelin levels also occurs in humans (17). Moreover, it has been shown that a transient surge in plasma ghrelin occurs in the prefeeding period in scheduled meal-fed sheep (75). 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 (86,93).

As the criterion for evaluation of lean and obesity in dog has not been established, relative lean and obesity beagle dogs were estimated by significant differences (average body weight ± two fold standard deviation vs normal dogs) from normal body weight. However, it seems to be important to know the distribution of body fat condition in lean and obesity dogs. Evaluation of the effect of obesity on the measurement of blood lipids and lipoproteins is important because hyperlipemia can result in interference with blood parameters. I measured some indexes for body fat condition, such as triacylglycerol, free fat acid, cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol in normal lean and obesity beagle dogs.

I 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, I examined whether ghrelin stimulates GH

release, and whether ghrelin-immunostained cells are localized in the dog stomach. Secondly, I investigated whether intravenous (iv) administration of ghrelin increases food intake in dogs, and determined peripheral ghrelin levels before and after feeding. Finally, I 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 x 0.8 m x 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 7.8 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 ethyl-enediaminetetraacetic 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 GH. Plasma GH was measured by radioimmunoassay (RIA) without extraction.

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 months of age. I 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

I 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. (35). The intra- and interassay coefficients of variation were 6.4% and 3.9% respectively.

RIA of ghrelin

RIA of ghrelin was performed by the method described by Hosoda et al (39). Synthetic rat [Tyr^{29}]-ghrelin [1–28] was radioiodonated by the lactoperoxidase method. The ^{125}I-labeled peptide was purified on a TSK ODS SIL 120A column (Tosoh Co. Ltd., Tokyo, Japan) by RP-HPLC. The radioimmunoassay incubation buffer was 50 mM sodium phosphate (pH 7.4) containing 0.5% canine serum albumin treated with N-ethylmaleimide, 80 mM NaCl, 25 mM EDTA, 0.05% NaN_3, and 0.5% Triton X-100. A diluted sample or standard solution (100 μl) was incubated for 24 h with 100 μl anti-ghrelin [1–11] antiserum (final dilution 1/620000). The tracer solution (16000 cpm in 100 μl) was added, and the mixture was again incubated for 24 h. Bound and free ligands were separated by a second antibody (200 μl). All procedures were done at 4 °C. All samples were treated with Sep-Pak C-18 cartridges to extract the ghrelin. Recovery experiments were always performed with sample assay using three points of references (high, middle and low levels) and radiolabeled ghrelin. The recovery rate was 92.2% ± 0.4% (S.E.M.). The intra- and interassay coefficients of variation were 5.1% and 2.4% at 50% binding. The dilution curves for the extracts of canine plasma and rat plasma paralleled the standard curves.

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. After that, plasma ghrelin levels were measured with a ghrelin RIA kit (Linco Research, St Louis, MO, USA). Without exception, all assay procedures were carried out in accordance with the protocol supplied by the manufacturers.

Measurement of blood chemistry related to body fat

The blood was collected after 12 hour-starvation from each lean, normal and obesity beagle dogs. The plasma was stored at –20 ℃ until clinical blood examination. The blood values of triacylglycerol, free fat acid, cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol were determined by clinical diagnostic reagents using Fuji Dry Chem. 3500 (Fuji film Co., Tokyo, Japan).

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 pre-treatment 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. (20). 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.

Statistical analysis

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. Data of blood chemistry were analyzed statistically by repeated measures ANOVA followed by the Student-Newman-Keuls test. Differences with a value of $P<0.05$ were considered significant.

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 1545 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

I 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 hema-toxylin 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 μg/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

μg/kg ghrelin, but only the highest dose resulted in a statistically significant change.

Establishment for radioimmunoassay of canine ghrelin

Typical standard curve for rat ghrelin and parallel displacement between canine plasma, rat plasma and rat ghrelin standard was presented in Fig. 4. Addition of either canine plasma or rat plasma resulted in displacement of ghrelin tracer in a dose-response manner. Regression analyses of these dose-response lines with plasma were not significantly different from the lines of the standard without the addition of plasma. These results suggested that it is possible to measure the canine ghrelin in serum using anti-rat ghrelin antiserum.

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. 5). 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. 6). 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±59.8 vs male 497±98.3, mean± S.E.M. n = 4).

Examination of blood chemistry related to body fat in lean and obesity dogs

As shown in Table 1, in obesity dogs, the values of triacylglycerol, free fat acid, cholesterol and high-density lipoprotein cholesterol were significantly higher than those of normal dogs. However, there was no significant difference in low-density lipoprotein-cholesterol levels. On the other hand, in lean dogs, free fat acid, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were significantly lower than those of normal dogs. There was no significant difference in triacylglycerol and cholesterol levels between lean and normal dogs. When those blood chemistries were compared between lean and obesity dogs, all parameters measured were significantly higher in obesity dogs than those in lean dogs.

Discussion

Injection of ghrelin at a dose of 5 μg/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 (8). The cDNA encoding the dog ghrelin precursor has been sequenced (84), 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 recorded in this study was considerably lower than that reported previously in healthy dogs in response to a dose of 2 μg/kg (8). Indeed, it has been shown that ghrelin strongly stimulates GH release in humans, whereas in dogs, either ghrelin is not be 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-immunostained 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 (84). The dog gastric mucosa has been investigated at the electron microscope level by Rindi et al. (63), 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 (52,86,94) and humans (92). 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. I 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 (51). 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 (75). When sheep are fed two or four times a day, the ghrelin levels rise just before each feeding (77). Plasma ghrelin levels showed a nocturnal rise that exceeded the meal-associated increase in lean human (98). 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 (75). 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 (76), 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 my 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 (17,81,87,92). 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 (88). Several studies have demonstrated that plasma ghrelin levels are inversely correlated with the body mass index (15,28,34,70), 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 (6). 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.

Obesity develops when energy intake consistently exceeds daily energy expenditure. Numerous environmental and social factors contribute to the formation of obesity. These include lack of exercise, overfeeding, or unbalanced diet. For example, ad libitum feeding of a high-fat diet is a well-known factor of obesity development. Genetic factors such as breed or physiological factors such as neutering have been associated with an increase in the risk of obesity. Drugs i.e. corticosteroids and progesterones or endocrine abnormalities have also been implicated as cause of obesity. Canine plasma as humans identified lipoproteins with the physical and chemical characteristics of low-density lipoprotein and high-density lipoprotein. The present study demonstrated that lean and obesity dogs showed the decrease and increase blood chemistry related to body fat, respectively. Especially, the increase of blood free fatty acid and triacylglycerol levels in obesity dogs indicated the hyperlipemia rather than simple increase of body weight. In addition, high levels of cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol also indicate the cholesteremia. Cholesterol and triglycerides are transported through plasma in special particles called lipoproteins. The reason for the hyperlipemia and cholesteremia in these beagle dogs is unknown in the present study. Long-term effects of hyperlipemia in dogs are unknown, but cholesteremia has been associated with ocular lesions and hypertriglyceridemia may induce acute pancreatitis. As all animals are kept in individual canine cage in long term, the over supplement of food and lack of exercise

may be one of the causes. On the other hand, the lean dogs might feel the some stresses. It has been well known that stress-induced release of corticotropin releasing hormone from hypothalamus is strongly inhibited food intake, and increase the energy expenditure.

It seems that those obesity accompanying hyperlipemia and cholesteremia is able to consider the morbid obesity. On the other hand lean accompanying hypoglycemia may be morbid lean. Whether or not obesity can be linked to plasma ghrelin levels needs to be clarified by further characterization of the pathophysiology of obesity and/or lean in dogs. Recently, abnormal circulating ghrelin levels have been reported in patients with anorexia nervosa (80) and Prader–Willi syndrome (15). 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.

Figures

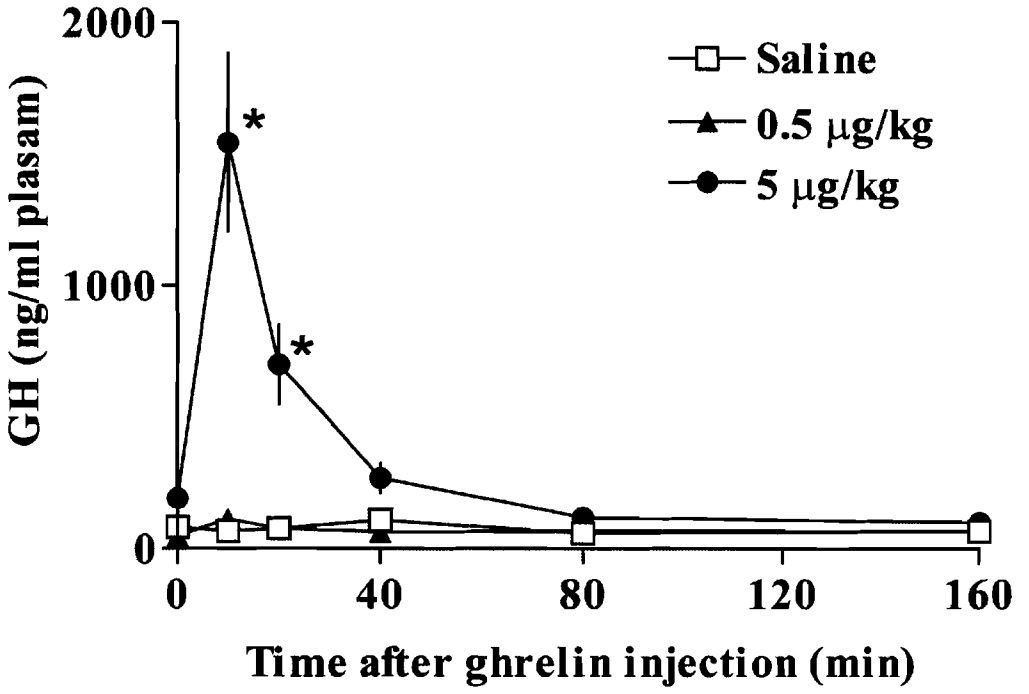

Fig. 1. Time course of changes in plasma GH levels after i.v. administration of ghrelin in beagle dogs. Symbols and vertical lines represent the mean value± S.E.M. of four healthy dogs. *P < 0.05, significantly different from the saline-treated and 5 μg/kg ghrelin-treated groups at each time point.

