Neurogastroenterol Motil (2009) **21,** 78–84 doi: 10.1111/j.1365-2982.2008.01179.x

Ghrelin regulates gastric phase III-like contractions in freely moving conscious mice

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Abstract *In humans and dogs, motilin regulates phase III contractions of migrating motor complex (MMC) in the interdigestive state, while ghrelin regulates MMC in rats. It still remains unclear whether ghrelin regulates phase III contractions of the mouse stomach. A miniature strain gauge transducer was sutured on the antrum to detect circular muscle contractions and gastric contractions of the interdigestive state were evaluated. Effects of ghrelin, a ghrelin receptor antagonist, and atropine on spontaneous gastric contractions were studied in freely moving conscious mice. Similar to the rat stomach, phase III-like contractions were observed in the interdigestive state, which disappeared immediately after the feeding. Ghrelin augmented spontaneous phase III-like contractions, while growth-hormone secretagogue receptor antagonists and atropine abolished the occurrence of spontaneous phase III-like contractions. The spontaneous phase III-like contractions were no more observed in vagotomized mice. These results suggest that ghrelin regulates phase III-like contractions in mice stomach via its own receptors. Ghrelin-induced gastric phase III-like contractions are mediated via vagal cholinergic pathways in mice. Our recording system of mice gastric motility may be useful to study the functional changes in gene knockout mice, in the future.*

Keywords *growth-hormone secretagogue receptor, vagotomy, vagus nerve.*

Abbreviations: GHS-R, growth-hormone secretagogue receptor; MI, motility index; MMC, migrating motor complex.

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Received: 26 March 2008
Accepted for publication: 27 June 2008

INTRODUCTION

Ghrelin, the endogenous ligand of the growth-hormone secretagogue receptor (GHS-R), was isolated from the rat stomach.[1] Besides stimulating GH release, ghrelin plays an important role in regulating the energy balance. Both central and peripheral administration of ghrelin stimulates food intake in humans[2] and rodents.[3]

Ghrelin also stimulates gastrointestinal (GI) motility.[4–7] Ghrelin administration induces phase III-like contractions in rat stomach.[7] Ghrelin also induces premature phase III contractions of migrating motor complex (MMC) of the stomach in humans.[5] We have recently showed that endogenous ghrelin is involved in mediating phase III-like contractions in the rat stomach.[8]

Mice are one of the most commonly used animals in laboratory. It still remains unclear whether ghrelin regulates interdigestive contractions of the stomach in mice.

To evaluate the spontaneous phase III-like contractions in mice, we invented a miniature transducer which fits the mice stomach. Using this animal model, we studied whether exogenous and endogenous ghrelin regulates spontaneous phase III-like contractions in conscious mice. We also studied whether ghrelin-induced phase III-like contractions are mediated via muscarinic receptors and vagal pathways.

MATERIALS AND METHODS

Animals

Male Swiss Webster mice weighing 25–30 g were kept in-group cages under conditions of controlled temperature (22–24 °C), humidity and light (12 h light cycle starting at 7:00 AM) with free access to laboratory chow and water. All experiments were started at 9:00 AM every day.

Protocols describing the use of mice were approved by the Institutional Animal Care and Use Committee

of Zablocki VA Medical Center at Milwaukee and carried out in accordance with the National Institute of Health 'Guide for the Care and Use of Laboratory Animals'. All efforts were made to minimize animal suffering and to reduce the number of animal in experiments.

Animal preparation

After an overnight fast, mice were anaesthetized with isoflurane (2%) (Baxter Healthcare Corporation, Deerfield, IL, USA). Through a midline laparotomy, the stomach was exposed and a miniature strain gauge transducer (5 × 4 mm) was implanted on the serosal surface of the gastric antrum. The wires from transducer were exteriorized through abdominal wall, ran under skin towards the back. Wires were protected by a protective jacket. After the surgery, mice were housed individually access to a standard diet and tap water. Mice were allowed to recover for 1 week before the study.

