TRANSGENIC EXPRESSION OF GHRELIN IN PANCREAS

Summary

We developed and analyzed two types of transgenic mice: rat insulin II promoter–ghrelin transgenic (RIP-G Tg) and rat glucagon promoter-ghrelin transgenic mice (RGP-G Tg). The pancreatic tissue ghrelin concentration measured by C-terminal RIA and plasma des-acyl ghrelin concentration of RIP-G Tg were about 1,000 times and 3.4 times higher than those of nontransgenic littermates, respectively. The pancreatic tissue n-octanoylated ghrelin concentration measured by N-terminal RIA and plasma active ghrelin concentration of RIP-G Tg were not distinguishable from those of nontransgenic littermates. RIP-G Tg showed suppression of glucose-stimulated insulin secretion. Arginine-stimulated insulin secretion, pancreatic insulin mRNA and peptide levels, β cell mass, islet architecture, GLUT2 and PDX-1 immunoreactivity in RIP-G Tg pancreas were not significantly different from those of nontransgenic littermates. Islet batch incubation study did not show suppression of insulin secretion of RIP-G Tg in vitro. Insulin tolerance test showed lower tendency of blood glucose levels in RIP-G Tg. Taking lower tendency of triglyceride level of RIP-G Tg into consideration, these results may indicate that the suppression of insulin secretion is likely due to the effect of des-acyl ghrelin on insulin sensitivity. RGP-G Tg, in

which the pancreatic tissue ghrelin concentration measured by C-RIA was about 50 times higher than that of nontransgenic littermates, showed no significant changes in insulin secretion, glucose metabolism, islet mass and islet architecture. The present study raises the possibility that des-acyl ghrelin may have influence on glucose metabolism.

TRANSGENIC EXPRESSION OF GHRELIN IN PANCREAS

Introduction

Ghrelin is a 28-amino-acid peptide with unique modification of acylation, which is essential for its biological action (1). Ghrelin was originally identified in rat stomach as an endogenous ligand for an orphan receptor, which has been so far called growth hormone secretagogue receptor (GHS-R) (1). Ghrelin expression is detected in the stomach, intestine, hypothalamus, pituitary gland, kidney, placenta and testis (2-6). Ghrelin is involved in a wide variety of the functions including the regulation of growth hormone (GH) release, food intake, gastric acid secretion, gastric motility, blood pressure and cardiac output (7-19).

Recently Date et al. reported that ghrelin is present in α cells of normal human and rat pancreatic islets (20). Volante et al. described ghrelin-expression in β cells of human islet (21). Wierup et al. and Prado et al. reported that ghrelin expressing cell is a new islet cell type distinct from α, β, δ and PP cells in human, rat and mouse islets (22-24). Although there was no apparent change of plasma insulin levels in ghrelin null mouse (25,26), which may indicate that ghrelin is not a direct regulator of insulin secretion in the physiological condition, there have been several reports of the effect of pharmacological dose of ghrelin on insulin secretion. Broglio et al., Egido et al. and Reimer et al. have reported that ghrelin has inhibitory effect on insulin secretion (27-30).

Adeghate et al., Date et al. and Lee et al. have reported that ghrelin stimulates insulin secretion (20,31,32). Salehi et al. has reported ghrelin has both inhibitory and stimulatory effects depending on its concentration (33). Therefore, there is still a lot of controversy about the localization of ghrelin in the pancreas and the effects of ghrelin on the insulin secretion. As for the effects of des-acyl ghrelin on insulin secretion, Broglio et al. has reported that acute des-acyl ghrelin administration has no effect on insulin secretion in human (34), but that it counteracts the inhibitory effect of n-octanoylated ghrelin on insulin secretion when co-administrated with n-octanoylated ghrelin (35).

Here we developed and analyzed two types of transgenic mice: rat insulin II promoter-ghrelin transgenic mice (RIP-G Tg) and rat glucagon promoter-ghrelin transgenic mice (RGP-G Tg). The purpose of this study was to clarify the effect of transgenic overexpression of ghrelin cDNA in pancreatic islets.

