Down-regulation of ghrelin receptors in the small intestine delays small intestinal transit in vagotomized rats

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Abstract. Vagal nerve injury may occur in esophageal and gastric surgeries. The aim of this study was to observe the effects of ghrelin on small intestinal motility upon vagal nerve injury and the possible co-relationship between changes in ghrelin receptor expression in the small intestine and delayed small intestinal transit after vagotomy. The effects of intraperitoneal administration of ghrelin (20, 40 and 80 μg/kg) and the ghrelin receptor antagonist [D-Lys^3]-GHRP-6 (1.5 μmol/kg) on small intestinal transit were studied in control and vagotomized rats *in vivo*. The effects of ghrelin (0.01, 0.1, 0.5, 1.0 and 2.0 μmol/l) on the contraction force of smooth muscle strips from the jejunum were studied in the presence or absence of carbachol (50 nmol/l) and [D-Lys^3]-GHRP-6 (10 μmol/l) *in vitro*. Ghrelin receptor expression was assessed in intestinal muscle layers by means of Western blotting. The results indicated that ghrelin dose-dependently increased small intestinal transit in the control and model rats. In addition, ghrelin enhanced smooth muscle strip contraction induced by carbachol. Ghrelin receptor antagonist [D-Lys3]-GHRP-6 blocked the effect of ghrelin. Ghrelin receptor expression in the small intestinal muscle layers was down-regulated in the vagotomized rats. Down-regulation of growth hormone secretagogue receptor 1a in small intestinal muscle layers, which affected the function of ghrelin, may be one of the mechanisms behind delayed small intestinal transit after vagotomy.

Introduction

Growth hormone secretagogue receptor type 1a (ghrelin receptor, GHS-R1a) is a specific G protein-coupled receptor (1). It has been found in tissues of the central nervous system, such as the hypothalamus and anterior pituitary gland (2,3), as well as in multiple peripheral organs and tissues (4,5), such as the stomach, intestine (6), pancreas (7) and kidney (8).

Ghrelin is an endogenous ligand for GHS-R1a (1). Ghrelin was initially identified because of its function of stimulating the release of growth hormone (9). Following this discovery, a wide variety of biological functions of ghrelin have been determined. It is known to stimulate appetite (10) and a positive energy balance (11); it has cardiovascular actions (12) and controls digestive motility (13-15).

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 (16) and the frequency of phase III of the interdigestive migrating myoelectric complex (MMC) (17). Furthermore, it induced fasting motor activity in fed rats (13). 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, its central effects are abolished (6,18,19). Peripheral effects of ghrelin may be caused by activation of ghrelin receptors on the vagal nerve (13) and gastrointestinal enteric plexus (17). Although expression of ghrelin receptors has been detected in the small intestine, changes in ghrelin receptor expression, which may affect the effect of ghrelin on small intestinal transit after the vagal nerve is severed have not yet been reported.

Better understanding of the effects of ghrelin on small intestinal transit and smooth muscle contraction and the association of expression levels of ghrelin receptors may help explain the phenomenon of delayed small intestinal transit after surgery involving trauma of the vagal nerve (20,21).

Materials and methods

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

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Preparation of the animal model. According to previously reported surgical procedures (22,23), all rats were anaesthetized with ketamine (100 mg/kg). After a midline incision to the upper abdominal wall, the lower part of the esophagus was exposed and the posterior branch of the vagal nerve was exposed and incised under a surgical microscope. During surgery a polyethylene microcatheter (inside diameter, 1.2 mm; outside diameter, 1.7 mm) was chronically implanted in the duodenum via the stomach, 1 cm distal to the pylorus, then fixed with sutures to the stomach wall. The catheter was tunneled subcutaneously to exit at the back of the animal's neck (24,25). Animals were housed individually and were given free access to food and water 6 h after surgery. Control animals underwent surgical procedures as described above, but vagotomy was not performed. The completeness of vagotomy was confirmed by morphological studies for cholinergic nerve fibres in the gastrointestinal tract as described in a previous study (23).

Small intestinal transit. Animals were allowed 3 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. Trypan blue is a non-absorbable enteral dye marker (22). 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 the 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 (22,26). Rats were sacrificed by cervical dislocation 30 min after administration of ghrelin. The distances of phenol red transit and the full length of the intestine (from pylorus to ileocecal valves) were calculated. Small intestine transit (%) was assessed by using the percentage ratio of trypan blue transit over the full intestinal length (25,27).

We also studied the effect of ghrelin (40 μg/kg) on small intestinal transit in control and vagotomized rats after rats were pretreated with [D-lys^3]-GHRP-6 (1.5 μmol/kg) for 10 min.