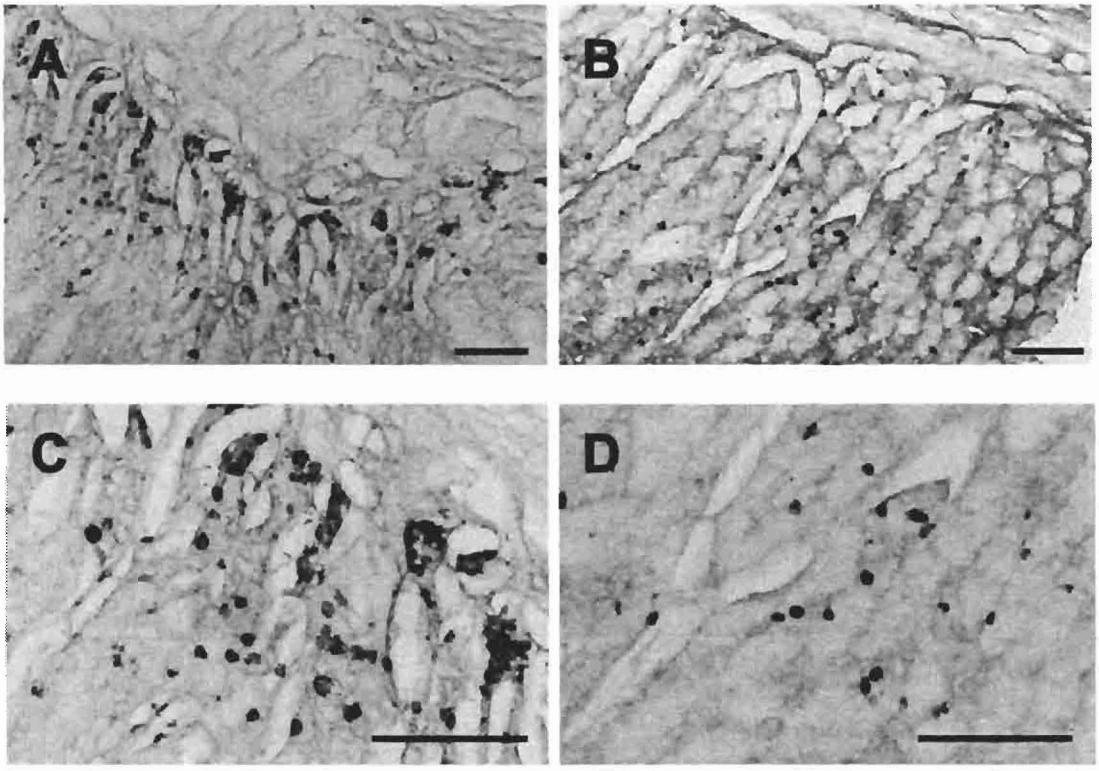

Fig. 2. Ghrelin-immunoreactive cells in the stomach of beagle dogs. Immunoreactive cells were detected in the oxyntic glands from the fundus (A and C) and body (B and D) region. Ghrelin-immunoreactive cells were restricted to the gastric mucosa. Many ghrelin cells were scattered from the glandular base to the glandular neck. Each lower panel (C and D) shows a higher magnification of the panel above (A and B respectively). The data shown are representative of all three animals studied. Bars = 100 μm.

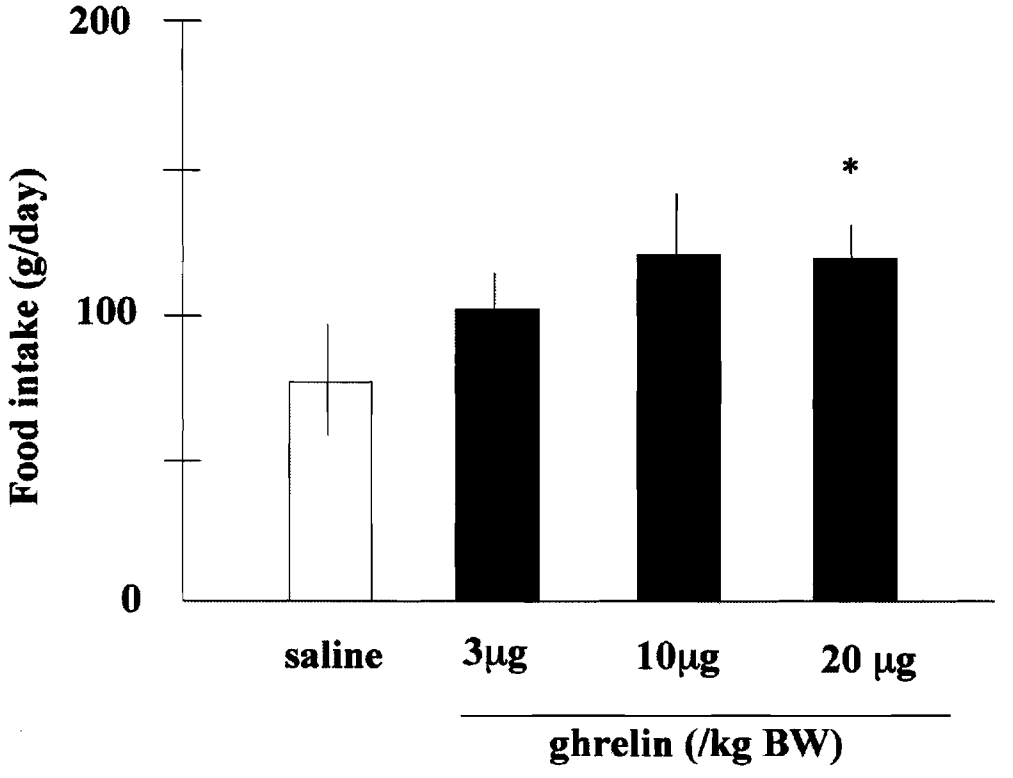

Fig. 3. Effect of a single i.v. injection of ghrelin on daily food intake in dogs. Daily food intake increased after treatment with ghrelin. Bars and vertical lines represent the mean values± S.E.M. (n = 6). *P < 0.05, significantly different from the saline-treated group. BW, body weight.

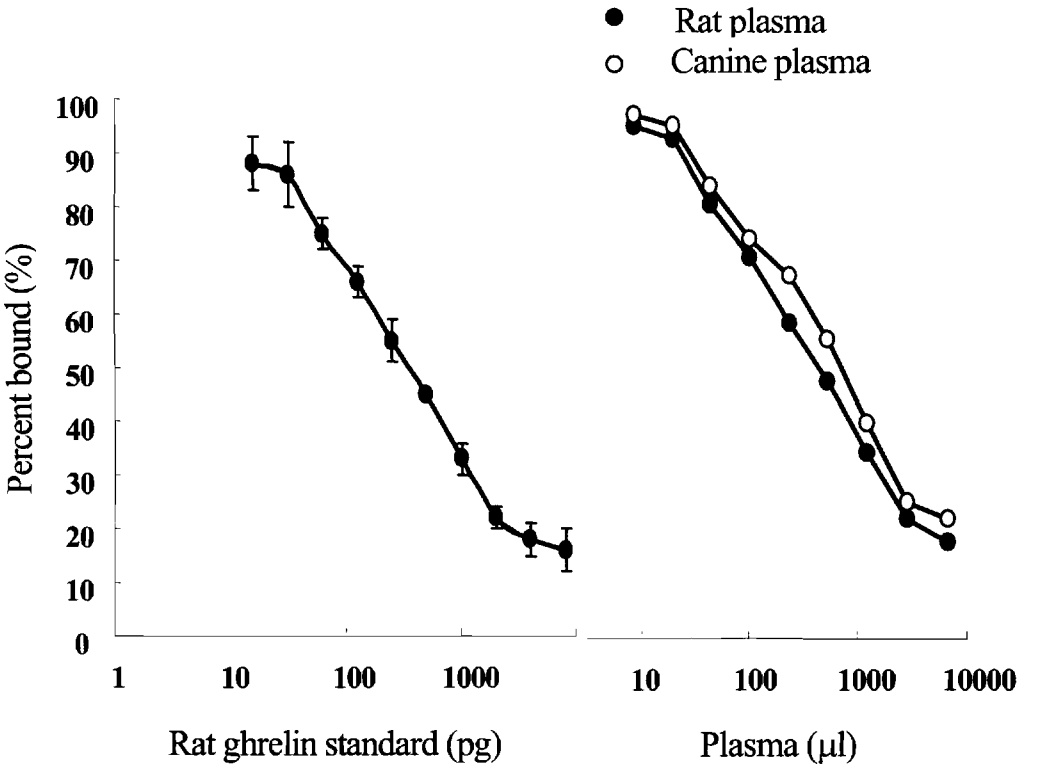

Fig. 4. Dose response lines of rat ghrelin standard (closed circle), rat plasma (closed square) and canine plasma (open circle) in the ghrelin radioimmunoassay. Both plasma samples was adjusted to 12.5 25, 50, 100, 200, 400, 800, 1600, 3200 μl. Each value represents the mean of triplicate determination.