Experimental Procedures

Generating RIP- and RGP-ghrelin transgenic mice. Mouse stomach cDNA library was constructed from 1 μg of mouse stomach poly $(A)^+$ RNA with cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Mouse ghrelin cDNA was isolated from this library, using rat ghrelin cDNA as a probe. A fusion gene comprising RIP and mouse ghrelin cDNA coding sequences was designed. The purified fragment (10 μg/ml) was microinjected into the pronucleus of fertilized C57/B6J mice (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) using standard techniques. Transgenic founder mice were identified by Southern blot analysis of tail DNAs using the mouse ghrelin cDNA fragment as a probe. RGP-G Tg was generated similarly. Transgenic mice were used as heterozygotes. Animals were maintained on standard rat food (CE-2, 352 kcal/100 g, Japan CLEA, Tokyo, Japan) on a 12 h light/12 h dark cycle. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain "ABC" Elite kit, Vector Laboratories, Burlingame, CA) as described previously (36). Serial

sections were used and the thickness of each section was 5 μm. Sections were incubated with anti-C-terminal ghrelin [13-28] (1:1000 at final dilution), anti-N-terminal ghrelin [1-11] (1:2000) (1) that recognizes the n-octanoylated portion of ghrelin, anti-glucagon (1:500), anti-insulin (1:500), anti-somatostatin (1:500), anti-pancreatic polypeptide (PP) (1:500) (DAKO, Glostrup, Denmark), anti-PDX-1 (1:2000) (kindly provided by Christopher V. E. Wright)(37) and anti-GLUT2 (1:200) (kindly provided by Bernard Thorens)(38) antisera. Quantification of β cell area was performed in insulin-stained sections by using Axio Vision (Carl Zeiss, Hallbergmoos, Germany) and Scion Image (Scion Corporation, Frederick, MD). Ten sections (200μm interval) for each mouse (n=5) were analyzed. The percentage of β cell area in the pancreas was determined by dividing the area of all insulin-positive cells in one section by the total area of the section.

Measurements of plasma and tissue ghrelin concentrations. Plasma was sampled from 10 weeks old RIP-G Tg and their nontransgenic littermates under ad-lib feeding states considering the promoter activity. From RGP-G Tg and their littermates, it was sampled after overnight fast. Blood was withdrawn from the retroorbital vein or the proximal end of the portal vein under ether anesthesia, immediately transferred to chilled siliconized glass tubes containing Na_2EDTA (1mg/ml) and aprotinin (1000

KIU/ml, Ohkura Pharmaceutical, Kyoto, Japan), and centrifuged at 4℃. Hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after separation of plasma. Plasma was immediately frozen and stored at −80℃ until assay. Plasma ghrelin concentration was determined by des-acyl ghrelin ELISA kit and active-ghrelin ELISA kit that recognizes n-octanoylated-ghrelin (Mitsubishi Kagaku Iatron, Tokyo, Japan).

As for measurement of tissue ghrelin concentration, pancreata or stomachs were taken from the 8 weeks old male mice. The rumen was removed from the stomach. Samples were diced and boiled for 5 minutes in the 10-fold v/w of water. Acetic acid was added to each solution so that the final concentration was adjusted to 1M, and the tissues were homogenized. The supernatants were obtained after centrifugation. Tissue ghrelin concentration was determined by radioimmunoassay (RIA) using anti-ghrelin [13-28] antiserum (C-RIA) and anti-ghrelin [1-11] antiserum (N-RIA) as described previously (39).

Measurements of body weight and food consumption. Mice were housed individually and were allowed free access to standard rat chow. Body weights of mice were measured weekly. Daily food intake was measured by weighing the pellets between 9:00 and 10:00 AM.

Measurements of % body fat and visceral/subcutaneous fat mass ratio (V/S ratio). Forty weeks old mice were anesthetized with pentobarbital. Percent body fat and visceral/subcutaneous fat mass ratio (V/S ratio) of mice were measured by Latheta LTC-100 (ALOKA, Tokyo, Japan).

Glucose and insulin tolerance tests. For the glucose tolerance test (GTT), after overnight fast, the mice were injected with 1.5 g/kg glucose intraperitoneally. For the insulin tolerance test (ITT), after 4 hours fast, mice were injected with 2.0 mU/g human regular insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) intraperitoneally. Blood was sampled from the tail vein before and 15, 30, 60, 90, and 120 min after the injection. Blood glucose levels were determined by glucose oxidase method using Glutest sensor (Sanwa Kagaku, Kyoto, Japan).