Motility recording

After a 24-h fasting with free access to water, gastric contraction was measured in conscious, freely moving mice. The wires from the transducer were connected to a recording system (Power-Lab model 8SP; ADI instruments, Colorado Springs, CO, USA) and gastric contractions were monitored for 2–3 h to observe spontaneous phase III-like contractions. Phase III-like contractions were defined as clustered contractions with amplitude of more than 1.5 g.

Effect of ghrelin administration on gastric motility in the interdigestive state

According to the studies of ghrelin administration to the mice,[9,10] acyl ghrelin (20, 40 and 100 μg kg^{-1}) was injected intraperitoneally (i.p.) 3 min after finishing the spontaneous phase III-like contractions. Motility index (MI) and peak amplitude of the contraction were compared 30 min before and after ghrelin administration. Saline-injected mice served as controls. Each mouse received a single dose of ghrelin, without a cross-over study.

We have previously showed that GHS-R antagonist [(D-lys3)GHRP6; 0.9 mg kg^{-1}] significantly attenuated spontaneous phase III-like contractions of the rat stomach.[8] We have also showed that (D-lys3)GHRP6 (0.9 mg kg^{-1}) blocked the effects of exogenously administered ghrelin (0.8 nmol kg^{-1} min^{-1}, i.v.) on phase III-like contractions in rats.[8] To study whether endogenous ghrelin is involved in mediating phase III-like contractions in mice, (D-lys3)GHRP6 (0.28 and 2.8 mg kg^{-1}, i.p.) was administered. Mean interval time and duration of phase III-like contractions were calculated from the two phase III-like contractions before and after the administration of a GHS-R antagonist.

To study whether cholinergic pathway is involved in mediating phase III-like contractions, atropine (500 μg kg^{-1}, i.p.) was administered. To evaluate the involvement of vagal nerve in mediating phase III-like contractions, subdiaphragmatic vagotomy was performed at the time of transducer-implantation. Under the dissecting microscope, the trunks of the subdiaphragmatic vagus were exposed and were dissected from the oesophagus. Sham surgeries were performed to expose simply the vagal trunks. Mice were allowed to recover for 1 week before the study.

Chemicals

Acyl ghrelin (Tocris Cookson Inc., Ellisville, MO, USA) and (D-lys3)GHRP-6 (Bachem, King of Prussia, PA, USA) were kept in powder form at −70 °C. Peptides were dissolved in sterile saline immediately before use. Atropine (Sigma, St. Louis, MO, USA) was kept in 4 °C and dissolved in saline immediately before use.

Statistical analysis

Results were shown as mean ± SE. ANOVA followed by Student's *t*-test was used to assess the difference among groups. A P-value < 0.05 was considered to be statistically significant.

RESULTS

Spontaneous phase III-like contractions in fasting state

Spontaneous phase III-like contractions were observed every 12–15 min in the antrum of mice. The duration of phase I-like contractions was 7–10 min, while the duration of phase III-like contractions was 4–6 min of the mice stomach. The frequency and the peak amplitude of phase III-like contractions were 2.09 ± 0.09 times per 30 min and 1.95 ± 0.02 g (n = 9) respectively. After feeding, the spontaneous phase III-like contractions were completely abolished and interdigestive motor pattern was changed to postprandial motor pattern (Fig. 1).