Organ bath. Control and model rats (n=6 per group) were sacrificed by cervical dislocation. Segments of the proximal intestine 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, x10). Circular muscle strips (length, 10 mm; width, 2 mm), stripped of mucosa and submucosa, 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. Values for 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 0.1 g and allowed to equilibrate for 40 min. The buffer was changed every 10 min. Measurements were carried out after 1 h of equilibration.

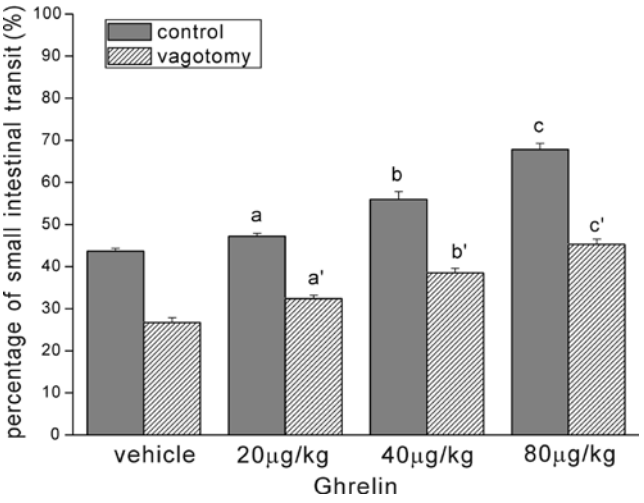

Figure 1. Effect of ghrelin (0, 20, 40 and 80 μg/kg) on small intestinal transit (%) in the control and vagotomized rats. $^{a}p<0.01$, $^{ab}p<0.01$, $^{bc}p<0.01$; $^{a'}p<0.01$, $^{a'b'}p<0.01$, $^{b'c'}p<0.01$; $^{aa'}p<0.01$, $^{bb'}p<0.01$, $^{cc'}p<0.01$, n=4 per condition. Mean ± SEM.

In this study, the effects of ghrelin (0.01, 0.1, 0.5, 1.0 and 2.0 μmol/l) on the contraction force of smooth muscle strips were studied in the presence or absence of carbachol (Cch; 50 nmol/l) and [D-Lys^3]-GHRP-6 (10 μmol/l). Results were expressed as the percentage of maximal contractile amplitudes induced by Cch (23).

Western blotting. Ghrelin receptor expression was evaluated by Western blotting in small intestinal muscle layers of the control and model rats (n=6 per group). Proximal intestinal muscle layers were stripped of mucous and submucous layers, lysed with RIPA lysate solution, skived and centrifuged for 10 min at 12000 x g and 4˚C. Liquid supernatants were collected for Western blotting. SDS-PAGE electrophoresis was performed with a 10% gel. Polyvinylidene fluoride (PVDF) membranes were blocked with 5% skim milk solution. GHS-R1a (44 kDa) (F-16) (goat anti-rat) was diluted in 5% dried skimmed milk solution and added to the PVDF membranes at a ratio of 1:100. Membranes were incubated at 4˚C overnight. Secondary antibodies (rabbit anti-goat) coupled by alkaline phosphatase (AP), were added to the PVDF membranes at a ratio of 1:200 and incubated for 2 h. NBT/BICP was used for color development. β-actin (43 kDa) (mouse anti-rat) was used as an internal control.

Drugs and chemicals. Rat ghrelin, carbachol and [D-Lys^3]-GHRP-6 (ghrelin receptor antagonist) were obtained from Tocris Cookson (Bristol, UK). GHS-R1a (F-16) (goat anti-rat) and β-actin (mouse anti-rat) were obtained from Santa Cruz (CA, USA). AP-conjugated second antibody (rabbit anti-goat) and AP-conjugated second antibody (goat anti-mouse) were obtained from Jackson Immuno Research Inc. (West Grove, PA, USA).

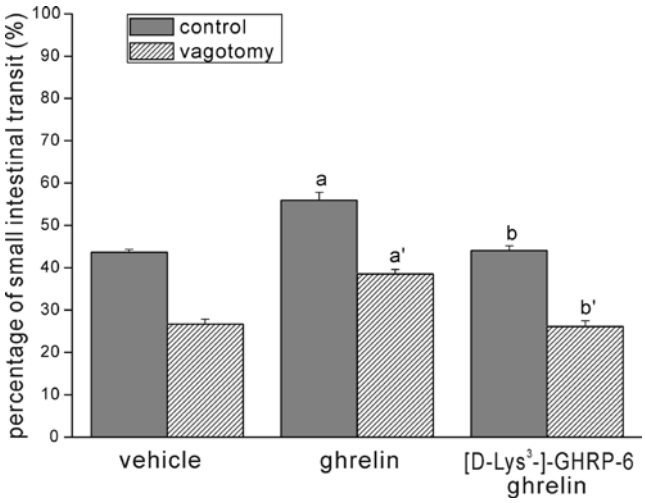

Figure 2. Effect of 40 μg/kg ghrelin on small intestinal transit (%) with and without pre-treatment with [D-Lys^3]-GHRP-6 (1.5 μM/kg) in the control and vagotomized rats. ^{ab}p<0.01, ^{b}p>0.05; $^{a'b'}$p<0.01, $^{b'}$p>0.05, n=4 per condition. Mean ± SEM.