Insulin release. After overnight fast, the mice were injected with 3.0 g/kg glucose or 0.25 g/kg L-arginine intraperitoneally. Plasma was sampled from the tail vein before and 2, 5, 15, 30, and 60 min after the injection using heparin coated tubes. The measurement of insulin concentration was carried out by ELISA using ultra sensitive rat insulin kit (Morinaga, Yokohama, Japan).

Pancreatic insulin concentration. As for measurement of pancreatic insulin concentration, pancreata were obtained from the mice under the ether anesthesia and

homogenized in acid-ethanol. The supernatants were used for assay after centrifugation.

Batch incubation of islet Under the pentobarbital anesthesia, Type IV collagenase (Worthington, Lakewood, NJ) dissolved in Hank's balanced salt solution (HBSS) (1.5 mg/ml) was injected into mouse pancreatic duct. Pancreas was removed and incubated at 37 ℃ for 14 minutes. After washing out collagenase by HBSS, islets were collected by Ficoll gradient and manually picked up so that the sizes of the islets were equal. Islets were incubated at 37 ℃ in RPMI1640 containing 10% FCS for 2 hours and then in Krebs-Ringer bicarbonate buffer (KRBB) containing 3.3 mM glucose and 0.2 % bovine serum albumin (BSA) for 30 minutes. 5 islets were incubated at 37 ℃ in 500 μl of KRBB containing 0.2% BSA and 3.3 or 8.7 or 16.7 mM glucose for 1 hour. After centrifugation, the supernatants were collected. Insulin concentrations in supernatants were determined by rat insulin kit (Morinaga, Yokohama, Japan).

Northern blot analysis and real time quantitative RT-PCR. Total RNA was extracted from pancreata using RNeasy mini kit (QIAGEN K.K., Tokyo, Japan). Filters containing 5 μg of total RNA were prepared. Northern blot analyses were performed as described previously (36) using the mouse insulin II cDNA and human β-actin cDNA (Clontech, Palo Alto, CA) as probes. To confirm that approximately

equal amount of total RNA was assayed in Northern blot hybridization analysis, the density of 18S rRNA in the gel and signal of β-actin in each lane was used. The hybridization signal intensity was quantitated using an image analyzer BAS-2500 (Fuji Photo Film, Tokyo, Japan). Reverse transcription (RT) was performed with random hexamer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real time quantitative PCR was performed with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The following primers and taqman probe were used: mouse GHS-R sense, 5 - CACCAACCTCTACCTATCCAGCAT-3 ; antisense, 5 - CTGACAAACTGGAAGAGTTTGCA-3 ; taqman probe, 5 -TCCGATCTGCTCATCTTCCTGTGCATG-3 , mouse ghrelin sense, 5'-GCATGCTCTGGATGGACATG-3'; antisense, 5'-TGGTGGCTTCTTGGATTCCT-3'; taqman probe, 5'-AGCCCAGAGCACCAGAAAGCCCA-3'.

Lipid measurements. Blood was collected from retroorbital vein of 35 weeks old RIP-G Tg and their nontransgenic littermates. After separation of serum, total cholesterol, triglyceride, free fatty acid, HDL-cholesterol levels in serum were determined by Cholesterol E-test Wako, Triglyceride E-test Wako, NEFA C-test Wako and HDL-cholesterol E-test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis. All values were expressed as means ± SE. Statistical significance of difference in mean values was assessed by repeated measures ANOVA or Student's t-test.

Results

Distribution of ghrelin in normal mouse pancreas. We first examined which cell type of islet cells expresses ghrelin in mouse by immunohistochemistry using anti-C-terminal ghrelin antiserum. In the most of the islets no ghrelin-like immunoreactivity was detected. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice (Fig.1A). Most of the ghrelin positive cells were also glucagon positive by serial section analysis (Fig.1 B) while most of the glucagon positive cells were not ghrelin positive.

