Involvement of NGF in the Rat Model of Persistent Muscle Pain Associated With Taut Band

Immunolocalization of Ghrelin in the Stomach of Sprague-Dawley Rat

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Running title: Ghrelin in the rat stomach

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Summary

To investigate the distribution of ghrelin in different regions of stomach in Sprague-Dawley rat, and attempt to compare with those of humans and other mammalian species in the present study, the stomach of rats was divided into five sections, cardia, fundus, greater curvature, lesser curvature and pylorus. Immunohistochemistry and Western blotting were performed to investigate the ghrelin-producing cells. The immunolocalization and protein levels of ghrelin differed significantly in different regions of stomach in rats. It was present at a high level in the greater curvature of the pars glandularis, and the lesser curvature. In the fundus and pylorus, no ghrelin immunoreactive cells were detected. In this study, we elucidated the distribution of ghrelin-producing cells in different regions of rat stomach in detail for the first time. It is further considered that the differences of ghrelin distribution in stomach of different species may induce different stimulatory effects on fat accumulation and metabolism.

Key words: Ghrelin; Stomach; Greater curvature; Immunolocalization; Sprague-Dawley rat

Introduction

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 (Date et al., 2002; Kojima and Kangawa, 2005; Nakazato et al., 2001; Tschöp et al., 2000; Wren et al., 2001). 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, indeed 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; Yakabi et al., 2008; Zhao and Sakai, 2008). However, the immunolocalization in the different regions of stomach in rats has not been elucidated in detail. Moreover, some inconsistent reports still exist about the immunolocalization of ghrelin in the stomach of rats and other species.

Morphologically, in rats, the stomach consists of forestomach and pars glandularis. 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), and not occurring oxyntic glands of stomach.

In this study, to investigate the distribution of ghrelin-producing cells in the different

regions of stomach in rats, the stomach was divided into cardia, fundus, greater curvature, lesser curvature and pylorus as humans. We attempted to compare with those of humans and other mammalian species, and discuss from functional viewpoint.

Materials and Methods

Animals

Male Sprague-Dawley rats (3- to 6-months old, body weight: 350-450 g) purchased from Clea-Japan, Inc. (Tokyo, Japan) were used in the experiment. 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. All rats were housed under controlled illumination (25±2 ^{o}C, 12:12 h light/dark cycle, lights on at 08:00) with standard F-2 chow (Nippon Funabashi Farm Co., Ltd., Chiba, Japan), and the pellets and water were supplied *ad libitum*.

All protocols for animal care and experimental procedures (including euthanasia) were reviewed and approved by the Animal Care Committee of Tokyo Medical University and were in accordance with the National Institute of Health guidelines.

Tissue preparation for immunohistochemistry

Tissue preparation for immunohistochemistry was performed as previously described (Yi et

al., 2004). Briefly, rats (n = 10) were first anesthetized with ether and then injected intraperitoneally with urethrane solution (sodium ethylcarbamate, 900 mg/kg). After the animals were completely anesthetized, perfusion was initiated with normal saline 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 divided into 5 sections, cardia, fundus, greater curvature, lesser curvature and pylorus, and then immersed in 4% PFA at 4 ^{o}C overnight. The fixed tissue was then routinely embedded in paraffin wax. The sections (5 μm) were cut and placed on gelatin-coated glass slides.

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, x0909, USA) for 1 h at room temperature (RT), incubated with the primary antibody overnight at 4 ^{o}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, k355, 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, Dannstadi, Germany).

The primary and secondary antibodies were rabbit anti-rat ghrelin antibody and biotinylated link anti-rabbit IgG, respectively, details described in Table 1.

The control experiments were performed as follows: (1) removal of primary antiserum; (2) substitution of primary antibody with 0.05 M Tris-BSA buffer. The controls were run on sections at the time of treating with the primary antibody.

Western blotting

Ghrelin protein levels in the rat stomach (n = 5) were measured using Western blotting. After the animals were completely anesthetized, the abdominal cavity was opened and the stomach was removed. The stomach of rat was divided into five sections, cardia, fundus, greater curvature, lesser curvature and pylorus and immediately stored in liquid nitrogen.