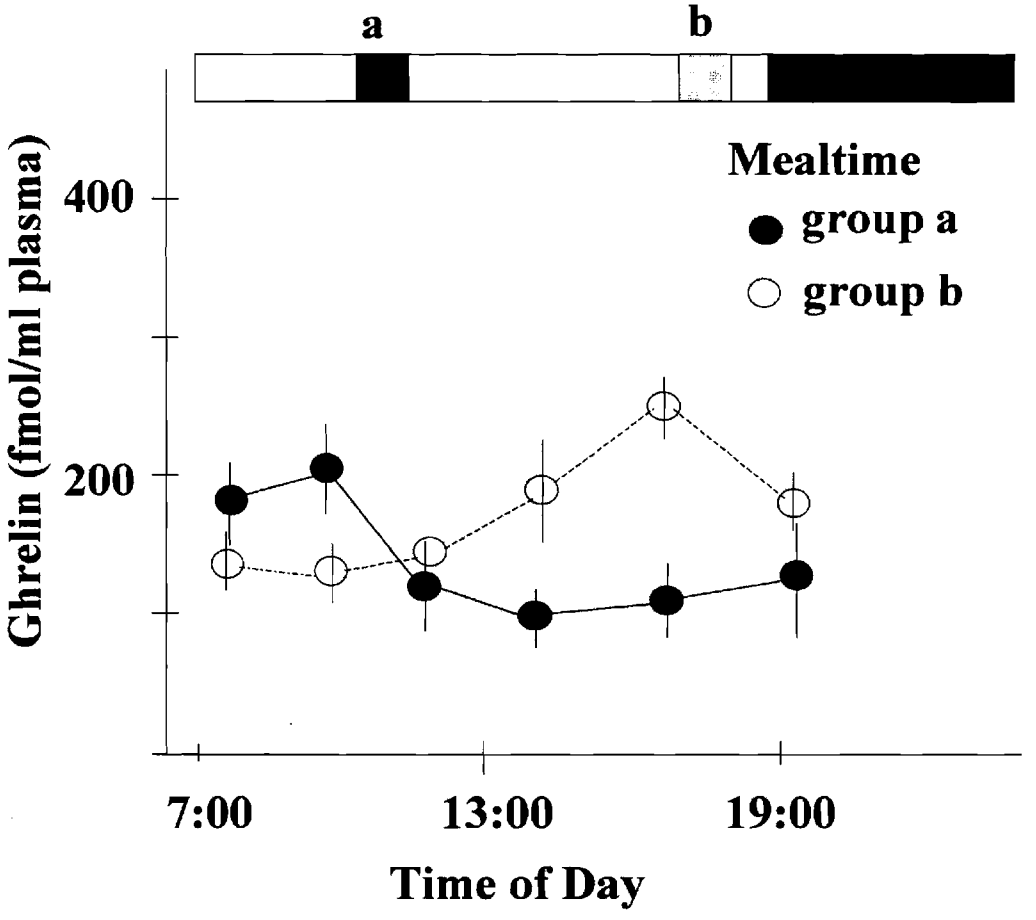

Fig. 5. Influence of daily feeding time on plasma ghrelin levels in dogs. The white and black bar represents the light and dark periods. Areas a and b represent the meal time in group a and group b respectively. Plasma ghrelin peaked just before the restricted daily feeding time at 1000 h (group a; closed 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± S.E.M. (n = 6).

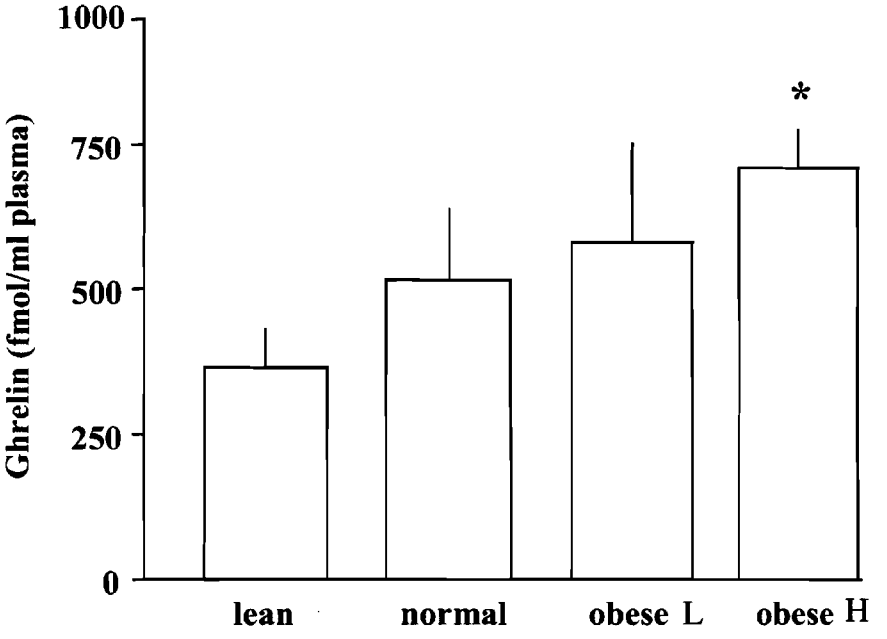

Fig. 6. Plasma ghrelin concentrations in lean, normal and obese dogs after overnight food deprivation. Bars and vertical lines represent the mean values± S.E.M. (n = 7). Young, female, adult, lean (mean body weight 7.2±0.2 kg), normal (mean body weight 10.1±0.2 kg), light obese (obese L; mean body weight 12.4±0.4 kg) and heavy obese (obese H; mean body weight 15.9±1.2 kg) beagle dogs were studied. *P < 0.05, significantly different from the normal.

Table 1 Comparison of biochemical value of blood between normal, lean and obesity beagle dogs (n=5).

	TG (mg/dl)	FFA (Eq/l)	CHO (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Lean	73 ± 4.65	1.69 ± 0.09[a]	116.5 ± 11.34	71.7 ± 3.90[a]	8.25 ± 0.75[a]
Normal	76 ± 4.44	1.79 ± 0.04	128.5 ± 6.34	79.7 ± 3.00	9.85 ± 0.15
Obesity	81 ± 3.01[b]	2.45 ± 0.15[a]	170.2 ± 12.80[a]	95.0 ±1.68[a]	11.75 ± 2.86[b]

a: <0.005 vs. normal b: <0.005 vs lean

TG: triacylglycerol, FFA: free fat acid, CHO: cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol

Chapter II: Relationship between growth and plasma concentrations of ghrelin and growth hormone in juvenile beagle dogs

Abstract

Although the release of growth hormone (GH) is known to be regulated mainly by GH-releasing hormone (GHRH) and somatostatin (SRIF) secreted from the hypothalamus, ghrelin also may be involved in GH release during juvenile period. I have examined plasma concentrations of acylated ghrelin, desacyl ghrelin, and GH in juvenile beagle dogs. Plasma acylated and desacyl ghrelin levels changed through aging; however, there was no closely correlation between ghrelin, body weight and circulating GH levels during juvenile period. The increase in body weight was essentially linear until 8 months of age, whereas plasma GH concentrations exhibited bimodal peaks for the meanwhile. The results suggest that ghrelin may not play internal cueing in GH secretion in juvenile beagle dogs.

Introduction

As in many other species, prepubertal growth and development play an important role in determining the onset of puberty or sexual maturity in dogs. Equally striking differences are observed in head shape, body proportions, hair coat, and behavior. Several hormones are involved in promoting growth and skeletal muscle development, and of these, growth hormone (GH), insulin, and thyroid hormone appear to be of major importance. Many earlier studies indicate that the secretion of GH during growth is attributable to alterations in hypothalamic activity. GH secretion is regulated primarily as a result of the interplay between hypothalamic GH-releasing hormone (GHRH) and somatostatin (SRIF) as well as input from other factors including nutritional intake and neural transmitters (58). In particular, GH secretagogues (GHS) are a group of synthetic compounds that induce GH secretion through the activation of the GHS receptor (GHS-R). Ghrelin, a recently discovered peptide hormone that is secreted mainly by the stomach, has been identified as the endogenous ligand of the GHS-R and has a potent GH-releasing effect (42). The discovery of ghrelin introduces another regulatory input into the hypothalamic GHRH/SRIF–pituitary GH axis. Since ghrelin has only recently been discovered, the information available on its intrinsic role during prepubertal growth and development is limited. I and other group have recently found that exogenous ghrelin injection in beagle dogs stimulates prompt GH release (8,99) and ghrelin-immunoreactive cells localize to the fundus and body of the stomach (63,99), but a physiological role of ghrelin in energy homeostasis during the growth phase has not yet been established. Thus, the present study was conducted to determine the juvenile growth patterns in beagle dogs, to measure changes in the plasma concentrations of GH, acylated ghrelin, and desacyl ghrelin, and to establish whether there is any correlation between these hormone levels and body weight (BW) increments in the prepubertal stages of growth in these animals.