Generation of RIP- and RGP-ghrelin transgenic mice. A fusion gene comprising RIP and mouse ghrelin cDNA coding sequences was designed so that ghrelin expression might be targeted to the pancreatic β cells (Fig. 2A). The ghrelin mRNA level of RIP-G Tg in pancreas determined by quantitative RT-PCR was about 215 times higher than that of nontransgenic littermates (215.3 ± 40.6 vs. 1.0 ± 0.025 arbitrary unit, n=5, $P<0.01$). There was also increment in ghrelin mRNA levels in brain of RIP-G Tg (242.6 ± 17.6 vs. 89.1 ± 27.3 arbitrary unit, n=5, $P<0.01$). To confirm the expression of ghrelin transgene in pancreatic β cells, we performed immunohistochemical analysis using anti-C-terminal ghrelin antiserum. C-terminal ghrelin-like immunoreactivity was observed in the nearly whole area of the islets of the RIP-G Tg (Fig.2C) while it

was only seen in the periphery of the islets of their nontransgenic littermates (Fig.1A). Immunohistochemical analysis using anti-N-terminal ghrelin antiserum showed the same staining pattern (Fig.2D), indicating that n-octanoylated-ghrelin may be produced in β cells of this transgenic mouse. We also stained the brain section of RIP-G Tg. No ghrelin-like immunoreactivity was detected either with anti-C-terminal or anti-N-terminal ghrelin antisera (data not shown). The pancreatic tissue ghrelin concentration of RIP-G Tg measured by C-RIA was about 1,000 times higher than that of their nontransgenic littermates (1024±108.9 fmol/mg vs. 1.2±0.1 fmol/mg, n=5, $P<0.01$). This concentration was about one third of the nontransgenic stomach concentration (3558.1 ± 51.0 fmol/mg, n=5). The pancreatic tissue ghrelin concentration of RIP-G Tg measured by N-RIA tended to be also higher than that of their nontransgenic littermates (0.054±0.017 fmol/mg vs. 0.038±0.006fmol/mg, n=5, NS; not significant), but it did not reach statistical significance. Plasma des-acyl ghrelin concentration of RIP-G Tg was about 3.4 times higher than that of nontransgenic littermates under the ad-lib feeding states (2805.5±236.4 vs. 825.9± 244.4 fmol/ml, n=5, $P<0.01$, Fig.2G). We also measured des-acyl ghrelin levels in portal vein of the mice. In the nontransgenic mice, the portal des-acyl ghrelin level was significantly higher than that in retroorbital vein. (1108.0±257.3 fmol/ml vs.

825.9±244.4 fmol/ml, n=5, P<0.05, Fig.2G) The des-acyl ghrelin concentration collected from portal vein of RIP-G Tg at the same time was much higher than that of nontransgenic littermates (3671.8±328.6 vs. 1108.0±257.3 fmol/ml, n=5, P<0.01, Fig.2G). The step-up of des-acyl ghrelin concentration from retroorbital vein to portal vein of RIP-G Tg was significantly higher than that of nontransgenic littermates. (866.3 ± 182.2 fmol/ml vs. 262.9 ± 59.8 fmol/ml, P<0.01, Fig.2H) Plasma n-octanoylated-ghrelin levels in retroorbital and portal vein of RIP-G Tg tended to be higher than that of their nontransgenic littermates (retroorbital; 78.5±13.4 vs. 66.1± 7.1 fmol/ml, n=5, NS, portal; 104.6±15.3 vs. 71.4±9.0 fmol/ml, n=5, NS), but it did not reach statistical significance.

We also generated RGP-G Tg, in which ghrelin expression is targeted to the pancreatic α cells (Fig.2B). The ghrelin mRNA level Tg in pancreas of RGP-G determined by quantitative RT-PCR was about 16 times higher than that of nontransgenic littermates (16.3±1.7 vs. 1.0±0.24 arbitrary unit, n=5, P<0.01). The ghrelin mRNA level in duodenum of RGP Tg was not statistically different from that of nontransgenic littermates (520.1±111.1 vs. 379.1±37.6 arbitrary unit, n=5, NS). The ghrelin mRNA level in brain of RGP Tg was not distinguishable from that of nontransgenic littermates (72.0 ± 6.4 vs. 71.8 ± 7.8 arbitrary unit, n=5, NS).