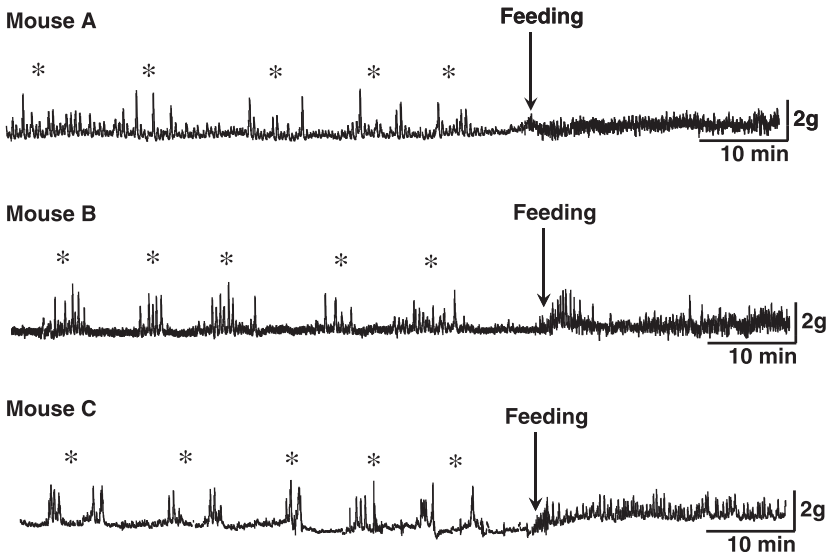

Figure 1 Gastric motor activities before and after feeding in three conscious mice. In a fasting state, regular contractions in the antrum were recorded, the contraction complex (phase III-like contractions) lasted 4–6 min, and the interval of phase II-I-like contractions is 12–15 min (n = 3). Immediately after the feeding, phase III-like contractions disappeared. The fasted state motor pattern was replaced to fed state motor pattern (*spontaneous phase III-like contractions).

Effect of ghrelin administration on gastric motility in the fasted state mice

Three minutes after finishing spontaneous phase III-like contractions, acyl ghrelin (20, 40 and 100 μg kg^{-1} in saline 50 μL, i.p.) were administered. I.p.-injection of saline (50 μL) did not affect spontaneous phase III-like contractions (Fig. 2A). Phase III-like contractions were augmented 2.25 ± 0.25 min after injection ghrelin (40 μg kg^{-1}) and the latency to induce phase III-like contractions was shortened to 1.45 ± 0.20 min by ghrelin (100 μg kg^{-1}) injection (Fig. 2B,C). Ghrelin (40–100 μg kg^{-1}) significantly increased the frequency (Fig. 3A), peak amplitude (Fig. 3B) and MI (Fig. 3C) of phase III-like contractions in a dose-dependent manner. Ghrelin at 20 μg kg^{-1} only significantly increased the frequency (Fig. 3A).

Effect of GHS-R antagonists on the spontaneous phase III-like contractions

Administration of a GHS-R antagonist, (D-lys3)GHRP6 (0.28 mg kg^{-1}, i.p.) failed to inhibit spontaneous phase III-like contractions (Fig. 4A), while (D-lys3)GHRP6 (2.8 mg kg^{-1}, i.p.) blocked spontaneous phase III-like contractions for more than 50 min (Fig. 4B).

Peak amplitude and MI of spontaneous contractions were significantly reduced by (D-lys3)GHRP6 (2.8 mg kg^{-1}, i.p.) (Table 1).

