Coordination of motilin and ghrelin regulates the migrating motor complex of gastrointestinal motility in *Suncus murinus*

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Mondal A, Xie Z, Miyano Y, Tsutsui C, Sakata I, Kawamoto Y, Aizawa S, Tanaka T, Oda S, Sakai T. Coordination of motilin and ghrelin regulates the migrating motor complex of gastrointestinal motility in *Suncus murinus*. Am J Physiol Gastrointest Liver Physiol 302: G1207–G1215, 2012. First published March 1, 2012; doi:10.1152/ajpgi.00379.2011.—Motilin and ghrelin are the gastrointestinal (GI) hormones released in a fasting state to stimulate the GI motility of the migrating motor complex (MMC). We focused on coordination of the ghrelin/motilin family in gastric contraction in vivo and in vitro using the house musk shrew (*Suncus murinus*), a ghrelin- and motilin-producing mammal. To measure the contractile activity of the stomach in vivo, we recorded GI contractions either in the free-moving conscious or anesthetized *S. murinus* and examined the effects of administration of motilin and/or ghrelin on spontaneous MMC in the fasting state. In the in vitro study, we also studied the coordinative effect of these hormones on the isolated stomach using an organ bath. In the fasting state, phase I, II, and III contractions were clearly recorded in the gastric body (as observed in humans and dogs). Intravenous infusion of ghrelin stimulated gastric contraction in the latter half of phase I and in the phase II in a dose-dependent manner. Continuous intravenous infusion of ghrelin antagonist (o-Lys3-GHRP6) significantly suppressed spontaneous phase II contractions and prolonged the time of occurrence of the peak of phase III contractions. However, intravenous infusion of motilin antagonist (MA-2029) did not inhibit phase II contractions but delayed the occurrence of phase III contractions of the MMC. In the in vitro study, even though a high dose of ghrelin did not stimulate contraction of stomach preparations, ghrelin administration (10−10−10−7 M) with pretreatment of a low dose of motilin (10−10 M) induced gastric contraction in a dose-dependent manner. Pretreatment with 10−8 M ghrelin enhanced motilin-stimulated gastric contractions by 10 times. The interrelation of these peptides was also demonstrated in the anesthetized *S. murinus*. The results suggest that ghrelin is important for the phase II contraction and that coordination of motilin and ghrelin are necessary to initiate phase III contraction of the MMC.

In many monogastric animals (including rodents, dogs, pigs, rabbits, and humans), the stomach and small intestine undergo a temporally coordinated cyclic motor pattern during the interdigestive state known as the migrating motor complex (MMC) (36). It has been established that the MMC consists of phase I (motor quiescence), phase II (preceding irregular contractions) and phase III (clustered potent contractions). It has been considered that phase III contractions of the MMC have physiological importance for clearance of secretions, debris, and microbes during fasting to enable the stomach to be prepared to receive the next meal (36), and the quiescent period (phase I) of the MMC allows muscles to rest and regenerate. Therefore, the physiological role of the MMC necessitates scientific interest. It has been well documented that gastric phase III is strongly associated with the peak of plasma motilin levels in humans (17, 37) and dogs (11, 14, 16), and intravenous administration of motilin causes gastric phase III-like contractions in humans (17) and dogs (14, 39). However, the mechanism controlling the MMC is incompletely elucidated.

Motilin was originally purified from porcine intestinal mucosa in the 1970s, and its molecular structure was determined to be a 22 amino acid polypeptide (4). Similarly, ghrelin is involved in the stimulation of growth hormone secretion (19) to modulate food intake (24). Also, the plasma peak of ghrelin is correlated with phase III-like contractions in rats (1). Intravenous infusion of ghrelin stimulates gastric contraction in rats (22) and mice (45), suggesting that ghrelin serves as an alternative to motilin with regard to gastrointestinal (GI) motility in motilin-lacking rodents (27). Although ghrelin does not induce gastric phase III contractions in dogs (25), it has been shown that ghrelin induces premature phase III contractions in the human stomach (32).