Immunohistochemical analysis showed ghrelin-like immunoreactivity in the periphery of the pancreatic islet of RGP-ghrelin transgenic mouse by both anti-C-terminal ghrelin and anti-N-terminal ghrelin antisera (Fig.2E, F). The pancreatic tissue ghrelin concentrations of RGP-G Tg measured by C-RIA were about 50 times higher than those of their nontransgenic littermates (48.9± 2.5 fmol/mg vs. 1.2±0.1 fmol/mg, n=5, P<0.01). The pancreatic tissue ghrelin concentration of RGP-G Tg measured by N-RIA tended to be higher than that of their nontransgenic littermates (0.076±0.019 fmol/mg vs. 0.038±0.006fmol/mg, n=5, NS), but it did not reach statistical significance. The plasma des-acyl ghrelin concentrations in retroorbital vein were not elevated in RGP-G Tg after over night fasting compared to nontransgenic littermates (661.6±38.0 vs. 1024.7±27.1 fmol/ml, n=5). The portal des-acyl ghrelin concentrations of RGP-G Tg were also indistinguishable from those of their nontransgenic littermate (1320.6± 164.7 vs. 1442.9±361.5 fmol/ml, n=5, NS). Plasma n-octanoylated-ghrelin levels in retroorbital and portal vein of RGP-G Tg were indistinguishable from those of their nontransgenic littermates (retroorbital; 98.3±18.7 vs. 133.5±25.3 fmol/ml, n=5, NS, portal; 154.3±20.7 vs. 198.9±34.9 fmol/ml, n=5, NS).

Body weight, food consumption and % body fat. There was no significant difference in body weight and food intake between RIP-G Tg and their nontransgenic

littermates (Fig. 3). Percent body fat and V/S ratio of RIP-G Tg were not different from those of nontransgenic littermates (Fig.2C, D). No significant changes were observed in RGP-G Tg, either (data not shown).

Glucose metabolism and insulin secretion. Although no significant differences in blood glucose levels were noted between RIP-G Tg and their nontransgenic littermates on the fasting state and intraperitoneal glucose tolerance tests (IPGTT) (Fig.4A, C), plasma insulin levels 2 and 30 minutes after the glucose injection were significantly decreased in RIP-G Tg compared to those in their nontransgenic littermates (Fig.4D). Suppression of insulin secretion was not observed in RIP-G Tg on intraperitoneal injection of arginine (Fig.4G). Blood glucose level of RIP-G Tg in insulin tolerance test tended to be lower than those of their nontransgenic littermates, but it did not reach statistical significance (Fig. 4H).

No significant differences in blood glucose or insulin levels were observed between RGP-G Tg and their nontransgenic littermates on the fasting state, ad-lib feeding or intraperitoneal (IP) glucose or arginine injection (Fig.4B, E, F and data not shown). Blood glucose levels on insulin tolerance test showed no differences between RGP-ghrelin and their nontransgenic littermates (data not shown).

Islet architecture and β cell mass. We studied the tissue sections of RIP-G Tg to

explore the effect of ghrelin on the islet architecture and β cell mass. There were no obvious abnormalities in the intra islet cytoarchitecture and cell number of insulin, glucagon, somatostatin and PP cells in the islets of the RIP-G Tg (Fig.5A, B, C, D). The intensity of staining of these four islet hormones in the islets of the RIP-G Tg was not apparently different from those of non transgenic littermates. The ratio of the β cell area to whole pancreas was not changed significantly (Fig.5I). We also studied the tissue sections of RGP-G Tg and found no significant differences (Fig.5E, F, G, H, J).

Expression of insulin mRNA and insulin content. Since RIP-G Tg showed suppression of insulin secretion, we examined pancreatic mRNA expression and peptide content of insulin in RIP-G Tg and their nontransgenic littermates by Northern blot analysis and RIA. The insulin mRNA in RIP-G Tg did not differ from those of their nontransgenic littermates (Fig.6A, B). No significant differences of insulin contents were observed between RIP-G Tg and their nontransgenic littermates (Fig.6C).