Materials and Methods

Healthy male and female beagle dogs were used for this study. All the dogs were kept in similar conditions throughout the study and were separately fed once a day a maintenance commercial canine laboratory diet (DS-A; Oriental Yeast, Chiba, Japan) that was formulated to contain 6.0% moisture, 24.7% crude protein, 8.2% crude fat, 7.0% crude ash, 3.9% crude fiber, 50.2% nitrogen-free extract, and 15.6kJ/g metabolizable energy. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. Whole blood samples were collected between 0900 and 1100 hours by jugular venipuncture after fasting overnight, transferred to ice-chilled tubes containing disodium ethylenediamine tetraacetic acid and 500 U aprotinin, and centrifuged at 4°C and 2000×*g*. Immediately after plasma collection, 100 μl of 1 N HCl was added per milliliter of plasma sample for use in an enzyme-linked immunosorbent assay (ELISA) for acylated and desacyl ghrelin. Plasma was then stored at –80°C until hormone analyses were performed. BW was recorded on the blood sampling days. Plasma acylated and desacyl ghrelin concentrations were determined using ELISA kits (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan) according to manufacturer's specifications. The minimum sensitivities of these kits were 2.5 fmol/m*l* and 12.5 fmol/m*l*, respectively, and the intra- and interassay coefficients of variation were both <10%. Since the kits were designed for use with rat, mouse, and human, I first verified that dog plasma contains suitable matrices. Plasma GH concentration was measured with the aid of commercial porcine/canine radioimmunoassay kits (Linco Research Inc, St Charles, MO, USA). The limit of sensitivity for the GH assay was 1 ng/ml, and the intra- and interassay coefficients of variation were both <7%.

Results

In the male dogs, the mean (± SEM) BW increased from 3.5 ± 0.3 kg at 2 months to 12.4 ± 0.5 kg at 12 months (Fig. 1-A). In the female dogs, the mean BW increased from 3.2±0.2 kg at 2 months to 11.4 ± 0.4 kg at 12 months (Fig. 2-A). All of the animals exhibited a healthy BW gain throughout the experimental period until reaching a mature BW by approximately 12 months of age, showing a sigmoid growth curve (Fig. 3-A). Although these animals do not grow at the same rate between birth and puberty, the increase in BW with time was essentially linear between 2 and 8 months of age.

The mean plasma acylated and desacyl ghrelin concentrations of these growing dogs are shown in Fig. 1-B, 2-B and 3-B. In the male dogs (Fig. 1-B), mean plasma acylated ghrelin concentrations decreased gradually from a start point of approximately 60.6 ± 8.3 fmol/ml at the beginning of the study until 8 months of age. A few episodic releases of acylated ghrelin were detected between 6 months and 12 months of age. In the female dogs (Fig. 2-B), mean acylated ghrelin concentrations reached a high of 84.3 ± 18.0 fmol/ml at between 4 and 5 months of age. Thereafter, acylated ghrelin levels decreased and remained at low a level, exhibiting only a slight increase, until the age of 10 months. There was a subsequent increase to 74.9 ± 14.2 fmol/ml at 11 months of age. The peak and nadir of desacyl ghrelin concentration was related to the corresponding plasma acylated ghrelin concentrations during the experimental period for both genders (Fig. 3-B).

Plasma GH concentrations were also determined from the blood sample collected at prepubertal stages of growth. In the male dogs (Fig. 1-C), GH levels were highest at 2 months of age. Thereafter, they steadily declined until 5 months of age and then increased from 6 to 7 months of age. Following this increase, the mean GH level decreased, remaining at a low level until 12 months of age. In the female dogs (Fig. 2-C), the GH concentrations at 2 months of age was 9.9 ± 0.9 ng/ml; the mean level declined thereafter and, other than a transient peak at 8

months of age, the concentration remained at approximately 2.0 ng/ml until the end of the study. The increase in BW with time was essentially linear between 2 and 8 months of age; plasma GH concentrations, however, exhibited a biphasic pattern during this period.

The temporal changes in the increase in BW and the secretion profiles of the three hormones were similar between the male and female dogs. It seems, therefore, that in our study gender had no significant effect on either parameter.

A significant correlation between the plasma acylated ghrelin and desacyl ghrelin levels was observed (Fig. 4). A negative linear correlation was found between BW and plasma acylated ghrelin levels and plasma GH levels of the beagle dogs (Figs. 5-A and 5-B), but regression lines of the relationship these parameters showed the low coefficient of correlation. There was no correlation between BW and plasma desacyl ghrelin levels, and GH and plasma acylated ghrelin levels (Figs. 5-C and 5-D).

Discussion

Body weight is regulated by the process of energy homeostasis, whereby total energy intake and expenditure are closely matched over long periods of time (18). Deviations from a genetically influenced level of defended body weight engage adaptive, centrally-mediated alterations in appetite and energy expenditure that resist weight change, rendering non-surgical weight-loss interventions relatively ineffective. Implicit in this regulatory feedback loop is the existence of adiposity signals that communicate the status of energy stores in the body to the brain. Leptin and insulin are accepted as adiposity hormones because they fulfill a series of criteria that should be met by any such agent (68). As a primary approach, I attempt to measure changes in the plasma GH and ghrelin; based on hypothesis that ghrelin also satisfies these criteria and may thus be a unique anabolic counterpart to leptin and insulin in energy homeostasis.

GH is an anabolic hormone that is essential for normal linear growth. In addition, GH has important metabolic effects on a variety of physiological systems throughout life. These nongrowth effects include facilitating the utilization of fat mass for energy stores, building and sustaining lean body mass, and maintaining bone mineral density. The beagle dogs used in this study exhibited an essentially similar prepubertal growth pattern to that reported for adult medium-sized breeds of dog, when comparing absolute BW with beagles of the same age (29). This is the first study to examine whether there is a relationship between acylated and desacyl ghrelin levels and BW increments during the prepubertal stages of growth in beagle dogs. There was significant relationship between the mean increase in BW of the animals used in the present study and the corresponding mean plasma acylated ghrelin and GH concentrations, throughout the experimental period. However there was only a low correction between BW and plasma acylated ghrelin levels and plasma GH levels.

Intriguingly, ghrelin's actions are not restricted to the GH axis. Ghrelin also functions as a

powerful orexigenic hormone; it stimulates feeding and increases body weight when administered either peripherally or centrally (52,86,94), and these effects appear to be independent of changes in GH (52,86). It is well known that the secretion of GH is exquisitely sensitive to perturbations in nutritional states; thus, ghrelin may be a critical hormonal signal of nutritional status to the GH neuroendocrine axis. In this paper I attempt our physiological studies designed to further understand how ghrelin interacts with the well established. It is known that exogenous administration of ghrelin stimulates GH release and appetite in beagle dogs (99). Previous investigations have suggested that ghrelin plays an important role in the regulation of metabolic balance. In the present study, the concentrations of acylated and desacyl ghrelin fluctuated between intermediate and high levels without any clear age-associated trend. The physiological significance of ghrelin in GH secretion and/or the prepubertal growth of juvenile dogs therefore remain unclear. Although further *in vivo* studies are required to establish the details of any correlation with various aspects of growth, it may be that ghrelin levels are also regulated by physiology of anabolism, feeding behavior, and nutritional homeostasis for GH secretion. In particular, the effects of ghrelin on the somatotroph remain to be classified.

One recent study (56) in which a GHS-R antagonist was used, revealed that circulating ghrelin in the peripheral blood may not play a role in generating pulsatile GH secretion. Moreover, deletion of ghrelin impairs neither growth nor appetite, indicating that ghrelin is not essential for GH secretion (78). Another study, however, demonstrated that the *in vivo* attenuation of GHS-R expression results in a reduction in food intake and growth, suggesting a physiological role of the ghrelin-GHS-R system in the secretory regulation of GH (71). My observations in juvenile dogs may not support the concept that plasma ghrelin plays a crucial role as an endocrine mediator of GH secretion.

Desacyl ghrelin, whose plasma concentrations in rats is at least 2.5-fold higher than that of acylated ghrelin (39), neither activates GHS-R nor exhibits endocrine activity (39,42,49). In

contrast, transgenic mice over expressing desacyl ghrelin are phenotypically smaller than the norm (2,4). This observation may indicate a role of desacyl ghrelin in the regulation of GH secretion. It was observed in the present study that plasma desacyl ghrelin levels were greater by about four-fold than that of acylated ghrelin, based on evaluations of individual animals.

The ratio of acylated to desacyl ghrelin is approximately the same in tissues that synthesize the hormone (e.g., stomach and pituitary) as it is in blood (20,43). This suggests that acylation occurs in the cells of origin and also that both acylated and desacyl ghrelin are produced by these cells, rather than that circulating desacyl ghrelin arises solely from degradation of the acylated form. A small but growing body of literature claims roles for desacyl ghrelin in adipogenesis (83), lipolysis (32,50), glucose homeostasis (32), cell proliferation (13), apoptosis (5), and cardiovascular function (7). These effects are hypothesized to be mediated by a putative alternate receptor (90), since desacyl ghrelin does not bind to or activate the classical ghrelin receptor, nor does it exert any endocrine or feeding effects (42,90). Data in mice and humans suggest that very high levels of desacyl ghrelin may inhibit some of the actions of acylated ghrelin (2,4,10,32).