It is now accepted that motilin and ghrelin are important in mediating phasic contraction of the MMC in some animals (including humans). Because the same family of peptides have common characteristics on the physiological response, it is reasonable to expect additional or synergic effects between ghrelin and motilin on GI motility. However, novel coordination of the peptides family upon the regulation of the MMC has not been revealed. This slow progression may be explained by the lack of suitable animal models. For example, mice and rats cannot be used for such studies because the presence of pseudogenes cause decreased production of motilin and its receptor (12). The mouse and rat genes encoding motilin and the motilin receptor were lost through independent mutations in existing genes and not by a disruptive chromosomal rearrangement that potentially could have removed both genes in a single event (12). Also, the characteristic features of gastric MMC among species are different. For example, the MMC cycle in rats is short (<20 min) compared with that of humans and dogs (usually observed every 90–120 min) (1, 8, 34).
We screened several laboratory animals that could be used as a new model for the mechanism of motilin and ghrelin-induced gastric contraction. We focused on house musk shrew (*Suncus murinus*). *S. murinus* has a visceral system that is very similar to that of humans and is a useful model of physiology and pathophysiology in the latter (41–44). The general appearance of the gastric mucosa of *S. murinus* is similar to that of the human gastric mucosa, and differs from some other widely used experimental animals, such as hamsters, rats, and mice (18). For example, the gastric mucosa consists of a glandular mucosa with well-developed luminal folds. Moreover, unlike the mouse and hamster, *S. murinus* has no forestomach (stratified squamous epithelial region). The pyloric and fundic regions are histologically distinguishable from each other by the presence of pyloric and fundic glands, respectively (18). We previously identified the cDNA sequence of suncus motilin and ghrelin in *S. murinus* using the PCR cloning method (13, 35). We also studied the contractile properties of the stomach in conscious free-moving *S. murinus* as well as in organ bath experiments. We found that *S. murinus* has almost identical GI motility and motilin responses as those found in humans and dogs (29, 35). We feel that *S. murinus* is the only small animal that can be used to study the effects of motilin and ghrelin on GI motility and to conduct a detailed analysis of the morphology and physiological mechanism.

We therefore investigated the effects of motilin and ghrelin (and their coordination) in regulation of the MMC of the GI motility in vivo and in vitro in this animal.

**MATERIALS AND METHODS**

**Ethical approval of the study protocol.** All procedures were approved and performed in accordance with the Committee on Animal Research of Saitama University (Saitama, Japan). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiment.

**Animals.** Experiments were carried out using adult male (10–30 wk of age) and female (5–30 wk of age) *S. murinus* of an outbred KAT strain established from a wild population in Kathmandu (Nepal), weighing between 50 and 100 g. Animals were housed individually in plastic cages equipped with an empty can for a nest box under controlled conditions (23 ± 2°C, 12:12-h lights on from 8 AM to 8 PM) with free access to water and commercial feeding pellets (no. 5P; Nippon Formula Feed Manufacturing, Yokohama, Japan). The metabolizable energy content of the pellets was 344 kcal/100 g. The pellets consisted of 54.1% protein, 30.1% carbohydrate, and 15.8% fat.

**Animal preparation for GI motility recording in vivo.** After fasting for 3 h, each animal was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Through a midline laparotomy, strain gauge force transducers were implanted on the serosal surface of the gastric body for recording the contractions of circular muscle. The wires from the transducer were exteriorized through the abdominal wall and run under the skin toward the back of the neck. An intravenous catheter was inserted into the right jugular vein and also exteriorized to the back of the neck. The catheter was filled with heparinized saline (100 units/ml) to prevent coagulation. The wires and catheter were protected by a protective jacket. Animals started to eat food 1 day after surgery. Food intake was similar to that in the nonoperated group (data not shown). In addition, the body weight of
the animals remained almost the same before and during the in vivo experiments (29). Motilin and ghrelin or other chemical compounds were administered on the 3rd day after surgery.

**Monitoring of GI motility in vivo.** The strain gauge force transducers used in the present study were made by our research team with appropriate modification of a previously reported method (15). Waterproofing and response properties were checked in all transducers before implantation. Amplified analog signals were converted with an analog-digital converter (model ADC-20; Pico Technology, St. Neots, UK), and then the digital signals were recorded on a personal computer. Spontaneous GI motility was recorded for 8–10 h in the fasting state. The definition of phase III contractions of the MMC in the conscious *S. murinus* was based upon that in dogs and humans; clustered contractions with amplitude of > 8 g and lasting > 5 min.

