Full Length Research Paper

Down-regulation of ghrelin receptors in the stomach delays gastric emptying in vagotomized rats

Wen-Cai Qiu, Cheng-Guang Yang, Jun Yan, Zhi-Gang Wang and Qi Zheng*

Department of General Surgery, the Affiliated Sixth Hospital of Medical School, Shanghai Jiaotong University, Shanghai 200233, China.

Ghrelin promotes gastric emptying and small intestinal transit when injected through central or peripheral channels in experimental animals. When the vagal nerve is cut off, the central effect of ghrelin is abolished, the peripheral effect is still observed. Furthermore, the function of ghrelin receptor expressing nerve cells in gastric muscle layers is affected. Expressional changes in ghrelin receptors may affect the effect of ghrelin. The effects of intraperitoneal administration of ghrelin (20, 40 and 80 µg/kg) on gastric emptying were studied in control and vagotomized rats *in vivo*. The effects of ghrelin (0.01, 0.1, 0.5 and 1.0 µmol/L) on the contraction force of smooth muscle strips from the stomach were studied *in vitro*. Ghrelin receptor expression was studied in gastric layers by means of immunohistochemistry. Ghrelin dose-dependently increased gastric emptying in control and model rats. In addition, ghrelin enhanced smooth muscle strip contraction induced by carbachol. Ghrelin receptor expression in stomach smooth muscle layers was down-regulated in model rats. Down-regulation of growth hormone secretagogue receptor 1a in gastric smooth muscle layers, which affected the effects of ghrelin, may be one of the mechanisms behind delayed gastric emptying after vagotomy.

Key words: Ghrelin, gastric emptying, growth hormone secretagogue receptor 1a, vagotomy; enteric plexus.

INTRODUCTION

Growth hormone secretagogue receptor 1a (ghrelin receptor, GHS-R1a) is a specific G protein-coupled receptor (van der Lely, 2004). It has been found in tissues of the central nervous system, such as the hypothalamus and anterior pituitary gland (Guan et al., 1997; Shuto et al., 2001), as well as in multiple peripheral organs and tissues (Kojima et al., 2001; Yokote et al., 1998), such as the stomach and intestine (Date and Ghrelin, 2000), pancreas (Kamegai et al., 2000), kidney (Mori et al., 2000) and so on.

Ghrelin is an endogenous ligand for GHS-R1a (van der Lely, 2004). Ghrelin was initially found because of its function of stimulating the release of growth hormone (Kojima et al., 1999). Following this discovery, a wide variety of biological functions of ghrelin were found. It is known to stimulate appetite (Dimaraki and Jaffe, 2006) and a positive energy balance (Asakawa, 2005); it has cardiovascular actions (Nagaya et al., 2003) and controls digestive motility (Fujino et al., 2003a; Yang et al., 2011; Kitazawa et al., 2011).

The effect of ghrelin on gastrointestinal tract motility has been of increasing interest. Both central and peripheral administration of ghrelin increased the gastric emptying rate (Trudel et al., 2002) and the frequency of phase III of the interdigestive migrating myoelectric complex (MMC) (Edholm and Ghrelin, 2004). Furthermore, it induced fasting motor activity in fed rats (Fujino et al., 2003a). Previous studies have indicated that ghrelin acts through nervous pathways. Centrally, ghrelin acts through activation of ghrelin receptors in the hypothalamus. When the vagal nerve is severed, central effects are abolished (Date, 2000; Kamiji et al., 2010; le Roux et al., 2005). Peripheral effects of ghrelin might be

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*Corresponding author. E-mail: sh6_zhengqi@126.com. Tel: +86-21-64369181. Fax: +86-21-64701361.

caused by activation of ghrelin receptors on the vagal nerve (Fujino et al., 2003a) and gastrointestinal enteric plexus (Edholm, 2004). Although expression of ghrelin receptors has been detected in the stomach, changes in ghrelin receptor expression, which might affect the effect of ghrelin on gastric emptying, after the vagal nerve is severed have not yet been reported.