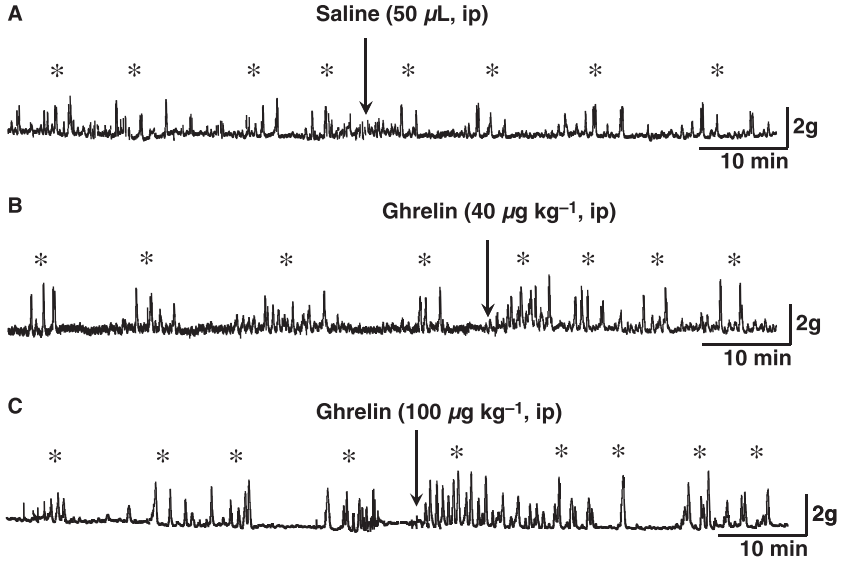

Figure 2 Effect of saline and ghrelin (40 and 100 μg kg^{-1}, i.p.) on gastric phase III-like contractions in the interdigestive state in conscious mice. Phase III-like contractions were augmented after ghrelin (40 and 100 μg kg^{-1}) injection (B,C), while saline injection had no effect on spontaneous phase III-like contractions (A) (*spontaneous phase III-like contractions).