It is tempting to speculate that desacyl ghrelin has physiologic functions, given that its concentrations in tissues and blood are nearly ten times those of acylated ghrelin (39), and I would like to think that nature is too frugal to synthesize a functionless peptide. Most of the data suggesting actions of desacyl ghrelin, however, come from pharmacologic or in vitro studies, and these observations may reflect effects of supraphysiologic doses of desacyl ghrelin crossreacting with heterologous receptors. Pending the discovery of a specific receptor for desacyl ghrelin, claims of physiologic roles for this entity remain conjectural. In dogs, the significance of any physiological role of desacyl ghrelin is not clear at this time. It is possible, however, that circulating desacyl ghrelin in dogs is regulated in the same manner as in humans and rodents.

The blood GH concentration primarily changes with growth, aging and lactation in mammals. The plasma GH concentration and secretory response to the GHS are high during the postnatal period, and become lower after puberty with increasing age. However, a limited number of studies have reported the basal level of GH in juvenile dogs. Eigenmann and Eigenmann (26) reported a mean±SEM GH level of 1.92 ± 0.14 μg/*l* for a group of 63 healthy adult dogs. A more recent report indicates that differences in final body size between medium-sized (beagles) and giant (Great Danes) dog breeds are associated with differences in GH release at a young age (29). During the entire observation period of that study, the basal plasma GH levels of the beagles remained at a stable level (29). In contrast, in the juvenile beagle dogs employed in the present study, the high GH levels observed exhibited a bimodal distribution, the peak being observed at 2 and 8 months of age. The position of the first peak is at a level similar to that observed until 7 weeks of age by Favier et al. (29). Because the experimental period of Favier et al. lasted from 6 until 24 weeks of age (c.a. 6 months), the second peak after the 24 weeks might have observed only in the current study. The neonatal period in humans is also characterized by relatively high GH concentrations (25). Neonatal hypersomatotropism in human beings is characterized by pulsatile GH secretion with a high pulse amplitude and a high pulse frequency (24,95). It is speculated that in my results the first excessive secretion of GH is also associated same manner with the immediate postnatal rise of GH secretion in the human newborn. The second peak observed at 8 months in the present study may be associated with the timing of changes in nutritional status or sexual maturity. In my observation, the plasma acylated and desacyl ghrelin levels were unaffected by the first, but not the second distribution of GH, therefore the reasons behind this rise remain enigmatic.

The results of this study demonstrate that the observed increase in BW is significantly correlated to the corresponding plasma acylated ghrelin and GH concentrations, but not desacyl ghrelin during the period of prepubertal growth in beagle dogs. However BW is not closely

correlated with plasma acylated ghrelin and GH levels. Although further *in vitro* and *in vivo* studies are required to establish the regulation of GHRH and SRIF secreted from the hypothalamus, the alterations of the GH response pattern and of acylated and desacyl ghrelin reported in the present study are valuable in the comparison of the relative contributions of the two hormones in growing beagle dogs.

Figures

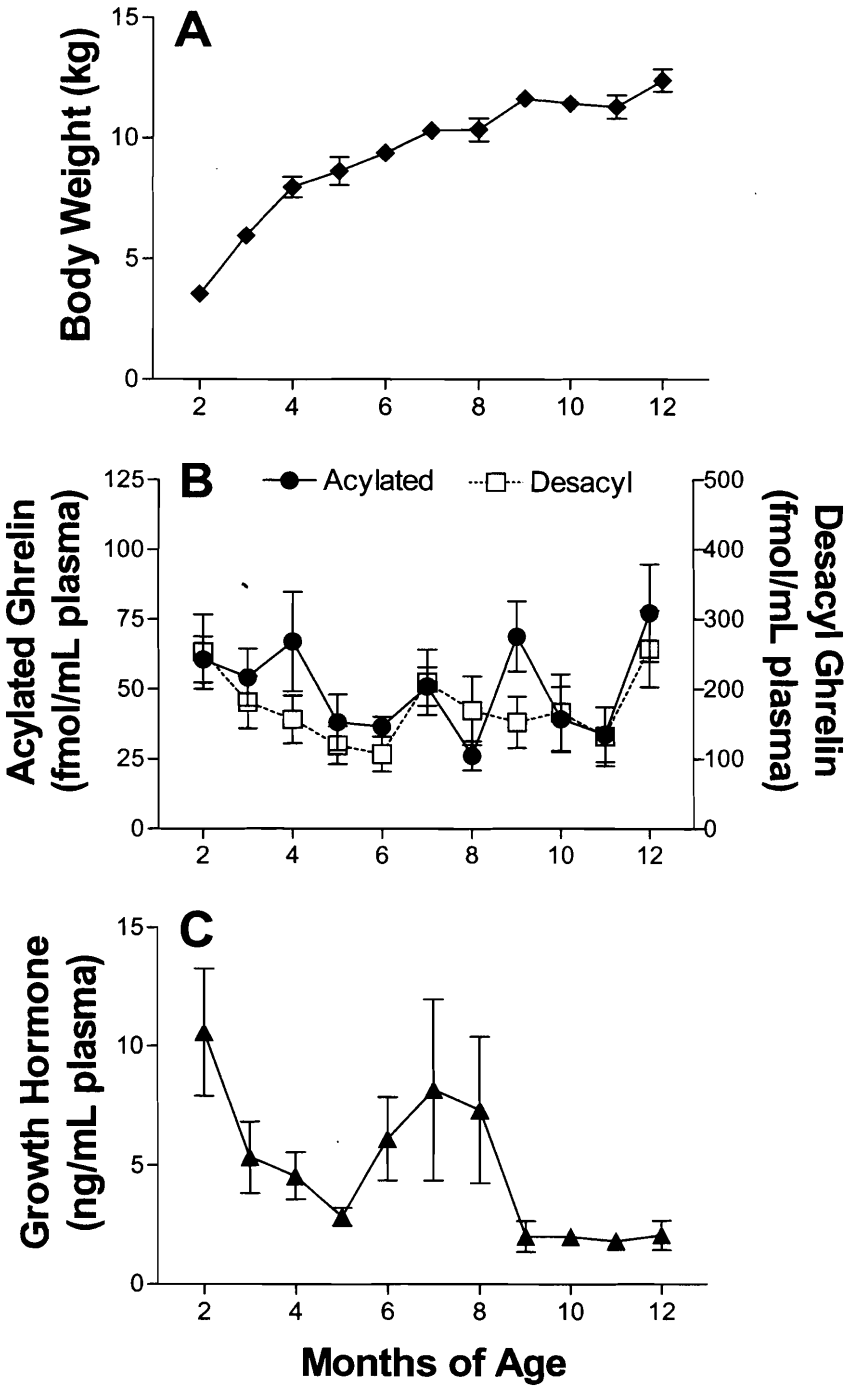

Fig. 1. Changes in body weight (A), plasma acylated ghrelin and desacyl ghrelin levels (B), and plasma growth hormone (GH) levels (C) with age in male beagle dogs. The symbols and vertical lines represent the mean and ±SEM. No. of animals studied per group were 5 males.