The aim of this study was to further study the effects of ghrelin on gastrointestinal tract motility and the changes in ghrelin receptor expression in gastric smooth muscle layers after vagotomy. Better understanding of the effects of ghrelin on gastric emptying and smooth muscle contraction and of the expression levels of ghrelin receptors may help explain the phenomenon of delayed gastric emptying after surgery involving trauma of the vagal nerve (Shafi and Pasricha, 2007).

MATERIALS AND METHODS

Animals

All animal procedures were conducted according to the ethical guidelines of Shanghai Jiao Tong University. Fifty-two male Sprague-Dawley rats (250 ± 50 g) were obtained from the Experimental Animal Center of the Shanghai Academia Sinica, China. Rats were housed in stainless steel cages at a controlled humidity (60 to 65%) and temperature (22 ± 2°C) with a normal 12:12 h light/dark cycle for at least 7 days before the surgical procedure (Tebbe et al., 2005a). Twenty-six male Sprague-Dawley rats were randomly selected as controls, the rest underwent vagotomy.

Preparation of animal model

According to previously reported operational procedures (Fujino et al., 2003b), all rats were anaesthetized with ketamine (100 mg/kg). After a midline incision of the upper abdominal wall, the lower part of the esophagus was exposed and the vagal nerve was exposed and incised under an operating microscope. Animals were housed individually and were given free access to food and water 6 h after surgery.

Control animals underwent surgical procedures as described previously, but vagotomy was not performed.

Measurement of the gastric emptying

Control and model rats were allowed 7 days to recover before measurements. During recovery, animals were trained daily to accept experimental conditions by injection of 0.2 ml Trypan blue semi-liquid test meal (50 mg/100 ml in distilled H_2O with 1.5% methylcellulose) followed by a 0.2 ml saline flush via the catheters. Measurements were performed in conscious animals after a 12 h fasting period. Ghrelin (20, 40 or 80 µg/kg) was administered intraperitoneally (i.p.) to vagotomized and control rats immediately after injection of 0.2 ml Trypan blue semi-liquid test meal via the catheter. Trypan blue injection was immediately followed by a 0.2 ml saline flush. This method was performed as described previously (Monnikes et al., 1993; Tebbe et al., 2005b). 20 min later, the rats were sacrificed. The stomach was clamped with a string above the lower oesophageal sphincter and a string beneath the pylorus to prevent leakage of Trypan blue. Gastric emptying was determined

spectrophotometrically, The stomach of each individual rat was cut just above the lower oesophageal sphincter and the pyloric sphincter. Trypan blue remains partly in the lumen of the stomach. The stomach and its contents were put in 5 ml of 0.1 mol/L NaOH. The stomach was minced, and then these samples contain the total amount of Trypan blue present in the stomach. The samples were further diluted to 10 ml with 0.1 mol/L NaOH and left at room temperature for 1 h. 5 ml of the supernatant was then centrifuged at 800 g for 20 min. The absorbance was read at a wavelength of 546 nm with a spectrophotometer (Shanghai Yixian company, China), and then Trypan blue content remained in the stomach was calculated. Percentage of gastric emptying of the rats was calculated as (infusion–remained)/infusion × 100%.

Effect of ghrelin on contraction of smooth muscle strips *in vitro*

Rats were killed by cervical dislocation. Segments of the proximal stomach were quickly removed and placed in cold Krebs solution (gassed with 95% O_2/5% CO_2). Preparation of smooth muscle strips was performed with the help of a microscope (magnification ×10). Circular muscle strips (length, 10 mm; width, 2 mm), stripped of mucosa and sub mucosa, were suspended vertically in a 5 ml organ bath chamber filled with Krebs solution containing 121.5 mM/L NaCl, 4.7 mM/L KCl, 2.5 mM/L $CaCl_2$, 1.2 mM/L $MgSO_4$, 1.2 mM/L KH_2PO_4, 25.0 mM/L $NaHCO_3$, and 5.6 mM/L Glucose. The organ bath chamber was gassed with 95% O_2/5% CO_2 and warmed to 37°C. One end of the muscle strip was fixed to a hook at the bottom of the chamber while the other end was connected by a thread to an external isometric force transducer (Harvard Apparatus, South Natick, USA) at the top. Figures of isometric tension obtained from the isometric force transducer were continuously recorded and stored on a computer by the SMUP-E biological signal processing system (Chengdu Equipment Factory, China). The strips were stretched to a tension of 1 g and allowed to equilibrate for 40 min. The buffer was changed every 10 min. Measurements were done when there were no fluctuations in the contractions of the strips.