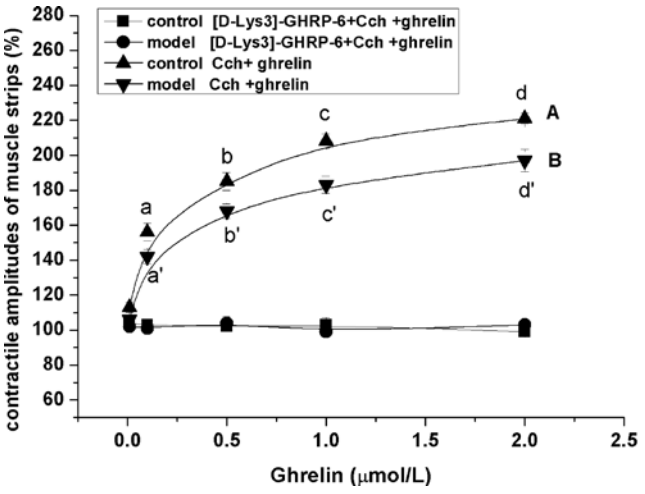

Figure 3. Effect curves (%) of ghrelin on small intestinal smooth muscle strips in the presence of carbachol (50 nmol/l) or [D-Lys^3]-GHRP-6 (10 μmol/l) + carbachol (50 nmol/l) in the control and vagotomized rats. In curve A, ^{a}p<0.01, ^{ab}p<0.01, ^{bc}p<0.01; in curve B, $^{a'}$p<0.01, $^{a'b'}$p<0.01, $^{b'c'}$p<0.01; between curve A and B, $^{aa'}$p>0.05, $^{bb'}$p<0.05, $^{cc'}$p<0.01, $^{dd'}$p<0.01, n=6 per condition. Mean ± S.E.M. Cch: carbachol.

Statistical analysis. Results are expressed as the mean ± SEM. Data were analyzed with the Origin 8.0 software. Photoshop 8.0.1 software and CorelDRAW X4 software were used to process the figures. Quantity 4.6.2 software was used to analyze the staining intensity. Data recordings were evaluated by one way analysis of variance (ANOVA) followed by Dunnett's test. p<0.05 was considered statistically significant.

Results

Effect of ghrelin on small intestinal transit. There was a statistically significant difference in small intestinal transit (%) between the control and vagotomized rats when no ghrelin was injected (42.73±0.53 vs. 25.32±1.02%; p<0.01, n=5 per condition). Ghrelin increased small intestinal transit in the control and model rats in a dose-dependent manner (Fig. 1). [D-Lys^3]-GHRP-6 blocked the effect of ghrelin (40 μg/kg) (Fig. 2).

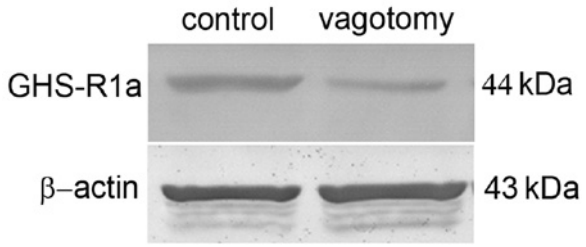

Figure 4. Expression of ghrelin receptors in small intestinal muscle layers in control and vagotomized rats as determined by Western blot bands.

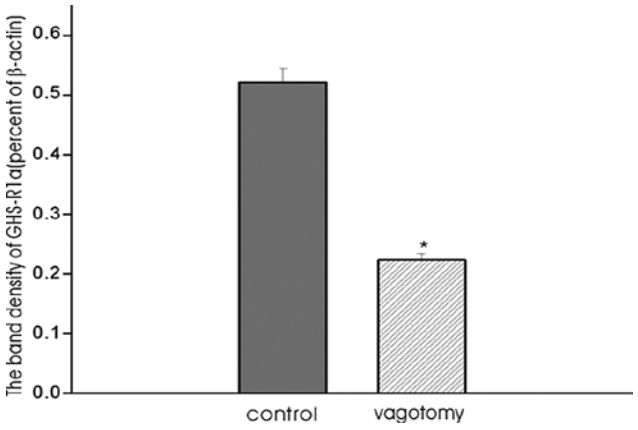

Figure 5. Results of the Western blot analysis of ghrelin receptor expression levels (percent of β-actin) in small intestinal muscle layers in control and vagotomized rats. *p<0.01, n= 6 per condition. Mean ± SEM.

