ORIGINAL ARTICLE

Distribution of ghrelin-producing cells in stomach and the effects of ghrelin administration in the house musk shrew (*Suncus murinus*)

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Abstract

OBJECTIVE: To investigate the distribution of ghrelin, an important appetite regulatory factor related to obesity, in the stomach of *Suncus murinus*, and attempted to elucidate the ghrelin-mediated regulatory effect in this animal.
METHODS: The stomachs of *Suncus murinus* were divided into 5 sections, cardia, fundus, greater curvature, lesser curvature, and pylorus, for investigating the ghrelin-producing cells by immunohistochemistry and Western blotting. Then *Suncus murinus* were randomized into two groups with ghrelin intraperitoneal injection (ghrelin-ip group) and saline intraperitoneal injection (control group), respectively. The effects of food intake and body weight were measured, and furthermore, the distribution of ghrelin in stomach was also investigated by immunohistochemistry and Western blotting.
RESULTS: The immunolocalization and protein levels of ghrelin differed significantly in different regions of the stomach of *Suncus murinus*. Furthermore, ghrelin administration did not change the rate of food intake, but resulted in an increase in body weight compared with the control group.
In this study, we elucidated the distribution of ghrelin-producing cells in the stomach of *Suncus murinus* in detail for the first time. Ghrelin intraperitoneal administration was found to induce an increase in body weight without changing food intake in this species.
CONCLUSION: Our study implied ghrelin showed a different regulatory function in *Suncus murinus* from other species. It is considered that ghrelin may be associated with obesity-resistance phenomenon in *Suncus murinus*.

INTRODUCTION

Insectivores (*Soricidae*) have been considered a primitive group and identified as one of the earliest eutheria by phylogenetic analysis. They are reported to possess features closer to those of primates than rodents on the basis of, for example,

mitochondrial DNA sequences (Oda and Kondo 1976; 1977). The house musk shrew, *Suncus murinus* (*S. murinus*), is one of the most common insectivores, and is distributed widely in Southeast Asia (Yi *et al.* 2003; 2004; 2005; 2007). The Japa-

nese shrew was established as a new laboratory animal species (Oda and Kondo 1976) and has been utilized for studies of cold intolerance, effects of high-fat diet, and brown adipose tissue (Suzuki *et al.* 2006a; 2006b; 2007). Furthermore, our studies and those of other groups have demonstrated that *S. murinus* has a visceral system that is very similar to that of humans and is a useful model of human physiology and pathophysiology (Yi *et al.* 2003; 2004; 2005; 2007).

Morphologically, the stomach of *S. murinus* has a single cavity, no forestomach, and consists of cardia, fundus, greater curvature, lesser curvature, and pylorus regions. The general appearance of the gastric mucosa of *S. murinus* is similar to the human gastric mucosa, and differs from some other widely used experimental animals such as hamsters, rats, and mice (Kanamori *et al.* 1989). Our recent studies identified obesity-resistance phenomenon in *S. murinus*, whose body weight did not change during growth (aging) from 2 months to 12 months old, and less visceral fat accumulated. In particular, mesenteric fat accumulation did not occur in this shrew (Yi *et al.* 2010).

Ghrelin was initially identified in the rat stomach as an endogenous ligand of the growth hormone secretagogue receptor. As a potent appetite stimulant, intracerebroventricular, intravenous and subcutaneous injections of ghrelin have been shown to increase food intake (Tschöp *et al.* 2000; Nakazato *et al.* 2001; Date *et al.* 2002; Kojima and Kangawa 2005). Ghrelin-producing cells have been detected in oxyntic glands of the rat stomach (Kojima *et al.* 1999). Ghrelin mRNA was found to be most highly expressed in the stomach among various human tissues, and the stomach is a major source of circulating ghrelin (Ariyasu *et al.* 2001). It has also been reported that ghrelin is predominantly produced in the stomach of rats and humans (Date *et al.* 2000); however, the distribution of ghrelin in the stomach and its physiological characteristics in *S. murinus* have not been elucidated in detail.

In this study, we investigated the distribution of ghrelin-producing cells in the stomach of *S. murinus*, and attempted to elucidate the effect of ghrelin-mediated regulatory in the species.