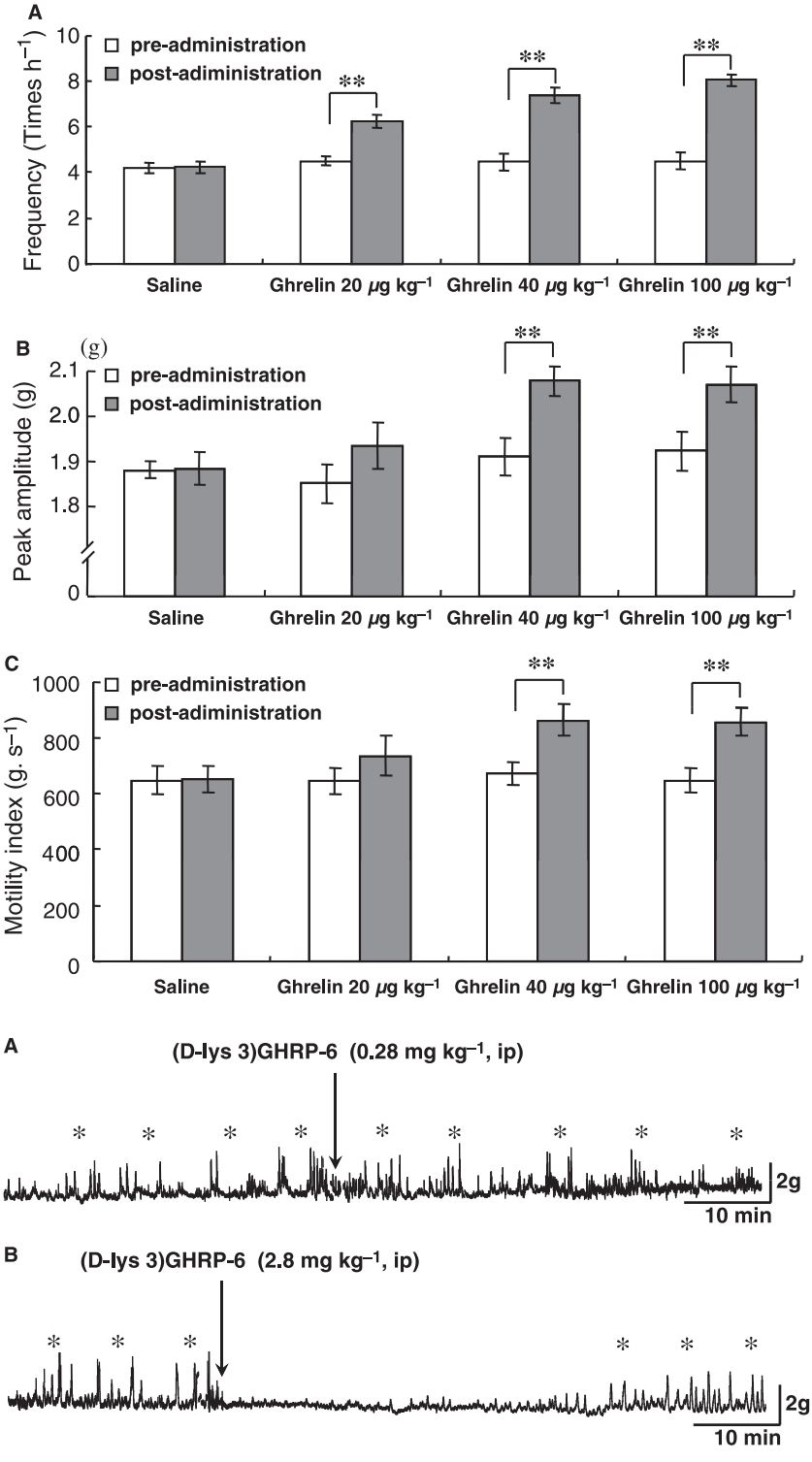

Figure 3 Effect of ghrelin (20, 40 and 100 μg kg^{-1} i.p.) on gastric phase III-like contractions in the interdigestive state in conscious mice. Frequency (A), ghrelin administration (20, 40 and 100 μg kg^{-1}) give dose-dependent effect on frequency of gastric contractions in conscious mice. Peak amplitude (B), ghrelin administration (40 and 100 $\mu\varpi$ g kg^{-1}) give stimulating effects on peak amplitude of gastric contractions in conscious mice, but no significant effects were observed for ghrelin(20 μg kg^{-1}) administration. Motility index (MI), ghrelin administration (40 and 100 μg kg^{-1}) give stimulating effects on MI of gastric contractions in conscious mice, but no significant effects were observed for ghrelin (20 μg kg^{-1}) administration (n = 4, *P < 0.05, **P < 0.01).

Figure 4 Effect of (D-lys3)GHRP6 on spontaneous phase III-like contractions of the mice stomach. (D-lys3)GHRP6 (0.28 mg kg^{-1}; i.p.) did not affect the spontaneous phase III-like contractions (A). In contrast, (D-lys3)GHRP6 (2.8 - mg kg^{-1}; i.p.) almost completely abolished spontaneous phase III-like contractions. The inhibitory effects of (D-lys3)GHRP6 (2.8 mg kg^{-1}) on spontaneous phase III-like contractions lasted more than 1 h (B) (*spontaneous phase III-like contractions).

Effect of atropine and vagotomy on gastric contractions

Administration of atropine (500 μg kg^{-1}) abolished spontaneous phase III-like contractions. Ghrelin (40 μg kg^{-1}) failed to cause phase III-like contractions in the presence of atropine (Fig. 5A).

In vagotomized mice, spontaneous phase III-like contractions were no more observed. MI of the spontaneous contractions was significantly reduced in

Table 1 Effect of (D-lys)GHRP-6 (2.8 mg kg^{-1}, i.p.) on frequency, peak amplitude and motility index (MI) of phase III-like contractions of mice stomach

	Frequency (times per 30 min)		Peak amplitude (g)		MI (g•s)	
	Before	After	Before	After	Before	After
Saline	2.09 ± 0.11	2.11 ± 0.12	1.84 ± 0.02	1.85 ± 0.03	625.6 ± 47.5	630.2 ± 45.4
(D-lys3) GHRP-6	2.38 ± 0.13	0.08 ± 0.02**	2.15 ± 0.08	0.3 ± 0.04**	618.4 ± 17.1	210.9 ± 11.6**

(D-lys)GHRP-6 almost completely abolished the spontaneous phase III-like gastric contractions (**$P < 0.01$, $n = 4$).