Western blotting analysis was performed as previously reported with certain modifications (Ohta et al., 2003). Briefly, snap-frozen tissue from rat 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 12000 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, Tokyo, Japan) by electroblotting. BSA/Tween-PBS (1% w/v) in Tween-PBS buffer was used to block filters for 3 hr at 4 °C. The primary antibody was added to the membranes for 1 hr at RT. After five washes in blocking buffer, the membranes were incubated with 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, Chicago, IL, USA) according to the supplier's recommendations. Thereafter, the developed membrane was exposed to X-ray film (Kodak, Wiesbaden, Germany). Ghrelin protein level measuring was made by determining the ghrelin/ß-actin ratio of the immunoreactive area by densitometry.

The primary and secondary antibodies were polyclonal rabbit anti-rat ghrelin/polyclonal rabbit anti-ß-actin and horseradish peroxidase-conjugated anti-rabbit IgG, respectively, the details described in Table1. Western blotting band intensity analyses were presented as raw volume by the software of Image Quant TL analysis toolbox (GE Healthcare, Tokyo, 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 /mm^2 (Singh et al., 2004).

Data are presented as the means ± SEM. Repeating measures ANOVA analyses were used to perform the test by JSTAT (version 10.0, Tokyo, Japan) software.

Results

Immunolocalization of ghrelin in the stomach

In the Sprague-Dawleg rats, the region showing the highest density of ghrelin immunoreactive cells was the lesser curvature (67 ± 7/mm^2), followed by the greater curvature of the pars glandularis (48 ± 3/mm^2). In the fundus (0 ± 0/mm^2) and pylorus (0 ± 0/mm^2), no ghrelin immunoreactive cells were detected (Figs. 1 and 2).

No immunoreactive cells (0 ± 0/mm^2) were detected in the gastric mucosa of rat stomach when antisera were absorbed with excessive ghrelin.

Ghrelin protein distribution in the stomach by Western blotting

In Western blotting analyses, the regions showing the highest concentration of ghrelin protein level occurred at the lesser curvature and greater curvature of the pars glandularis, with values of 19.31×10^3 and 17.31×10^3 raw volume respectively. While in the fundus, pylorus and

149 cardia, no ghrelin protein was detected (Fig. 3).

150

151 ## Discussion

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153 In this study, the distribution of ghrelin-producing cells in the different regions of stomach in
154 Sprague-Dawleg rats was investigated in detail. It was found that ghrelin-producing cells
155 were absent throughout the fundus, and presented at a high level in the greater curvature of
156 the pars glandularis, and the lesser curvature; and Western blotting analysis also showed the
157 same results.

158 Regarding the immunolocalization of ghrelin in the different regions of stomach in rats,
159 Lee et al. (2002) reported the highest ghrelin expression in the rat stomach fundus. Sakata et
160 al. (2002) also reported that distribution of ghrelin-producing cells appeared in fundic region
161 of stomach in postnatal rats. However, the fundus of rats is located in the forestomach, which
162 has no gastric glands, and the mucosal layer is covered by stratified squamous epithelia in rats
163 (Kurohmaru, 1985). It is possible that the greater curvature was confused with the fundus in
164 rats in these reports. Katayama et al. (2007) reported that mRNA of ghrelin was expressed
165 predominantly in the lower body and pyloric gland of rat stomach, but little or not in the
166 upper body and cardia. Date et al. (2000) also reported that ghrelin cells were distributed from
167 the neck to the base of the rat oxyntic gland and were infrequent in the pyloric gland. In their

report, the glandular stomach of rats was divided into the fundus and pylorus; other regions, including the greater and lesser curvatures, were not mentioned in detail.

Except for rats, distribution of ghrelin-producing cells in stomach of the humans and other mammalian species has also been reported. Recently, Stengel et al. (2010) reported ghrelin-O-acyltransferase-immunoreactive cells were mainly distributed throughout the middle portion of the oxyntic glands in mice, whereas in rats they were localized mainly in the lower portion of the glands. In humans, it was also reported that the distribution of ghrelin-immunoreactive cells were found in all regions of stomach, but mainly in the fundic region (Tanaka-Shintani and Watanabe, 2005). The results were similar to our recent study in the house musk shrew, *Suncus murinus*, one of the most common insectivore species.

Furthermore, our recent study has demonstrated *Suncus murinus* has natural obesity-resistant phenomenon (Yi et al., 2010; Li et al., 2010), and it was considered that the different distribution in different regions of stomach in this shrew may be associated with obesity-resistant phenomenon, the relation with fat accumulation and metabolism will need to be investigated further in rats.