MATERIAL AND METHODS

Animals

House musk shrew, *Suncus murinus*

Male laboratory-bred *S. murinus* (4 weeks old, 49.86±2.09 g) were obtained from a closed breeding colony at our laboratories. The mother colony, JIc: KAT-c, is maintained at the Central Institute for Experimental Animals, Nagoya, Japan. The animals were housed and handled in accordance with the *Guide for the Care and Use of Laboratory Animals* and the *Guide for the Care and Use of Experimental Animals* of the Canadian Council on Animal Care. Briefly, all shrews were kept separately after weaning (20 d after birth) in plastic cages equipped with a wooden nestbox containing paper strips in a conventionally conditioned animal room: 28±2°C, no humidity control, and 12:12 h light/dark cycle, lights on at 08:00, with commercial feeding pellets for trout (a specific and natural food of the insectivore *S. murinus*; Oriental Yeast Co., Ltd., Bioindustry Division, Chiba, Japan). The pellets and water were supplied *ad libitum* (Yi *et al.* 2003, 2005, and 2007).

Ghrelin intraperitoneal administration

Eight-week-old male *S. murinus* (n=14) were randomized into two groups with similar body weights (66.35±0.96 g): the ghrelin intraperitoneal injection group (ghrelin-ip group, n=7) and the saline intraperitoneal injection group (control group, n=7); assays were performed according to the manufacturer's protocol. Ghrelin reagent (stored at –20°C; Peptide Institute Inc., Osaka, Japan) was dissolved in distilled water (0.1 mmol/l) and then stored at 4°C. Immediately before the injection, the peptides were diluted in vehicle solution consisting of sterile 0.15 M NaCl solution to a final concentration of 1 nmol/ml. The peptide solution was kept on ice for the duration of the experiment. Ghrelin intraperitoneal administration was performed in accordance with Tschöp *et al.* (2000), Wren *et al.* (2001), and Rüter *et al.* (2003). Briefly, each animal in the ghrelin-ip group of *S. murinus* received an intraperitoneal injection at a dose of 0.25 nmol/every animal, at 17:30 every day for six weeks. The control group received an intraperitoneal injection of saline at 17:30 every day. During ghrelin administration, food intake was monitored every day and body weight was monitored every week in the two groups. After completing the ghrelin administration, the stomachs of the two groups were used for histological analysis (n=4) and Western blotting (n=3).

Tissue preparation for immunohistochemistry

Tissue preparation for immunohistochemistry was performed as previously described (Yi *et al.* 2004). Briefly, 3-, 6- and 12-month-old male *S. murinus* (n=5 each, mean weights 70.45±4.18 g, 74.25±5.02 g and 68.10±4.79 g, respectively) were first anesthetized with ether, and then injected intraperitoneally with urethrane solution (sodium ethylcarbamate, 900 mg/kg). After the animals were completely anesthetized, the abdominal cavity was opened, and a catheter was inserted retrograde into the abdominal aorta immediately above its bifurcation into the common iliac arteries. Perfusion was initiated with normal saline containing heparin (10 IU/ml), and thereafter with 0.01 M PBS (pH 7.4) containing 4% paraformaldehyde (PFA). The whole stomach, including the esophagus-cardia junction and part of the pylorus, was then removed and immersed in 4% PFA at 4°C overnight, and the fixed tissue was then routinely embedded in paraffin wax. Sections (5 μm) were cut and placed on gelatin-coated glass slides.

Similarly, for ghrelin-administered shrews (ghrelin-ip and control groups), after the perfusion fixing, the

stomachs were removed for tissue preparation as above procedures.

Immunohistochemistry

Immunohistochemical procedures were performed as previously described (Yi *et al.* 2004). Briefly, after rinsing the fixed tissue specimens in 0.01 M PBS (pH 7.4), endogenous peroxidase activity was inhibited by 30-min incubation in methanol containing 0.3% (v/v) hydrogen peroxidase. After rinsing in PBS, the sections were blocked with Protein Block Serum-Free (DAKO Cytomation, USA) for 1 h at room temperature (RT), incubated with the primary antibody overnight at 4 °C in a humidified chamber, and then with the secondary antibody for 1 h at RT. Subsequently, the avidin-biotin-complex technique (ABComplex/HRP; DAKO, Denmark) was performed by incubating the sections with ABC complexes for 30 min at RT, and then treating them for 1 min with 3-3-diaminobenzidine and 0.005% H_2O_2, which acted as chromogens. The sections were counterstained with Harris hematoxylin for 50 s, dehydrated in a graded ethanol series and xylene, and mounted under coverslips with Entellan neu (Merck, Germany).