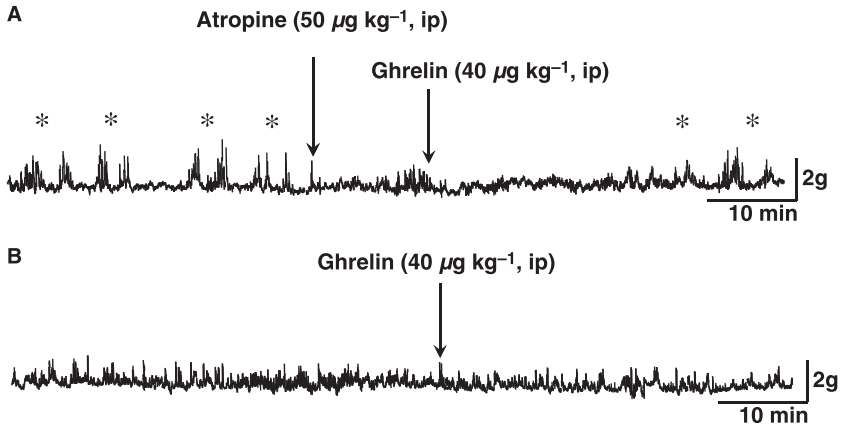

Figure 5 Effect of atropine (A) and vagotomy (B) on gastric contractions in the fasted state in mice. Injection of atropine (50 μg kg^{-1}) abolished spontaneous phase III-like contractions. Administration of ghrelin (40 μg kg^{-1}) failed to stimulate gastric contraction (A) (*spontaneous phase III-like contractions, representative tracing from four atropine-treated rats). In vagotomized mice, no spontaneous phase III-like contractions were observed. Administration of ghrelin (40 μg kg^{-1}) failed to stimulate gastric contractions in vagotomized mice (B).

vagotomized mice (435.9 ± 13.6 g•s, $n = 4$), compared to that of sham-operated mice (636.6 ± 35.3 g•s, $n = 4$, $P < 0.01$). Ghrelin (40 μg kg^{-1}) failed to cause any phase III-like contractions on vagotomized mice (Fig. 5B).

DISCUSSION

Ghrelin has a sequence similarity with motilin. Receptors of motilin and ghrelin also share a marked sequence homology.[11] It has been well established that motilin administration causes phase III contractions in the interdigestive state in humans and dogs.[12,13] However, motilin administration failed to stimulate gastric emptying and GI transit in rats.[14]

Ghrelin augments interdigestive gastric contractions in conscious rats.[7] Ghrelin also induces premature phase III contractions of the human stomach,[5] while others showed that ghrelin administration failed to stimulate gastric phase III contractions in conscious dogs.[15] These suggest that species differences are present in motilin- and ghrelin-induced interdigestive contractions of the stomach. However, it remains unknown whether ghrelin is involved in mediating interdigestive contractions of the mice stomach.

It has been well established that GHS-R is involved in regulating GH release and feeding behaviour in rodents.[9,16] However, recent studies showed that ghrelin is not a critical endogenous factor or has redundant role in the regulation of food intake and gastric emptying in mice.[10] Mutant mice with a deletion of the ghrelin gene did not show impaired growth or appetite.[17,18] The knockout mice are not dwarf and show normal increases in bodyweight gain and food intake.

Growth-hormone secretagogue receptor antagonist [(D-lys3)GHRP6] abolished the spontaneous phase III-like contractions in rat stomach,[8] suggesting that the endogenous ghrelin regulates interdigestive gastric contractions in rats. In this study, a GHS-R antagonist also abolished spontaneous phase III-like contractions in mice stomach. This suggests that endogenous ghrelin regulates gastric phase III-like contractions through GHS-R in mice.

It has been shown that (D-lys3)GHRP6 at 10 μmol L^{-1}, but not at 1 μmol L^{-1}, induced biphasic contractions of the rat fundic strips *in vitro*. (D-lys3)GHRP6-indcued contraction was markedly reduced by the 5-HT_{2B} receptor antagonist. This suggests that pharmacological dose of (D-lys3)GHRP6 (10 μmol L^{-1}) stimulates muscle contractions via 5-HT_{2B} receptors of the rat stomach.[19] However, this study showed that (D-lys3)GHRP6 (0.28 and 2.8 mg kg^{-1}, i.p.) itself caused no muscle contractions of the mice stomach *in vivo*. This indicates that (D-lys3)GHRP6 does not interact with 5-HT_{2B} receptors of the mouse stomach.