In this study, 0.01, 0.1, 0.5 or 1.0 µmol/L of ghrelin was administrated to the strips in the presence or absence of carbachol (40 nmol/L). The effect of ghrelin on muscle strip contraction was expressed as the percent change from the mean of three pre-drug responses to carbachol (Edholm, 2004).

Paraffin sections

Ghrelin receptor expression in the stomach wall was evaluated in control and vagotomized rats. Negative controls were set up. Animals were killed with an overdose of ketamine. A 5 cm proximal stomach fragment was prepared and fixed with 4% paraformaldehyde for two days. Full-thickness stomach wall was used for paraffin slices. Fixed tissue samples were embedded in paraffin and cut at 5 µm thickness, then adhered to glass slides for staining. Slides were incubated with 10% fetal bovine serum for 60 min. Then, primary antibodies to ghrelin receptors, GHS-R1a (F-16) (goat anti-rat), were diluted in 1×PBS and added to the samples at a ratio of 1:100. Samples were then incubated at 4 °C overnight. Second antibodies (rabbit anti-goat), which were coupled by horseradish peroxidase (HRP), were diluted in 1×PBS and added to the slides at a ratio of 1:200. DAB was used for color development. Hematoxylin was used to counterstain samples. Samples were dehydrated through ascending graded ethanol, cleared in xylene and coverslipped. Lastly, the slides were studied under a microscope (×20).
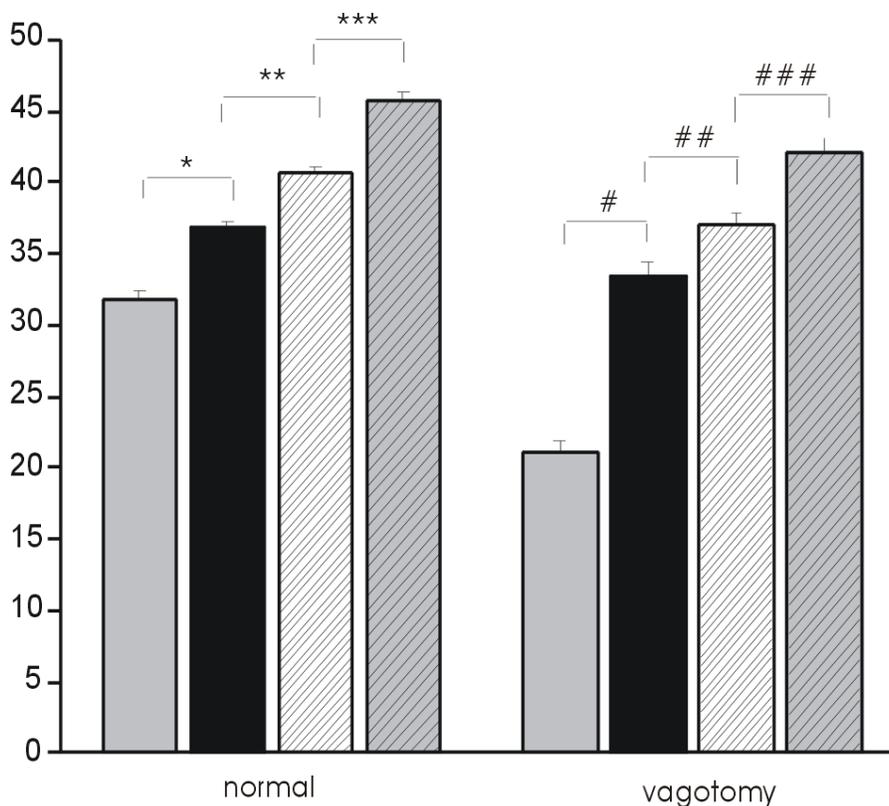
Figure 1. The effect of ghrelin (20, 40 and 80 µg/kg) on gastric emptying in control and vagotomized rats. Gray bar represents control (no injection of ghrelin); black bar represents 20 µg/kg ghrelin; striated bar represents 40 µg/kg ghrelin; gray and striated bar represents 80 µg/kg ghrelin. *, $p < 0.001$; **, $p < 0.001$; ***, $p < 0.001$; #, $p < 0.001$; ##, $p < 0.05$; ###, $p < 0.005$; n=6 per condition. Mean ± S.E.M.