The primary antibody was rabbit anti-rat ghrelin antibody (polyclonal, no. KR069; Trans Genic Inc., Japan), diluted at 1:2 500. The secondary antibody was biotinylated link anti-rabbit and anti-mouse IgG (no. K1491; DAKO Cytomation, USA), diluted to be ready to use.

The control experiments were performed as follows: 1) removal of primary antiserum; 2) substitution of primary antibody with 0.05 M Tris-BSA buffer; and 3) adsorption control: substitution of primary antibody with antiserum pre-adsorbed with various hormones at a concentration of 10^{-6} M. These controls were run on sections at the time of treating with the primary antibody.

Western blotting

Ghrelin protein levels in the stomach were measured by Western blotting in 6-month-old *S. murinus*, as well as in ghrelin-ip and control groups of *S. murinus*. Briefly, after the animals were completely anesthetized (injected intraperitoneally with urethrane solution, sodium ethylcarbamate, 900 mg/kg), the abdominal cavity was opened, and the stomach was removed. For *S. murinus* (no ghrelin treatment), the stomachs were divided into 5 sections, cardia, fundus, greater curvature, lesser curvature, and pylorus, and immediately stored in liquid nitrogen, respectively. However, for ghrelin-ip and control groups, the stomachs were not divided, immediately stored in liquid nitrogen for Western blotting, and the total ghrelin protein levels in the stomach were analyzed.

Western blot analysis was performed as previously reported with certain modifications (Ohta *et al.* 2003). Briefly, snap-frozen tissue from *S. murinus* or rats stomach was homogenized in lysis buffer (10 mM phosphate buffer, pH 7.2, 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 1 µg/ml leupeptin, 1 µg/ml chymostatin). Insoluble material was removed by centrifugation at 12 000 g for 30 min. Approximately 50 µg of cellular protein extract was loaded into a well, separated electrophoretically through a 10% SDS-polyacrylamide gel, and transferred onto Sequi-Blot PVDF membrane (Atta, USA) by electroblotting. BSA/Tween-PBS (1% w/v) in Tween-PBS buffer was used to block filters for 3 hr at 4 °C. A 1:200 dilution of specific primary rabbit polyclonal antibody against ghrelin (polyclonal, NO: F2607, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a 1:200 dilution of rabbit polyclonal anti-β-actin (polyclonal, NO: B1308, Santa Cruz, USA) antibody was added to the membranes for 1 hr at RT. After five washes in blocking buffer, the membranes were incubated with a 1:10 000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (NO: 329616, Amersham Biosciences, UK) diluted as the secondary antibodies for 1 hr at RT. The membranes were finally washed five times in washing buffer; the signals were detected by chemiluminescence using ECL Plus (Amersham Pharmacia Biotech, USA) according to the supplier's recommendations. Thereafter, the developed membrane was exposed to X-ray film (Kodak, Wiesbaden, Germany). Comparisons between different treatment groups were made by determining the ghrelin/β-actin ratio of the immunoreactive area by densitometry.

Western blotting band intensity analyses were presented as raw volume by the software of Image Quant TL analysis toolbox (GE Healthcare, Japan).

Statistical analysis

All sections with ghrelin immunostaining were evaluated. Only nucleated cells with distinct cytoplasmic or surface staining were counted. Positively stained cells were counted in a fixed field of vision. The results are expressed as positive cells /mm² (Singh *et al.* 2004).

Data are presented as the means ± SEM. Repeating measures ANOVA analyses were used to evaluate differences between groups. Relationships between variables were analyzed using student t-test. And the software: JSTAT Version 10.0 is used in our experiment for ANOVA analyses and student t-test. Values of *p*<0.05 were considered significant.