PDX-1 and GLUT2 immunoreactivity. We examined the immunoreactivity of PDX-1, and GLUT2 in RIP-G Tg. The staining intensities of PDX-1 and GLUT2 in the RIP-G Tg (Fig.7A, C) were not apparently different from those in the nontransgenic littermates (Fig.7B, D).

Batch incubation of islets. The insulin secretion from isolated islet of RIP-G Tg by

batch incubation was indistinguishable from that of nontransgenic littermates, in 3.3 or 8.7 or 16.7 mM glucose conditions (Fig. 9).

Lipid metabolism. Plasma total cholesterol level of RIP-G Tg tended to be lower than those of nontransgenic littermates, but it did not reach statistical significance (total cholesterol;85.4±6.9 vs. 79.4±7.5 mg/dl, n=6, NS). Plasma triglyceride level of RIP-G Tg tended to be lower than that of nontransgenic littermates, but it did not reach statistical significance (154.5±11.0 vs. 136.9±10.3 mg/dl, n=6, NS). Free fatty acid level and HDL-cholesterol level of RIP-G Tg were not significantly different from those of non transgenic littermates free fatty acid; 0.44±0.05 vs. 0.48±0.07 mEq/L, n=6, NS, HDL-cholesterol; 46.1±2.3 vs. 44.9±3.4 mg/dl, n=6, NS).

Expression of GHS-R mRNA. To rule out possible down-regulation of GHS-R due to chronic exposure to high-level ghrelin, we measured the expression level of GHS-R mRNA in pancreas and pituitary by real time quantitative RT-PCR. There were no significant differences in GHS-R mRNA levels between RIP-G Tg and their nontransgenic littermates either in pancreas (Fig.8A) or in pituitary (Fig.8B).

Discussion

In wild-type mouse, no ghrelin-like immunoreactivity was detected in most of the islets. C-terminal ghrelin-like immunoreactivity was observed in the periphery of minor proportion of islets of wild type mice, which is consistent with the previous report (24). By the serial section analysis, most of the ghrelin producing cells also showed glucagon-like immunoreactivity. These findings indicate that ghrelin was expressed in minor proportion of mouse pancreatic alpha cells. Expression of ghrelin was not detected in pancreatic β cells of wild type mice.

In the present study we developed RIP-G Tg, in which pancreatic ghrelin concentration measured by C-RIA was approximately 1,000 times higher than that of nontransgenic littermates. By immunohistochemistry using anti-C-terminal ghrelin [13-28] antiserum we detected C-terminal ghrelin-like immunoreactivity in almost whole area of islets. Therefore, since ghrelin was not detected in β cells of control mice by immunohistochemistry, ghrelin transgene driven by RIP was considered to be expressed in β cells.

We also found about 3 times higher expression level of ghrelin mRNA in the brain of RIP-G Tg compared to that of nontransgenic littermates, which could not be detected by immunohistochemistry. Although small amount of ghrelin has been

reported to be expressed in brain, which can be detected by immunohistochemistry only after colchicine treatment (1), there have been controversies on whether this small amount of ghrelin in the brain has biological role. Since the food intake of RIP-G Tg was not different from that of nontransgenic littermates, the ghrelin produced by transgene in the brain seems not to show bioactive effect of n-octanoylated ghrelin.

By immunohistochemistry using anti-ghrelin [1-11] antiserum that recognizes the n-octanoylated portion of ghrelin, ghrelin-like immunoreactivity was also demonstrated in nearly whole area of islets of RIP-G Tg, indicating the production of n-octanoylated-ghrelin in β cells. This finding indicates that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. This is reasonable since α and β cells are pancreatic endocrine cells derived from common precursor cells (40). Since N-RIA/C-RIA ratio of the pancreatic tissue ghrelin concentration of RIP-G Tg was much lower than that of the stomach (0.0053 % vs. 11.67 %, $P<0.01$), the ability of acylation in β cell might be lower than that of in ghrelin-producing cell in the stomach (X/A-like cell). It is possible that exocrine pancreatic enzymes might interfere with the results although these were inactivated by boiling before extraction. The other possibility is that because of the formalin fixation of ghrelin in the tissue section the epitope recognized by immunohistochemistry using