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253

Figures legends

Fig. 1. The longitudinal section of the stomach (upper) and immunolocalization of ghrelin in the stomach (under) of Sprague-Dawleg rat. Regions locating at the right and left of the line (in longitudinal section) show the forestomach (fs) and pars glandularis (pg) respectively. (a) fundus, (b) greater curvature, (c) lesser curvature, (d) pylorus. Arrows indicate ghrelin immunoreactive cells. ca, cardia; du, duodenum; fu, fundus; gc, greater curvature; lc, lesser curvature; and py, pylorus. Scale bars = stomach, 1200 μm; fu and others, 200 μm.

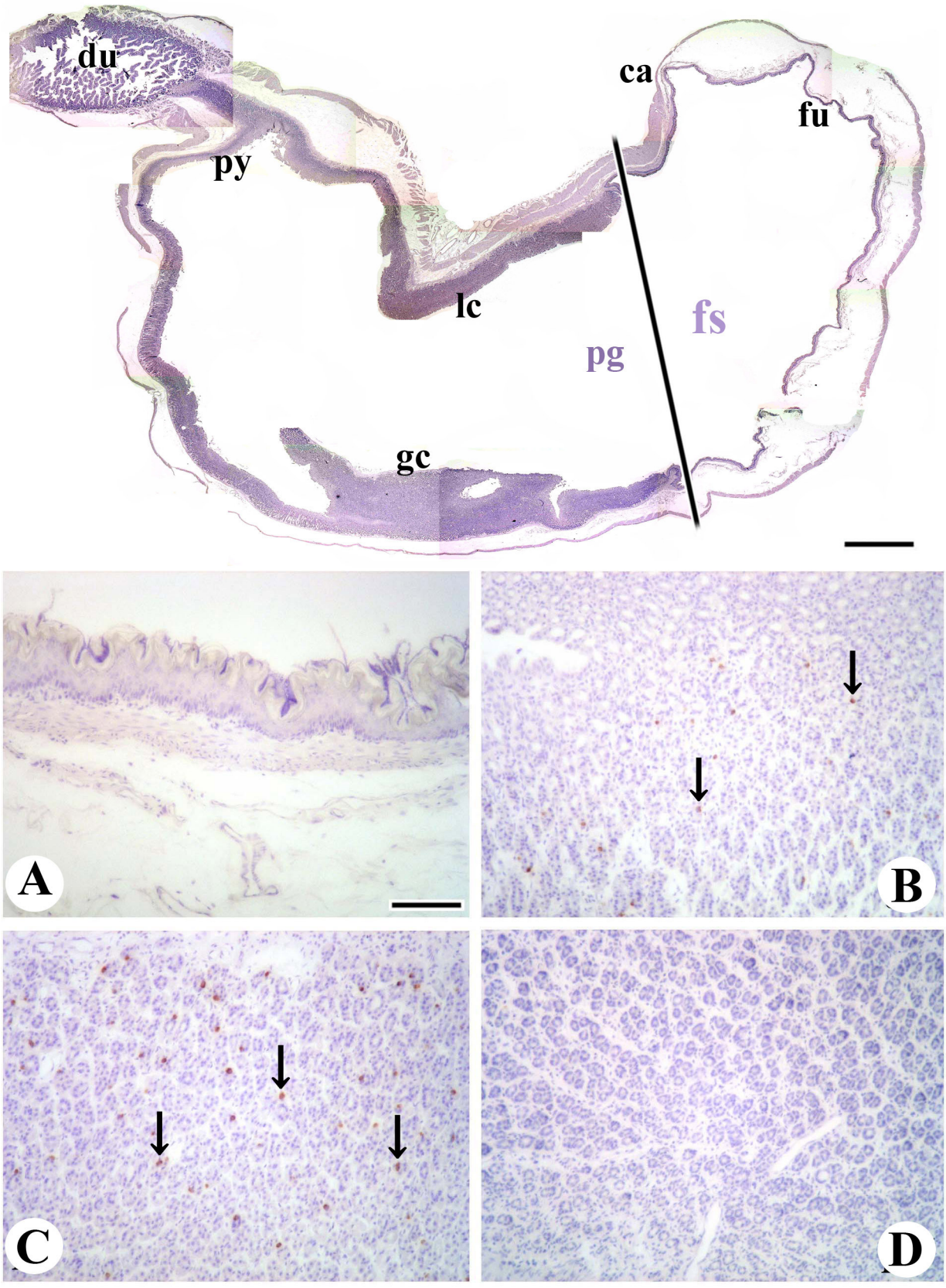
Fig. 2. Relative quantitation of ghrelin-producing cells in the stomach of Sprague-Dawleg rats (/mm^2) from the immunoreaction. Data are presented as the mean ± SEM.