Fluorescent staining

Fluorescent staining of ghrelin receptors was studied in gastric smooth muscle layers of control and model rats. Negative controls were set up. Muscle layers of the proximal stomach were stripped of mucous and submucous layers, stretched to 150% and fixed on a platform. They were subsequently fixed with 4°C acetone for 15 min. The acetone was washed off with 1×PBS. Muscle tissues were then incubated with 10% fetal bovine serum for 60 min. Then, GHS-R1a (F-16) (goat anti-rat) with 0.5% Triton X-100 was diluted in PBS and added to the muscle tissues at a ratio of 1:100. Tissues were incubated at 4°C for two days. Second antibodies (rabbit anti-goat), which were coupled by Fluorescein (FITC, green fluorescence) or Rhodamine (TRITC, red fluorescence), were diluted in 1×PBS and added to the fixed muscle tissues at a ratio of 1:200. Tissues were then incubated for half a day in darkness. DAPI was used as a counterstain. Finally, the tissues were moved from the platforms to glass slides and coverslipped with 50% glycerol. The slides were observed and scanned under a laser confocal microscope (Olympus, FV-1000, Japan).

Statistical analysis

Results are expressed as mean ± SEM. Data were analyzed with the Origin 6.0 software. Photoshop 8.0.1 software was used to procedure figures. Quantity 4.6.2 software was used to analysis staining intensity. Data recordings were evaluated by one way analysis of variance (ANOVA) followed by Dunnett's test. A probability (p) of less than 0.05 was considered statistically significant.

RESULTS

Effect of ghrelin on gastric emptying

There was a statistically significant difference in gastric emptying between control and vagotomized rats when no ghrelin was injected (32.4 ± 0.62% vs. 19.2 ± 0.76%, $p < 0.001$, n=5).