RESULTS

Immunolocalization of ghrelin in the stomach

The distribution of ghrelin immunoreactive cells differed significantly in different regions of the stomach. The region showing the highest density of cells was the fundus (37±5/mm²), followed by the greater curvature (15±2/mm²) and the lesser curvature (10±3/mm²). In cardia and pylorus regions, ghrelin immunoreactive cells were not detected (Figures 1 and 2).
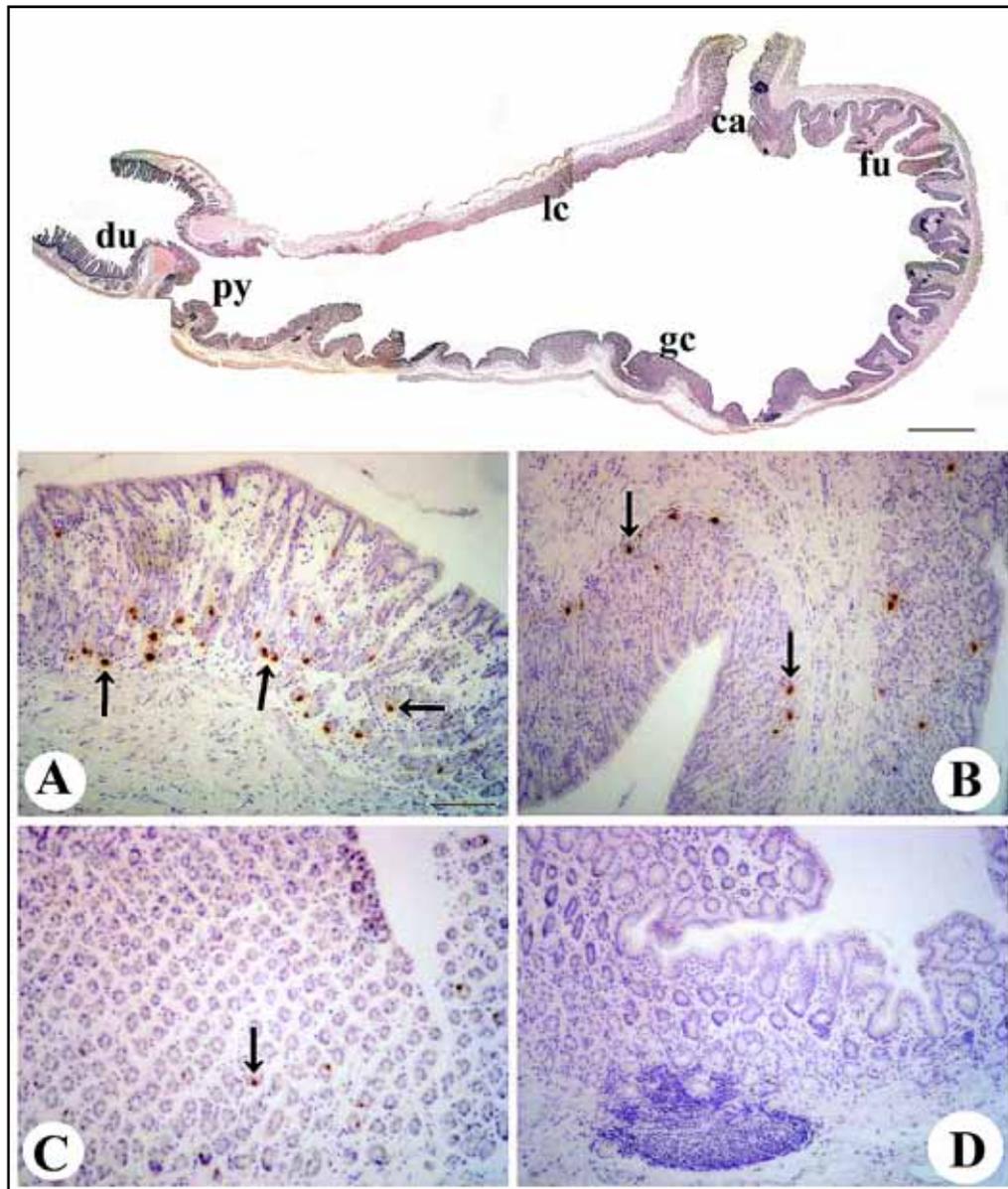
Fig. 1. The longitudinal section of the stomach (upper) and immunolocalization of ghrelin in the stomach (under) of *S. murinus*. A, B, C and D are fundus (fu), greater curvature (gc), lesser curvature lc), and pylorus (py), respectively. Arrows indicated ghrelin immunoreactive cells. ca, cardia; du, duodenum. Scale bars = stomach, 1 000 µm; the magnification of B, C, and D is the same of A , 200 µm.

No immunoreactive cells were detected in the gastric mucosa of *S. murinus* stomach when antisera were absorbed with excessive ghrelin (data not shown).