Fig. 3. Levels of ghrelin in the stomach of Sprague-Dawleg rats by Western blotting. Western blotting band intensity analysis results are also presented. st, stomach; ca, cardia; fu, fundus; gc, greater curvature; lc, lesser curvature; and py, pylorus.

Table 1. Antibodies used in this study.

Antibodies	Code	Source	Dilution	Purpose
Rabbit anti-rat ghrelin	No: KR069	Trans Genic Inc., Japan	1:2500	IHC
Biotinylated link anti-rabbit IgG	No: K1491	DAKO Cytomation, USA	Ready to use	IHC
Rabbit polyclonal anti-rat ghrelin	No: F2607	Santa Cruz Biotechnology, USA	1:200	WB
Rabbit polyclonal anti-ß-actin	No: B1308	Santa Cruz Biotechnology, USA	1:200	WB
Horseradish peroxidase-conjugated anti-rabbit IgG	No: 329616	Amersham Biosciences, UK	1:10000	WB

IHC, Immunohistochemistry; WB, Western blotting.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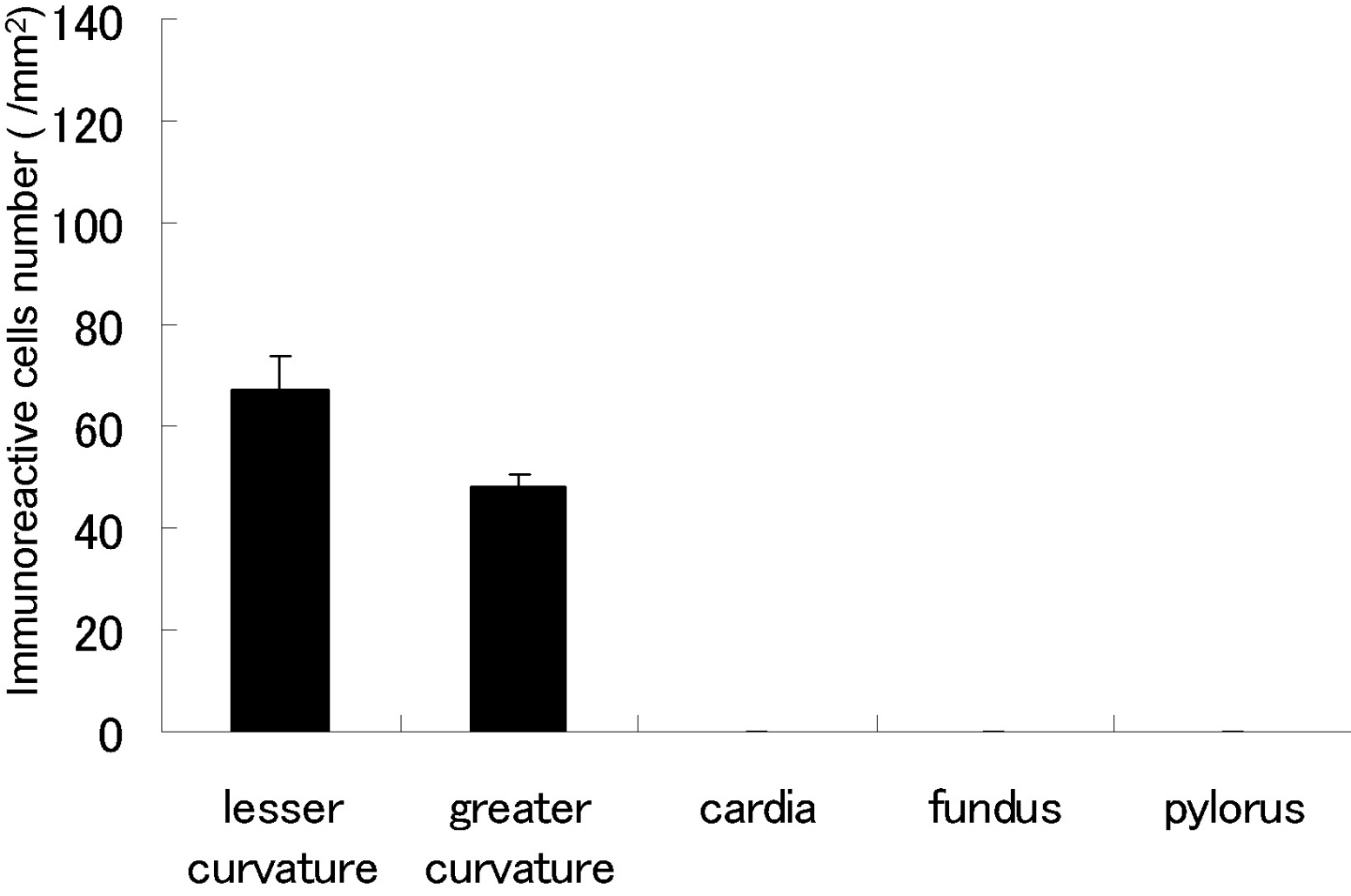
Immunoreactive cells number (/mm2)
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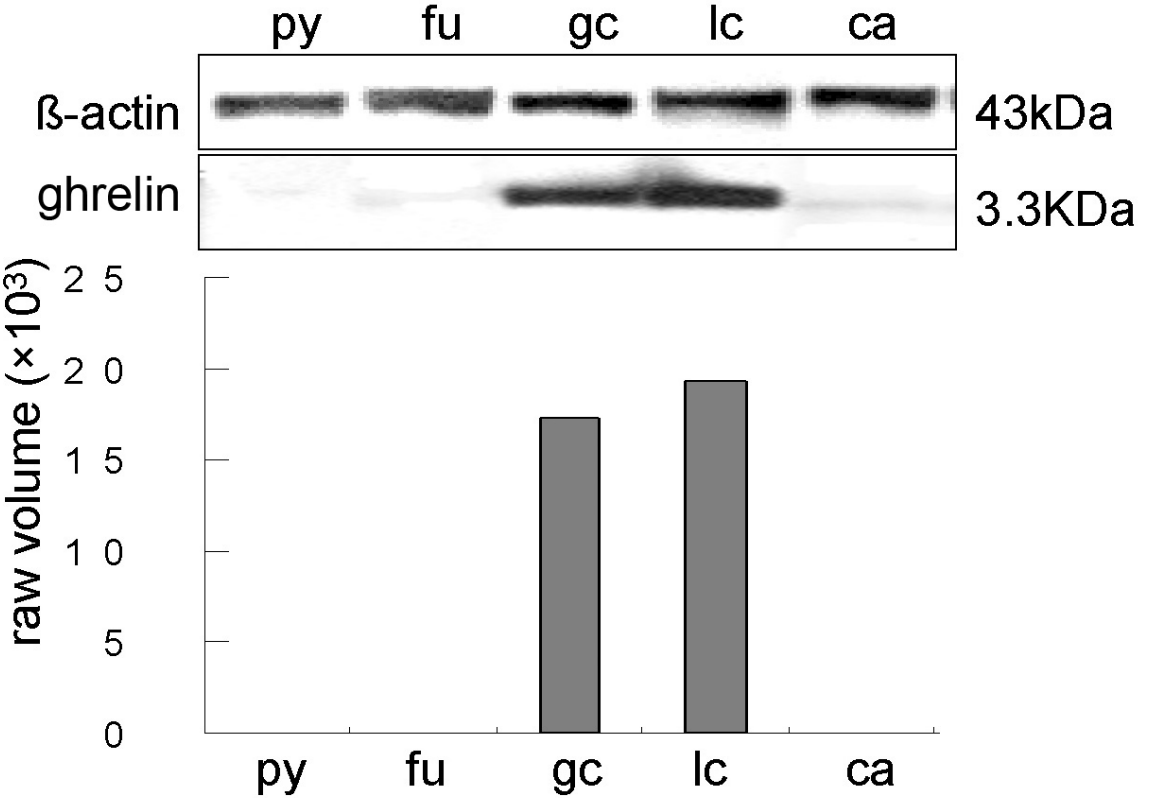
120
100
80
60
40
20
0
lesser curvature
greater curvature
cardia
fundus
pylorus


py
fu
gc
lc
ca
ß-actin
43kDa
ghrelin
3.3KDa
raw volume (×10³)
25
20
15
10
5
0
py
fu
gc
lc
ca