Ghrelin increased gastric emptying in control and model rats in a dose-dependent manner (Figure 1). In the control group, when 20, 40 and 80 µg/kg ghrelin was injected, the gastric emptying increased from 32.4 ± 0.62 to 36.4 ± 0.44, 39.8 ± 0.45 and 46.8 ± 0.55% respectively. In vagotomized rats, when 20, 40, 60 µg/kg ghrelin was injected, the gastric emptying increased from 19.2 ± 0.76 to 32.6 ± 0.98, 37.8 ± 0.81 and 41.8 ± 0.93%, respectively.
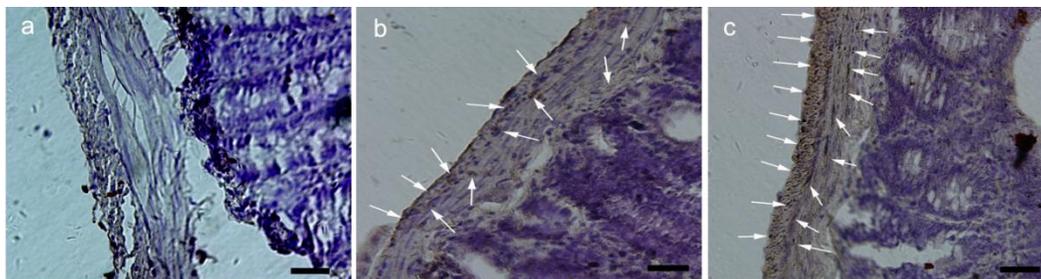
Figure 2. Paraffin sections; (a) there were no brown or brownish yellow granules in stomach wall in negative controls; (b) ghrelin receptors staining in model rats, brown or brownish yellow granules were observed in muscle layers (white arrow); (c) ghrelin receptors staining in control rats, brown or brownish yellow granules were observed in muscle layers (white arrow). Scale bar = 20 μm.
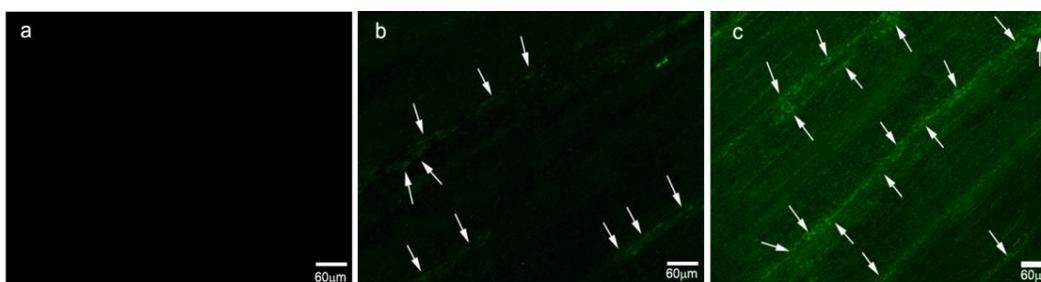

Figure 3. Ghrelin receptors fluorescence staining in gastric muscle layers in control and vagotomized rats; (a) Negative control ghrelin receptors staining; (b) Ghrelin receptors fluorescence staining in model rats; green fluorescent intensity was observed in muscle layers; green fluorescence was mainly located on some cells between smooth muscle bundles (white arrow); (c) Ghrelin receptors fluorescence staining in control rats; green fluorescent intensity was observed in muscle layers, green fluorescence was mainly located on some cells between smooth muscle bundles (white arrow).

Effect of ghrelin on smooth muscle strip contraction *in vitro*

Ghrelin did not increase smooth muscle strip contraction in the absence of carbachol. However, if carbachol (40 nmol/L) was applied, ghrelin did increase smooth muscle strip contraction. 0.01 µmol/L ghrelin had no effect on smooth muscle strip contraction. Contractile response to ghrelin at 0.1 µmol/L was 156 ± 3% of the mean of three pre-drug responses to carbachol (n=6, $p < 0.01$). Contractile response to ghrelin at 0.5 µmol/L was 185 ± 7% (n=6, $p < 0.05$ vs. 0.1 µmol/L). Contractile response to ghrelin at 1.0 µmol/L was 218 ± 4% (n=6, $p < 0.05$ vs. 0.5 µmol/L).

Paraffin sections

There were no brown or brownish yellow granules in muscle layers after the negative control staining (Figure 2a). Ghrelin receptors were mainly located on the circular muscle layer and the longitudinal muscle layer in the control animals (Figure 2b). Brown or brownish yellow

granules indicate locations of ghrelin receptors. The concentration of ghrelin receptors in gastric smooth muscle layers was lower in vagotomized rats than in controls (Figure 2c).

Immunofluorescence

There was no green fluorescence after negative control staining (Figure 3a). In the control group, ghrelin receptors were mainly located on some cells that were banded along smooth muscle bundles, while there was a lower fluorescence intensity on smooth muscle fascicles (Figure 3b and c). A lower fluorescence intensity was observed in muscle layers of vagotomized rats than in those of controls (Figure 4).