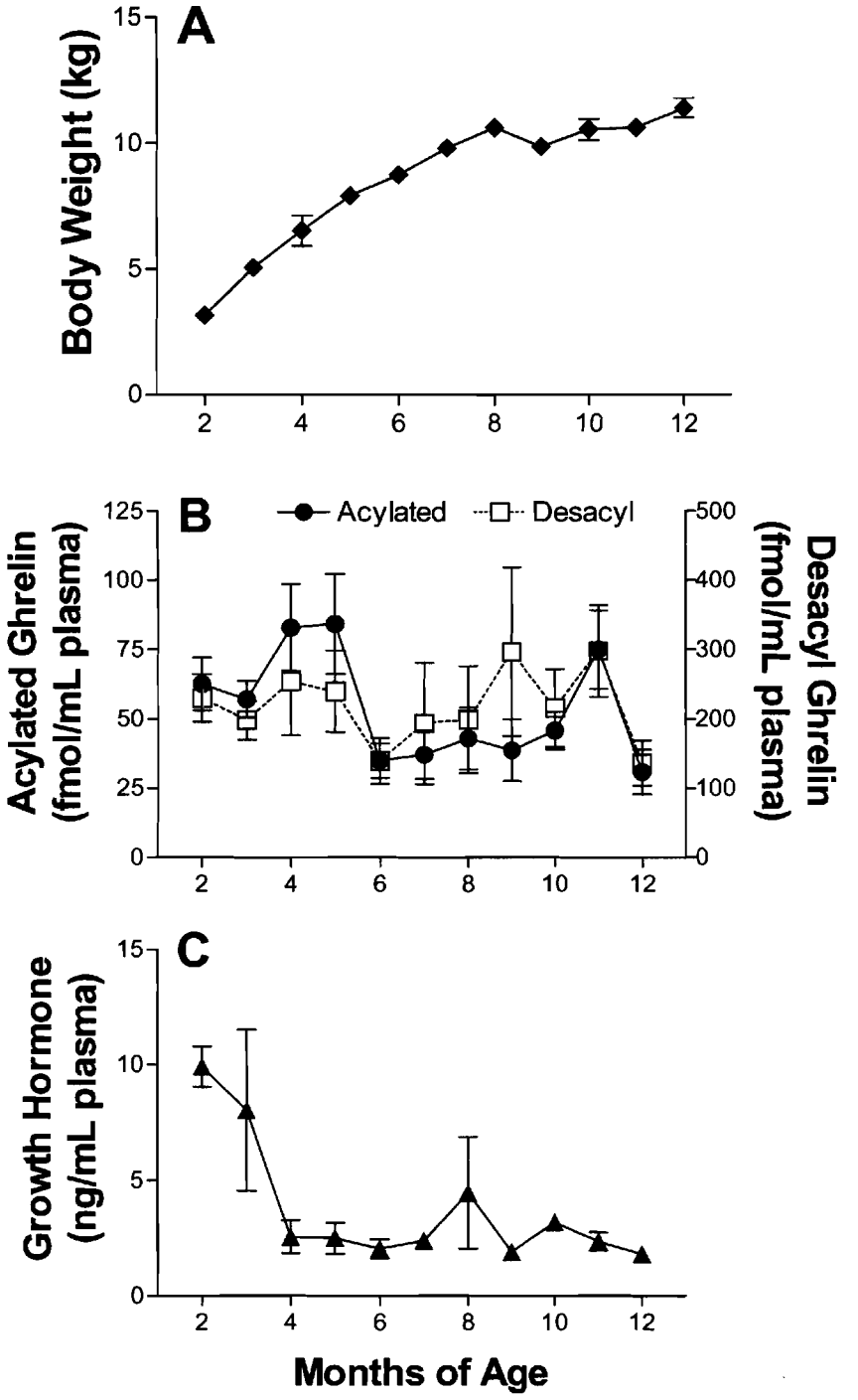

Fig. 2. Changes in body weight (A), plasma acylated ghrelin and desacyl ghrelin levels (B), and plasma growth hormone (GH) levels (C) with age in female beagle dogs. The symbols and vertical lines represent the mean and ±SEM. No. of animals studied per group were 5 females.

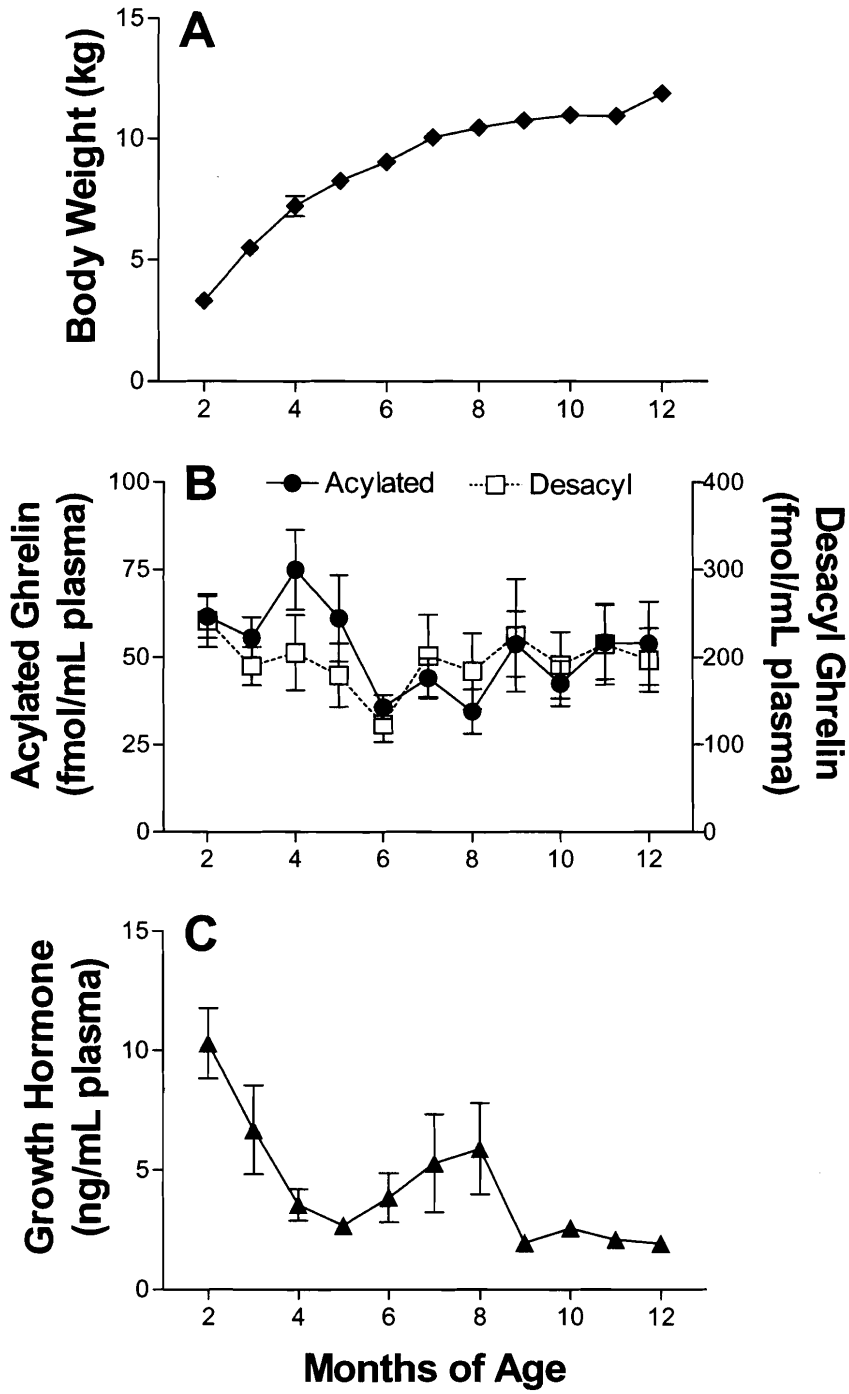

Fig. 3. Changes in body weight (A), plasma acylated ghrelin and desacyl ghrelin levels (B), and plasma growth hormone (GH) levels (C) with age in beagle dogs. The symbols and vertical lines represent the mean and ±SEM of five males and five females, respectively (n=10).

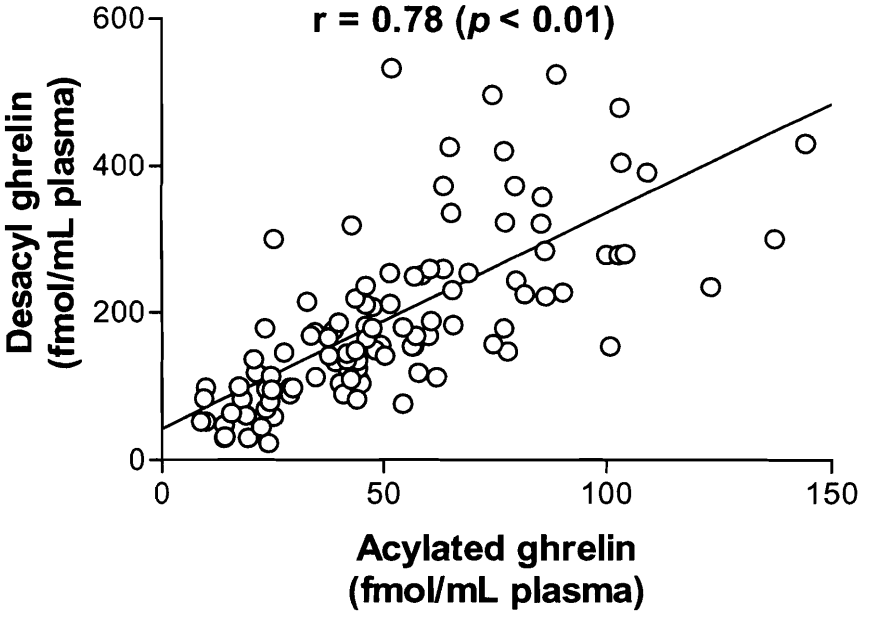

Fig. 4. Correlation between plasma acylated ghrelin and desacyl ghrelin levels in beagle dogs ($r = 0.78, p < 0.01$; Spearman).