Ghrelin protein distribution in the stomach by Western blotting

In Western blotting analyses, the highest concentration of ghrelin protein level was found in the fundus, in which Western blotting band intensity analysis gave a value of 9.50×10^3 raw volume, followed by the greater curvature and lesser curvature, with corresponding values of 6.76×10^3 and 5.50×10^3 raw volume, respectively (Figure 3).

Effects on food intake, body weight, and expression of ghrelin in the stomach of S. murinus after intraperitoneal administration of ghrelin

In ghrelin-ip group, after 6 weeks of intraperitoneal administration of ghrelin, food intake did not markedly change compared with the control group ($p > 0.05$; Figure 4A); however, body weight gain showed a slight increase three weeks after injection; 5 weeks after injection, the increment rose to 3.88%; and 6 weeks after injection, it rose to 5.37% (Figure 4B).

Furthermore, in the ghrelin-ip group, the number of ghrelin immunoreactive cells (28 ± 3/mm^2) was clearly reduced compared with the control group (58 ± 6/mm^2).

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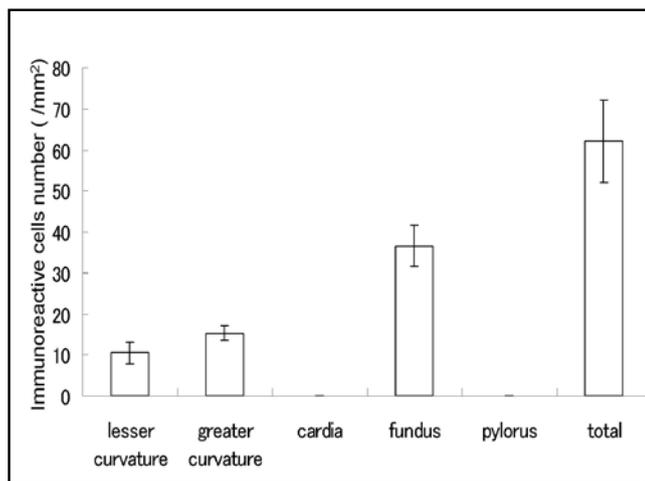
Fig. 2. Relative quantitation of ghrelin-producing cells in the stomach of *S. murinus* (/mm²) from the immunoreaction. Data are presented as the mean ± SEM. ** ($p<0.01$).
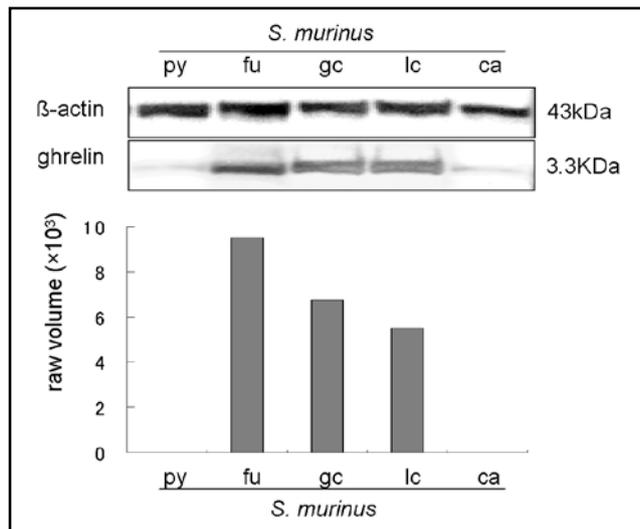
Fig. 3. Levels of ghrelin in the stomach (st) of *S. murinus* by Western blotting. Western blotting band intensity analysis results are also presented. ca, cardia; fu, fundus; gc, greater curvature; lc, lesser curvature; py, pylorus.
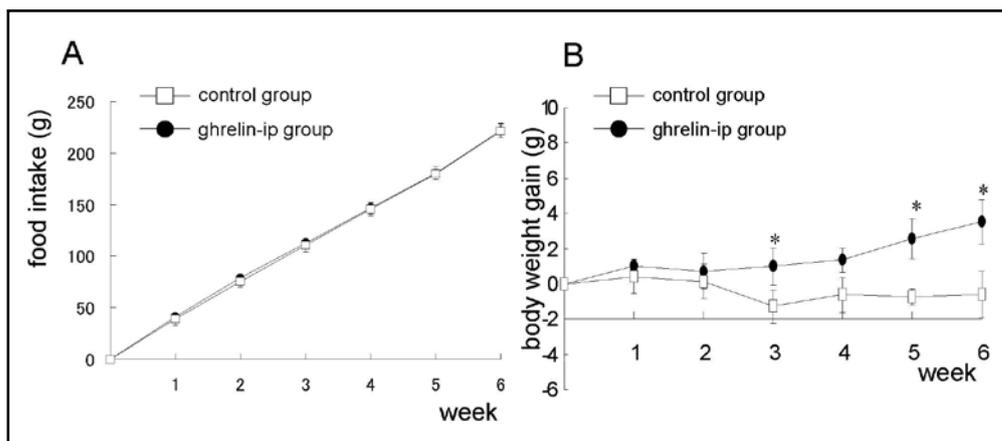
Fig. 4. Effects on food intake (g) (A) and body weight (g) (B), respectively, in *S. murinus* after ghrelin intraperitoneal administration. Data are presented as the mean ± SEM. * ($p<0.05$).