We speculated that the cells banding along smooth muscle bundles with round or oval nuclei were probably nerve cells in the small intestinal enteric plexus. For identifying these cells, we stained them with antibodies to growth hormone secretagogue receptor 1a (Figure 5a) and antibodies to neurofilament heavy polypeptide (Figure 5b). Neurofilament heavy polypeptide is a
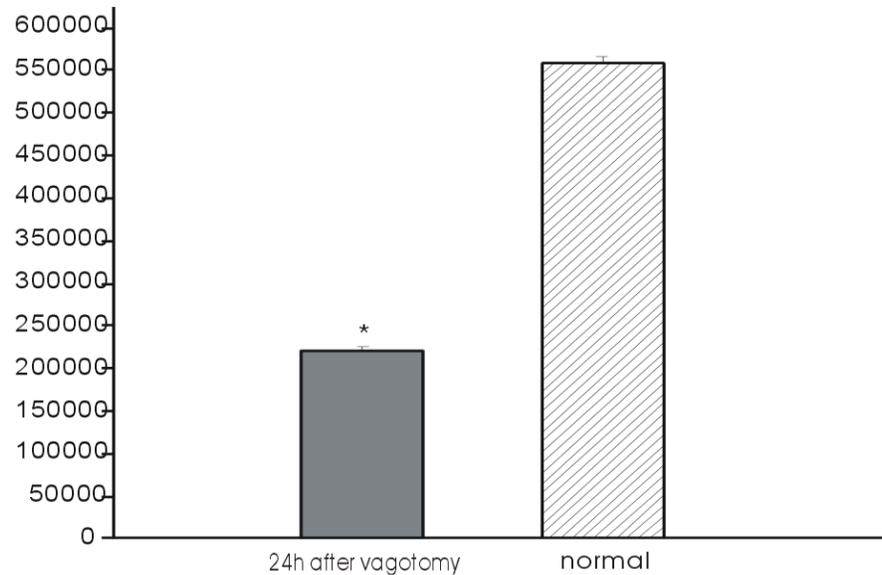
Figure 4. The analysis of ghrelin receptors staining fluorescent intensity in stomach muscle layers in controls and vagotomized rats. A lower fluorescent intensity was observed in muscle layers in model rats (gray bar) when compared with controls (striated bar) (*, $p < 0.001$, n= 6). Mean ± S.E.M.
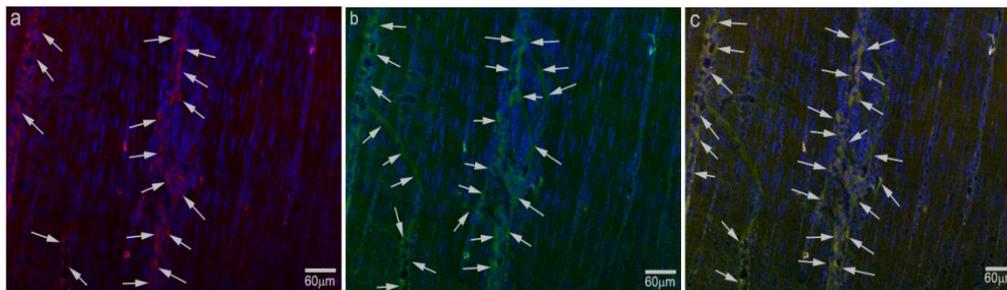

Figure 5. Identification of the location of ghrelin receptors by ways of double fluorescent staining in gastric muscle layers in rats. (a) Ghrelin receptors fluorescence staining (red fluorescence, white arrow); (b) Neurofilament heavy polypeptide fluorescence staining (green fluorescence, white arrow); (c) Ghrelin receptors and neurofilament heavy polypeptide double fluorescence staining; tan fluorescence (white arrow) were located on some cells with round or oval nuclei between smooth muscle bundles. DAPI stain cell nucleus (blue fluorescence).

neuron-specific marker. The results of staining with two kinds of antibodies indicated that both were located on the same cells (Figure 5c). Hence, we identified these cells to be nerve cells in the small intestinal enteric plexus.