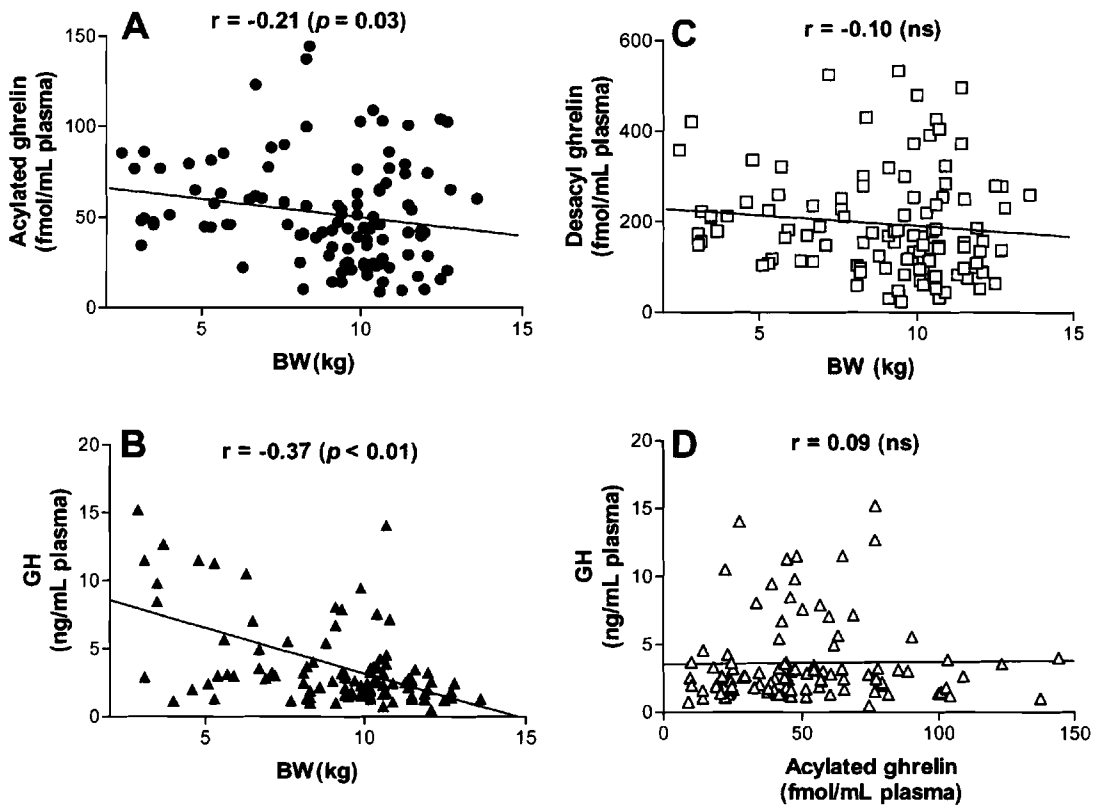

Fig.5. A, B and C: Relationships of BW with plasma acylated ghrelin, desacyl ghrelin, and GH levels in beagle dogs. D: Relationship between plasma acylated ghrelin and plasma GH levels in beagle dogs.

General Discussion

Food intake behavior and appetite stimulation are one of the most important physiological functions to maintain life. If we elucidate the mechanism of food intake behavior, it will be of benefit to human and animal medicine. Through neuronal network systems in the lateral hypothalamus, food intake is initiated as a basic behavioural drive. The ventro-medial part is related to the limitation of food intake. In addition to this central nervous action, gastric emptying seems to have an important role. It has been shown that decerebrate rats are capable of terminating their eating upon distension of the stomach with activation of stretch receptors (33). However, we usually finish our meals long before the stomach is fully distended, indicating that early metabolic signalling, which occurs during actual food intake, is involved in meal termination.

As regards the short-term regulation of energy intake, structures in the brain are primarily in control of the single meal as regards its volume, energy content and duration. This feedback mechanism from the gut involves a host of peptide hormones located at various points along the gastrointestinal tract. In addition to a direct central action on their brain receptors, signals mediated from peripheral receptors are conveyed through the vagus nerve to the NTS in the brainstem (62). The peripheral and central vagus systems sense mechanical distension and chemical stimulation by different nutrients. Not only do these signals affect sensory nerve fibres directly, they are also released to the circulation as a hormonal response. Peripheral signalling from the gastrointestinal tract and pancreas, with orexigenic as well as anorexigenic properties, seems to exert short-term influences, whereas signalling from adipose tissue and other tissues is considered to exert long-term regulatory mechanisms in order to ensure basal metabolic needs (9).

About 25 years ago, the initial report of a GHS introduced a new regulatory pathway for GH release to accompany the known GH-releasing hormone (GHRH) pathway. Since then, the

ghrelin story started with synthetic GHS that represented the dream of small molecules for the treatment of GH deficiency and/or as anabolic treatment for antiageing intervention.

In the first chapter, I investigated the distribution of ghrelin in the stomach and its possible role using beagle dogs. 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.

After the ghrelin discovery and the demonstration of its orexigenic action, the new dream in terms of clinical perspectives is the possibility that ghrelin analogues acting as agonists or antagonists are useful for the treatment of cachexia, eating disorders and obesity. Ghrelin is one of the first peptides able to modulate appetite and food intake even in canines and other mammals. I describe that ghrelin is a potent GH secretagog (GHS) also 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. Even though there have been many scientific breakthroughs in the understanding of the regulation of food intake and energy disposal throughout the last few decades, new treatments have not reached our hands. This is partly due to the complex neuronal circuitry in the central nervous system and periphery that regulate energy deposition and expenditure, and the difficulty of extrapolating findings in experimental animals; mostly rodents to humans. This plus contributing genetic and environmental factors, the discovery of novel disease targets has been tardy and unfruitful. Despite these hurdles, abdominal obesity clearly represents a major contributing factor and also initiator of the metabolic syndrome; defined by the World Health Organization (WHO) as insulin resistance plus two of the five additional criteria of hypertension,

high triglycerides, low HDL cholesterol, high body mass index (BMI), and high urinary albumin excretion, treating obesity is a heavily pursued area for academic and industrial researchers and several new chemical entities are currently being investigated for their treatment of obesity. Many excellent reviews have recently been published outlining the potential therapeutic targets for the treatment of obesity.

Circulating ghrelin levels are negatively associated with body mass index; ghrelin secretion is increased in anorexia and cachexia, reduced in obesity and normalized by recovery of ideal body weight (3,15,19,38,80,88). Thus, ghrelin changes in response to variations in the nutritional state are opposite to those of leptin and it has been suggested that both hormones may act as signals of the metabolic balance to the central nervous system (38,73,98). The only exception to the negative association between body mass index and ghrelin secretion is the Prader–Willi syndrome where obesity is surprisingly associated with ghrelin hypersecretion (15). In humans, circulating ghrelin levels are increased by energy restriction and decreased by food intake indicating that ghrelin secretion is mostly regulated by metabolic signals (3,17,88). Ghrelin secretion is decreased by either intravenous or oral glucose load as well as during euglycaemic–hyperinsulinaemic clamp and even after insulin-induced hypoglycaemia (10,47,70). The inhibitory influence of overexposure to insulin on ghrelin secretion is consistent with the strong negative association between ghrelin and insulin levels that had been predicted by the negative correlation between ghrelin levels and body mass index (70). However, it is still unclear whether insulin and glucose play a direct inhibitory role in ghrelin secretion (11,67).

The Roux-en-Y gastric bypass is one of the most effective and commonly performed procedures for severe obesity. The initial report by Cummings et al. (19) that, following gastric bypass, circulating ghrelin is drastically diminished has led to intense investigation into a possible role of ghrelin in the effects of obesity surgery. In contrast to what occurs after gastric bypass, plasma ghrelin normally increases following nonsurgical weight loss (88) and is

proportional to lean body mass. Hence, a reduction of ghrelin, and a consequent reduced stimulant for food intake, might well contribute to the weight loss of gastric bypass surgery. Initially, it was proposed that the exclusion of the fundus region of the stomach, the site that produces the majority of circulating ghrelin, was the mechanism through which gastric bypass resulted in lower ghrelin levels. However, numerous follow-up studies have either contradicted such findings or reported no change in ghrelin following surgery. Normally, ghrelin levels rise prior to or in preparation for a meal and decline immediately postprandially (17). To examine the contribution of the stomach in this response, Williams et al. (91) examined whether distension of the stomach and/or nutrient stimulation were required for the prandial changes in plasma ghrelin. Only when the infused calories (glucose) were allowed to pass through the pylorus into the foregut did ghrelin levels change, indicating that postgastric feedback is necessary for changes in ghrelin to occur postprandially. No changes in ghrelin were seen when glucose was held in the stomach by use of a pyloric cuff (91). These findings argue against the hypothesis that exclusion of the stomach lumen from ingested nutrients is the cause of reduced ghrelin following gastric bypass surgery. More investigation is required to determine the mechanisms underlying changes in ghrelin secretion following gastric bypass surgery.

Levin et al. (45) studied that the effects of ghrelin on the stomach by studying the effect of ghrelin iv infusion on gastric emptying of a non-nutrient as well as nutrient liquid meal, and basal and pentagastrin-stimulated gastric acid secretion in awake rats. Ghrelin decreases pentagastrin-induced acid secretion and increases gastric emptying of a non-nutrient liquid while basal acid secretion and gastric emptying of a nutrient liquid were not affected. Moreover, it was provided the existence of motilin, ghrelin and their respective receptors in the myenteric plexus of the guinea pig (96) and suggested that both peptides may play a role in the activation of the enteric nervous system and hence in the regulation of gastrointestinal motility. Thus, ghrelin seems to not only effect food intake but also gastric motor and secretory function indicating a

multifunctional role for ghrelin in energy homeostasis.

Because the gastrointestinal signals that govern food intake are components of a complex integrated and redundant control system, it is not surprising that knockout or natural mutation models often provide inconclusive information as to a peptide's contribution to the regulation of feeding. Smet et al. (72) reported that the role of ghrelin in the regulation of the energy balance was investigated by comparing food intake, respiratory quotient, and heat production between wild-type (ghrelin+/+) and ghrelin knockout (ghrelin-/-) mice. Absence of ghrelin did not affect gastric emptying, and the bell-shaped dose-response curves of the acceleration of gastric emptying by exogenous ghrelin were not shifted between both strains. It may concern that ghrelin is not an essential regulator of food intake and gastric emptying, but its loss may be compensated by other redundant inputs.