Effect of ghrelin on smooth muscle strip contraction in vitro. Ghrelin did not increase smooth muscle strip contraction in the absence of carbachol. However, when carbachol (50 nmol/l) was applied, ghrelin did increase smooth muscle strip contraction, and a statistically significant difference in smooth muscle strip contractile amplitudes was noted in the control and vagotomized rats with an increased dose of ghrelin (Fig. 3). [D-Lys^3]-GHRP-6 blocked the effect of ghrelin (Fig. 3), but no significant difference was noted in the smooth muscle strip contractile amplitudes between the control and vagotomized rats (p>0.05; n=6 per condition).

Western blot analysis. Ghrelin receptor expression was confirmed in the small intestinal muscle layers (Fig. 4). Lower expression of ghrelin receptors was observed in the small intestinal muscle layers of the vagotomized rats when compared with that of the controls (Fig. 5).

Discussion

In esophageal and gastric surgery, there is often a delay in gastrointestinal motility, and trauma of the vagal nerve sometimes is involved (20,21,28). When the vagal nerve is injured, the delay in gastrointestinal transit increases (29-31). This delay in small intestinal transit might be caused by

both central and peripheral mechanisms. When the vagal nerve is severed, the function of motor nerves that regulate small intestinal motility is impaired. This may be the main factor leading to delayed small intestinal transit. Ghrelin can promote gastrointestinal tract motility if administered through central or peripheral channels (13,15-17). Ghrelin exerts its role by activating ghrelin receptors (GHS-R1a) in central or peripheral tissues (2-5). Therefore, we aimed to study the role of ghrelin receptors in delayed small intestinal transit.

In the present study, only the posterior branch of the vagal nerve was severed. The anterior branch of the vagal nerve dominating the liver and anterior stomach was preserved. This accurately cut motor nerves dominating small intestinal motility while it had little effect on gastric motility and metabolism of the liver. Thus, we could observe the effect of vagotomy on growth hormone secretagogue receptor 1a expression in small intestinal muscle layers with little or no effect on other ventral organs.

In previous studies, administration of ghrelin through the central nervous system had a pronounced effect on appetite and motility of the gastrointestinal tract (32-35). These effects are carried out through the vagal nerve (36). When the vagal nerve was cut, these effects were abolished (18). Peripheral administration of ghrelin also enhanced motility of the gastrointestinal tract (14,15,17,37). In our experiment, ghrelin dose-dependently increased the small intestinal transit in control and model rats *in vivo*. The ghrelin receptor antagonist [D-Lys^3]-GHRP-6 blocked the effect of ghrelin. These results suggest that ghrelin affects small intestinal motility by activating ghrelin receptors (GHS-R1a).

Concentrations of 20, 40 and 80 μg/kg ghrelin were physiological stimulus doses. Under physiological conditions, plasma levels of ghrelin in rats are 500-2000 pmol/l (38). Fujino *et al* (13) found that injection of 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 small intestinal transit. This suggests that ghrelin directly promotes small intestinal transit without participation of the vagal nerve *in vivo*.

In vitro, ghrelin played a gastroprokinetic-like role when smooth muscle strips were stimulated by an electrical field (39). This phenomenon suggests ghrelin functions only when nerve impulses come into being or membrane potential is altered. Ghrelin was found to enhance smooth muscle strip contraction in the presence of carbachol (40). This suggests that ghrelin amplifies the effect of carbachol; it is just an adjuvant. When carbachol was absent, ghrelin did not promote smooth muscle strip contraction. It is possible that the function of the nerve cells in the 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. When nerve cells are activated, ghrelin may function by activating ghrelin receptors on the nerve cells. Further studies should be carried out to clarify this mechanism.

The effective doses of ghrelin were different *in vivo* than *in vitro*. *In vitro*, a 50-fold higher concentration was needed to promote smooth muscle strip contraction than *in vivo*. We speculated that there was a gradually decreasing concentration from the organ bath chamber to nerve cells in the muscle layers. In addition, delayed tissue fluid exchange *in vitro* might affect the diffusion of the experimental medicine.

Western blot analysis and ghrelin receptor staining both indicated that there was a decreasing trend in ghrelin receptor expression in the vagotomized rats. These results can explain those from the *in vivo* and *in vitro* experiments.

In conclusion, ghrelin increased small intestinal transit in control and vagotomized rats. Ghrelin affected small intestinal motility by activating ghrelin receptors in intestinal muscle layers without participation of the vagal nerve. After vagotomy, expression levels of ghrelin receptors were down-regulated. Ghrelin receptor down-regulation affected the effect of ghrelin. This may be one of the mechanisms behind delayed small intestinal transit after surgery resulting in trauma of the vagal nerve.

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