In particular, in the fundus region, the number of immunoreactive cells was 35 ± 2/mm² in the control group but only 15 ± 3/mm² in the ghrelin-ip group (Figure 5).

Moreover, in Western blotting analysis, the level of ghrelin protein in the ghrelin-ip group was found to have decreased markedly compared with that in the control group in overall (Figure 6). Western blotting band intensity analysis gave values of 15.57×10^3 and 5.11×10^3 raw volume in the whole stomach of the control group and the ghrelin-ip group, respectively (Figure 6).

DISCUSSION

In this study, the distribution of ghrelin-producing cells in the stomach of *S. murinus* was investigated for the first time. It was found that the distribution of ghrelin immunoreactive cells differed significantly in different regions of the stomach in *S. murinus,* the region with the highest concentration was the fundus, followed by the greater curvature and the lesser curvature; Western blotting analysis also showed the same results. Date *et al.* reported that ghrelin cells were distributed from the neck to the base of the rat oxyntic gland, were infrequent in the pyloric gland, and that the glandular stomach of rats was divided into the fundus and pylorus (Date *et al.* 2000); however, other regions, including the greater and lesser curvatures, were not mentioned. Lee *et al.* also reported that ghrelin production occurs in the rat gastrointestinal tract with the highest ghrelin expression in the rat stomach fundus (Lee *et al.* 2002). However, the stomach of rats consists of forestomach and pars glandularis, and the forestomach contains the fundus and part of the greater curvature, which has no gastric glands, and the mucosal layer is covered by stratified squamous epithelia (Kurohmaru 1985). It is
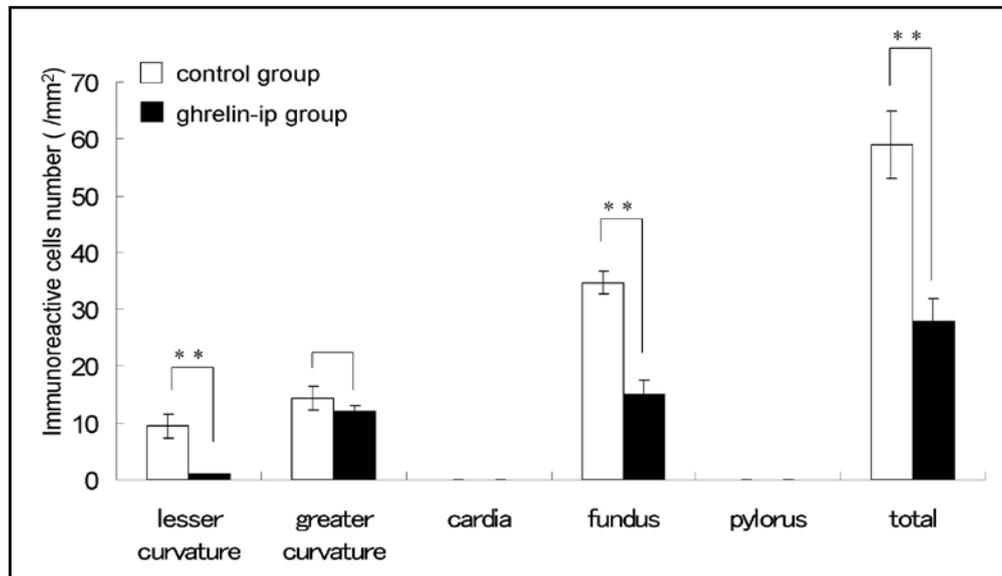
Fig. 5. Changes in number (/mm²) of ghrelin-producing cells in the stomach of *S. murinus* after 6 weeks of ghrelin intraperitoneal administration by immnuoreaction. Data are presented as the mean ± SEM. ***** (*p*<0.05).

possible that the greater curvature was confused with the fundus in rats in above reports.