Rubino et al. (65) presented that an experimental model of proximal bowel bypass with full preservation of the stomach, restores meal-induced suppression of acylated ghrelin and reduces food intake and weight gain in obese Zucker rats. Intriguingly, the operation does not alter food intake in normal Wistar animals. In obese Zucker rats, refeeding is associated with a paradoxical 30% increase of acylated ghrelin levels over fasting concentrations, suggesting that these obese animals are resistant to the meal-induced decrease of circulating acylated ghrelin. Altogether, these results indicate an intestinal contribution to the regulation of the dynamic ghrelin response to eating and suggest that an abnormal signaling from the proximal bowel may be involved in the pathogenesis of hyperphagia and obesity.

Prader-Willi syndrome (PWS) has a biphasic clinical phenotype with failure to thrive in the neonatal period followed by hyperphagia and severe obesity commencing in childhood among other endocrinological and neurobehavioral abnormalities. Stefan et al. (74) studied a transgenic PWS deletion mouse model established to evaluate endocrinological and metabolic abnormalities. An increase in plasma ghrelin levels occurs in postnatal TgPWS mice and

appears to begin at the onset of severe hypoglycemia but is not directly coincident with hypoinsulinemia. These findings are consistent with known regulators of ghrelin expression and secretion because both glucose and insulin have been shown to suppress ghrelin levels (16). By postnatal day 5, ghrelin levels in TgPWS mice are approximately 3-fold higher than in WT littermates, suggesting that high ghrelin levels in TgPWS might be a physiological adaptive mechanism in an attempt to increase feeding via its actions on the arcuate nucleus (38) to ameliorate the rapidly worsening failure to thrive. However, either this signal is unrecognized due to an unknown mechanism, or it may be too late to elicit a physiological response. The finding of high ghrelin levels in TgPWS mice echoes observations in PWS children and adults, in whom plasma ghrelin levels are 2.5- to 4.5-fold higher than those in normal lean and obese controls (15). PWS patients metabolically do not sense the degree of adipose tissue, and hence their lean body mass is in a starvation state that induces ghrelin production (37). In accordance with high ghrelin levels of obese dog, it may be revealed with morbid body weight condition with in a hyperlipemia or hypoglycemia.

The result of the second chapter demonstrate that the observed increase in BW is significantly correlated to the corresponding plasma acylated ghrelin and GH concentrations, but not desacyl ghrelin during the period of prepubertal growth in beagle dogs. However BW is not closely correlated with plasma acylated ghrelin and GH levels. At present, the only clinical perspective for ghrelin and GHS in the neuroendocrine field is that they could represent a reliable provocative test for the diagnosis of GH deficiency.

The nonacylated form of ghrelin, desacyl ghrelin, also exists at significant levels in both stomach and blood (39). In blood, desacyl ghrelin circulates in amounts far greater than acylated ghrelin. It is often observed that not only active, but also inactive, forms of peptide hormones exist in our body. Because the clearance rates of inactive forms of peptide hormones are often reduced, their half-lives are often longer than those of their respective active forms. Desacyl

ghrelin does not replace radiolabeled ghrelin at the binding sites of acylated ghrelin in hypothalamus and pituitary and showed no GH-releasing and other endocrine activities in rats. Thus one question is whether there is a specific receptor for desacyl ghrelin and whether desacyl ghrelin has specific functions distinct from those of acyl-modified ghrelin. Baldanzi et al. (5) have suggested the existence of another ghrelin receptor in the cardiovascular system. They showed that ghrelin and desacyl ghrelin both recognize common high-affinity binding sites on H9c2 cardiomyocytes, which do not express the ghrelin receptor GHS-R. Moreover, it has been reported that desacyl ghrelin shares with active acyl-modified ghrelin some nonendocrine actions, including the modulation of cell proliferation and, to a small extent, adipogenesis (13). Further study is required to determine whether desacyl ghrelin is biologically active and binds to an as-yet-unidentified receptor.

As another example, despite the third amino acid residue of ghrelin, serine (Ser^3), is modified by an acyl group; this modification is essential for ghrelin biological activity, there has not been any report detailing the mechanism of ghrelin acyl modification. Nishi et al. (53) reported that ingested medium-chain fatty acids (MCFAs) and medium-chain triglycerides serve as a source of fatty acids in the acyl modification of ghrelin. Ingestion of MCFAs (n-hexanoic, n-octanoic, and n-decanoic acid) or medium-chain triglycerides (glyceryl trihexanoate, glyceryl trioctanoate, and glyceryl tridecanoate) increased the stomach concentrations of ghrelin bearing an acyl group with the corresponding carbon chain length, i.e. n-hexanoyl ghrelin, n-octanoyl ghrelin, and n-decanoyl ghrelin. Ingestion of the corresponding faty acids in dogs, however, did not been previously reported. It may be speculated that acyl-modified and desacyl ghrelin with an intact C-terminal peptide sequence was enhanced by ingestion of fatty acids in canine ghrelin production.

It is unclear how information on the nutritional status is transmitted to the ghrelin-producing cells in the stomach and how it affects ghrelin secretion. However, the circulating ghrelin

concentration is known to increase during fasting and to decrease postprandially. The circulating ghrelin concentration is high in situations of nutritional deficiency (starvation) and low in situations of nutritional plenty (free access to food or infusion of nutrients) in rats (61). The actual presence or absence of food in the gastrointestinal tract seems irrelevant. This report also showed that long-term administration of nutrients solution to rats causes hyperlipemia (elevated serum cholesterol, free fatty acids triglycerides and phospholipids) (61) and that the hyperlipemia reflects not only retention of infused lipids in the blood stream but also an altered fat metabolism, resulting in greatly increased serum levels of HDL triglycerides and moderately reduced levels of HDL cholesterol and phospholipids. The findings of obese dogs seem to support the view of long-term infusion rats. It will be required that function and population of endocrine cells in oxyntic mucosa of the stomach are investigated across the rat, dog and human.

By using ghrelin and BMI-28163, a full competitive antagonist of the GHS-R1a receptor, the role of endogenous ghrelin, GH secretion and food intake were monitored in rats (101). Neither peripheral nor central BIM-28163 injection modified GH peak number, GH nadir, or IGF-I levels. In this protocol, food intake is not strongly modified and water intake is unchanged. Subcutaneous infusion of BIM-28163 did not change plasma leptin and insulin levels evaluated at 1200 and 1600 h. On the contrary, central BIM-28163 infusion slightly increased leptin and significantly increased insulin concentrations. Another potential explanation of the discrepant results using BIM-28163 may rely on the central or peripheral location of the ghrelin systems involved. Central ghrelin probably stimulates food intake and GH secretion through neural mechanisms involving neuronal circuitry. However, peripheral ghrelin can also modulate food intake and GH secretion via vagal afferent fibers (22). Blockade of the vagal afferent, either through vagotomy or through perivagal capsaicin application, totally blocks peripheral ghrelin effects on feeding, whereas it only attenuates the stimulation of GH secretion. The fact that BIM-28163 treatment, either icv or sc, does not change feeding behavior is surprising. The

mechanisms by which ghrelin modifies food intake remain to be defined and require further investigation.

As a current hot topic from Medical Letter on the CDC & FDA via NewsEdge Corporation on February 2006, Tranzyme Pharma, a biopharmaceutical company developing therapeutics for treating gastrointestinal and metabolic disorders, announced that the U.S. Food and Drug Administration (FDA) has cleared its investigational new drug application for TZP-101, a new chemical entity originating from Tranzyme's small molecule macrocyclic chemistry. TZP-101 is a selective ghrelin receptor agonist with potent gastroprokinetic properties that represents the first in its class to enter into a clinical trial. Tranzyme Pharma is developing TZP-101 as a mechanism-based therapy for postoperative ileus and other gastrointestinal motility disorders. Animal data suggest that ghrelin accelerates gastric emptying, enhances small bowel transit, and reverses delayed gastrointestinal transit stemming from surgery or opioid therapy. Clinical studies have shown that exogenous administration of ghrelin peptide accelerates gastric emptying and stimulates interdigestive motility in healthy volunteers and gastroparesis patients. In preclinical studies, TZP-101 has shown exceptional in vivo efficacy. The gastroprokinetic activity of TZP-101 has been demonstrated in animal models measuring gastric emptying in naive rats, and in the treatment of rats with delayed gastrointestinal transit caused by high caloric intake (i.e. a model of gastroparesis), abdominal surgery (i.e. a model of postoperative ileus), and pharmacological means (i.e. morphine). Importantly, concurrent studies in rats have demonstrated that TZP-101 does not elicit GH release at gastroprokinetic doses, in contrast to other ghrelin agonists. However crucial mechanism of TZP-101 is unknown for the differential effects between GH release and gastroprokinetic. The phase I trial will be a single-center, randomized, double-blind, placebo-controlled, dose-escalation study designed to evaluate safety, tolerability, pharmacokinetic, and pharmacodynamic parameters of TZP-101. Postoperative ileus is the impairment of gastrointestinal motility that routinely occurs after major surgeries and

contributes significantly to postoperative morbidity, and prolonged hospitalization. The pathophysiology of postoperative ileus is multifactorial and its duration correlates with the degree of surgical trauma.

The wide spectrum of ghrelin actions requires a systematic understanding of its physiology before speculating about potential therapeutic perspectives of diseases and animal industries in which the role, if any, of ghrelin is still unclear. There remain many interesting questions regarding clinical application in veterinary medicine. However, it is clear that the ghrelin story has so far provided us with an impressive amount of knowledge that is changing our understanding of some aspects of neuroendocrinology and even of clinical medicine in human.

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