DISCUSSION

Gastrointestinal operations often result in trauma of the vagal nerve (Shafi and Pasricha, 2007; Tebbe et al., 2005a; Fujino et al., 2003b). Also, there often is a delay in gastric emptying after surgery. When the vagal nerve is injured, the delay in gastric emptying and small intestinal transit increases (Noguchi et al., 2003; Van der Mijle et al., 1994; Hellström, 1986). The delay in gastric emptying might be caused by both central and peripheral mechanisms. When the vagal nerve is severed, the function of motor nerves that regulate gastric motility is impaired. This may be the main factor leading to delayed gastric emptying. Ghrelin can promote gastrointestinal tract motility if administrated through central or peripheral channels (Fujino et al., 2003a; Kitazawa et al., 2011; Trudel et al., 2002; Edholm, 2004). Ghrelin exerts its role by activating ghrelin receptors (GHS-R1a) in the central or peripheral tissues (Guan et al., 1997; Shuto et al.,

2001; Kojima et al., 2001; Yokote et al., 1998). Therefore, we aimed to study the role of ghrelin receptors in the occurrence of delayedgastric emptying.

In previous studies, administrating ghrelin through the central nervous system had a pronounced effect on appetite and motility of the gastrointestinal tract (Kamiji et al., 2008; Kobashi et al., 2009; Seoane et al., 2003; Tups et al., 2004). These effects were performed through the vagal nerve (Ammori et al., 2008). When the vagal nerve was cut off, these effects were abolished (Kamiji et al., 2010). Peripheral administration of ghrelin also enhanced motility of the gastrointestinal tract (Yang et al., 2011; Kitazawa et al., 2011; Edholm, 2004; Kobelt et al., 2005). In our experiment, ghrelin dose-dependently increased the gastric emptying in control and model rats *in vivo*. These results suggest that ghrelin affects gastric motility by activating ghrelin receptors in stomach muscle layers indepently from the central action mediated by the vagal nerve.

20, 40 and 80 μg/kg ghrelin were physiological stimulus doses. Under physiological conditions, plasma levels of ghrelin in rats are 500 to 2000 pmol/L (Murakami et al., 2002). Fujino et al. found that injection 1.5 nmol of ghrelin per rat resulted in an approximately 600 pmol/L increase in plasma ghrelin concentrations. Supporting this result, when 80 μg/kg ghrelin was applied in the present study, the plasma concentration was approximately 2400 pmol/L, which approached physiological concentrations. Hence, experimental results were of biological relevance. In vagotomized rats, the vagal nerve was severed while sympathetic nerves were preserved. Under these conditions ghrelin still promoted gastric emptying. This suggests that ghrelin directly promotes gastric emptying indepently from the central action mediated by the vagal nerve *in vivo*.

In vitro, ghrelin enhanced smooth muscle strip contraction in the presence of carbachol. This suggests that ghrelin amplifies the effect of carbachol. When carbachol was absent, ghrelin did not promote smooth muscle strip contraction. It is possible that the function of nerve cells in smooth muscle strips was suppressed. Carbachol may directly promote smooth muscle strip contraction, or it may activate nerve cells in smooth muscle strips by direct stimulation or stretching stimulation. When nerve cells are activated, ghrelin may play its role by activating ghrelin receptors on the nerve cells. Further studies should be performed to clarify this mechanism.

Ghrelin receptors (GHS-R1a) have previously been found in the gastric and the small intestine (Dassa et al., 2003). The results of paraffin section staining and fluorescent staining indicated that ghrelin receptors were mainly located on the muscle layers. Especially after fluorescent staining, we could clearly observe the location of ghrelin receptors in stomach muscle layers. Ghrelin receptors were mainly located on nerve cells in the stomach intestinal myenteric plexus.

In conclusion, ghrelin increased gastric emptying in control and vagotomized rats. Ghrelin affected gastric motility by activating ghrelin receptors on nerve cells in the myenteric plexus without participation of the vagal nerve. After vagotomy, expressional levels of ghrelin receptors were down-regulated. Ghrelin receptor down-regulation may affect the effect of ghrelin. This might be one of mechanisms behind the delayed gastric emptying after surgeries resulting in trauma of the vagal nerve.

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