It this study, the distribution of ghrelin-producing cells or ghrelin protein levels in the stomach of *S. murinus* showed different from other species (Ariyasu *et al.* 2001; Date *et al.* 2000; Lee *et al.* 2002). It was considered that it may have different stimulatory effects on food intake and body weight in these species. To date, although the fact that ghrelin administration can increase food intake and body weight in rats and other species has been demonstrated (Tschöp *et al.* 2000; Nakazato *et al.* 2001; Date *et al.* 2002), its effects on *S. murinus* have remained unknown.

In this study, food intake did not markedly change after ghrelin administration in *S. murinus*, while body weight increased slightly and the concentration of ghrelin in the stomach decreased after ghrelin administration. Hayashida *et al.* reported that ghrelin concentration in the stomach decreased, while it increased in plasma (Hayashida *et al.* 2002). This inverse relationship between the ghrelin concentrations in the blood and stomach might be due to the increased plasma ghrelin concentration inhibiting the expression of ghrelin in the stomach. Intraperitoneal ghrelin administration was found to increase the plasma ghrelin concentration, and then ghrelin directly reached the paraventricular nucleus (Shimbara *et al.* 2004), causing ghrelin expression in the stomach to be reduced.

Although food intake did not clearly change after intraperitoneal administration of ghrelin to *S. murinus*, body weight showed a slight increase. Tschöp *et al.* (2000) also reported that ghrelin treatment did not change food intake in wild-type mice, while body weight gain was induced. Recent data also indicated that ghrelin acts in the hypothalamus by altering fatty
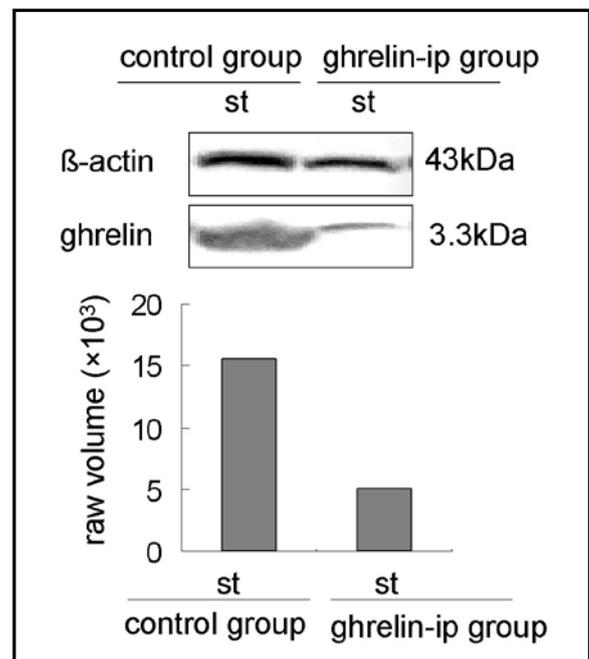
Fig. 6. Changes in level of ghrelin in the stomach (st) of *S. murinus* after 6 weeks of ghrelin intraperitoneal administration by Western blotting analysis. Western blotting band intensity analysis results are presented on the right.

acid metabolism (Mendieta-Zerón *et al.* 2008). It is considered that ghrelin intraperitoneal administration also induced body weight gain through metabolic changes in *S. murinus*. The metabolic changes induced by ghrelin led to an efficient metabolic state resulting in increased body weight despite food intake remaining constant (Tschöp *et al.* 2000).

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Furthermore, body weight may also have been induced by visceral fat accumulation in *S. murinus*, especially in the retroperitoneum and epididymis. This will be investigated in further experiments by *in vivo* micro-computerized tomography to automatically quantify visceral and subcutaneous fat distribution throughout the body.

In conclusion, the distribution of ghrelin-producing cells in the stomach of *S. murinus* was studied in detail. The ghrelin-mediated regulatory effect was also investigated by the ghrelin intraperitoneal administration in this shrew, although the mechanism of the ghrelin-mediated regulatory effect in *S. murinus* requires further investigation. As an important appetite regulatory peptide, ghrelin may be associated with obesity-resistant phenomenon in *S. murinus*.

ACKNOWLEDGEMENTS

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