The orexigenic effect of ghrelin is mediated via the vagal nerve, and circulating ghrelin acts predominantly on the stimulation of feeding and secretion of GH via the gastric vagal afferents in rats.[20] In this study, atropine and vagotomy abolished spontaneous phase III-like contractions as well as ghrelin-induced phase III-like contractions of the mice stomach. These indicate that both of endogenous and exogenous ghrelin-induced phase III-like contractions are mediated via vagal cholinergic pathways of the mice stomach.

It has not well been established whether vagus nerve regulates the occurrence of gastric phase III contractions in dogs. Gastric phase III contractions are completely abolished by acute bilateral cooling of the cervical vago-sympathetic nerve trunk in dogs.[21] As sympathetic receptor blockers do not affect the inhibitory effect of vago-sympathetic blockage, it is suggested that vagal innervation plays a major role in mediating gastric phase III contractions in dogs.[22]

It remains controversial whether bilateral truncal vagotomy alters interdigestive gastric motor activity in dogs. Spontaneous myoelectric activity (measured by serosal electrodes) of the stomach is not altered after truncal vagotomy in the interdigestive state.[23] In contrast, the same group showed, using force transducers, that chronic vagotomy (2 weeks after vagotomy) reduces the motor activity of gastric phase III contractions without affecting MMC cycle in dogs.[24]

We have successfully established the recording system of gastric contractions in freely moving conscious mice. This study, for the first time, demonstrated that phase III-like contractions exist in mice and that endogenous ghrelin regulates gastric phase III-like contractions through GHS-R. Ghrelin regulates interdigestive contractions through vagal cholinergic pathways in conscious mice.

Recently, ghrelin knockout mice[10] and ghrelin receptor knockout mice[9] have been developed. Our recording system of mice gastric motility may be useful to study the functional changes in gene knockout mice in the future.

Perspectives

Previous reports suggest that gastric and intestinal phase III contractions are regulated by the different mechanisms in dogs. Plasma motilin level is highly associated with the appearance of gastric phase III in dogs.[25] In contrast, phase III contractions in the small intestine sometimes occur without a concomitant increase in plasma motilin concentration.[26] Motilin antiserum inhibits the occurrence of phase III contractions only in the stomach, not in the intestine.[27] After duodenectomy, no obvious phase III contractions were observed in the antrum, but migrating phase III contractions were observed in the upper jejunum in dogs.[28] Acute vagal nerve blockade abolished gastric phase III without affecting intestinal phase III in dogs.[21,22] These suggest that vagal nerve regulates gastric, but not intestinal, phase III contractions in dogs.

Endogenously released ghrelin can regulate spontaneous phase III-like contractions of the stomach in rats.[8] Our recent study showed that spontaneous phase III-like contractions of the antrum are completely disappeared 7 days after bilateral truncal vagotomy. In contrast, intestinal phase III-like contractions are not altered after vagotomy.[29] This suggests that gastric phase III-like contractions are also mediated via vagal nerve in rats.

In contrast, interdigestive myoelectric activity of the stomach and intestine is not altered after truncal vagotomy in dogs.[23] Chronic vagotomy (2 weeks after vagotomy) reduces gastric phase III contractions, but does not affect MMC cycle in dogs.[24]

It is likely that the adaptive mechanism following vagotomy may develop to maintain gastric phase III contractions. We have previously shown that the pressure increase evoked by gastric distension (the accommodation reflex) was blocked by acute truncal vagotomy in rats. However, the accommodation reflex was fully restored 4 weeks after vagotomy in rats.[30] The adaptive mechanism following vagotomy may explain the discrepancy between the effects of acute and chronic blockade of vagal activity on gastric phase III contractions.

This study demonstrated that phase III-like contractions were completely disappeared 7 days after vagotomy of the mouse stomach.

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