Similarly, phase I was recognized as a period of motor quiescence and phase II was recognized as a period preceding irregular contractions.

**Drugs used.** Acetylcholine chloride (Sigma Aldrich, St. Louis, MO) was dissolved in distilled water. Synthesized motilin from *S. murinus* (Scrum, Tokyo, Japan), active human ghrelin (Asubio Pharma, Hyogo, Japan) and D-lys3-GHRP6 (Bachem, Torrance, CA) (2, 33, 45) were dissolved in 0.1% BSA/PBS. MA-2029 (kindly donated by Chugai Pharmaceutical, Tokyo, Japan) (26, 31) was dissolved in 0.9% (physiological) saline. In the antagonist experiment, the stomachs were equilibrated with atropine sulfate (10⁻⁶ M; Merck) for 30 min and then treated with *Suncus* motilin.

**Administration of ghrelin at different phases of GI motility in the fasting state.** Administration of acyl ghrelin (0.1, 0.5, 1, and 5 µg·kg⁻¹·min⁻¹ iv) was started at 50% (first half of phase I) and 70% (latter half of phase I) of the duration of recent phase I and was continued for 10 min. B: typical examples of the effects of intravenous administration of ghrelin (0.1, 0.5, 1, and 5 µg·kg⁻¹·min⁻¹) in the latter half of phase I of the MMC. Ghrelin at each dose stimulated gastric motility in the latter half of phase I. *Phase III contraction peak.*

**Fig. 2. Effects of ghrelin administration on gastric motility in the latter half of phase I of the MMC.** A: an intravenous infusion of ghrelin (1 µg·kg⁻¹·min⁻¹) was started at 70% of the duration of recent phase I and was continued for 10 min. B: typical examples of the effects of intravenous administration of ghrelin (0.1, 0.5, 1, and 5 µg·kg⁻¹·min⁻¹) in the latter half of phase I of the MMC. Ghrelin at each dose stimulated gastric motility in the latter half of phase I. *Phase III contraction peak.*

**Fig. 3. The motility index (MI; %) of ghrelin administration in the first half and latter half of phase I of the MMC.** The MI was calculated as the area under the curve (AUC). Percentages of the AUC in ghrelin-induced contractions compared with that of adjacent spontaneous phase III contractions. Bars in the graphs represent means ± SE. *P < 0.05 vs. first half; †P < 0.05 vs. saline (*n* = 5).
(latter half of phase I) of the duration of recent phase I and was continued for 10 min. Similarly, administration of ghrelin (0.1 and 0.2 μg·kg⁻¹·min⁻¹) was initiated 10–15 min after starting spontaneous phase II contractions in the fasting S. murinus. Quantified ghrelin-induced gastric motility was represented by the motility index (MI). In this study, the MI during 10-min infusion of motilin was defined as the percentage of the area under the curve for the 10-min duration of adjacent phase III contractions.

Effect of antagonist of motilin and ghrelin on gastric contractions in vivo. Administration of d-Lys3-GHRP6 (ghrelin receptor antagonist) and MA-2029 (motilin receptor antagonist) was initiated 10–20 min after starting of phase II contractions in the fasting S. murinus. d-Lys3-GHRP6 (6 mg·kg⁻¹·h⁻¹) and MA-2029 (1 mg·kg⁻¹·h⁻¹ iv) were continuous infused for 120 min. Saline was administered as a control. d-Lys3-GHRP6 and MA-2029 were prepared in each experiment as per manufacturer instructions.

Preparation of isolated whole stomach in vitro. After the induction of anesthesia, animals were killed by decapitation. The stomachs were immediately placed into freshly prepared Krebs solution (composition in mM: 118 NaCl, 4.75 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.8 NaH₂PO₄, 25 NaHCO₃, and 11.5 glucose; pH 7.2). After trimming and washing of tissue, stomachs were mounted in 10 ml water-jacketed organ baths and initially loaded with weights (~1.0 g). The temperature of the solution was maintained at 37 ± 0.5°C, and the solution was continuously aerated with a mixture of 95% O₂-5% CO₂.

Study of gastric contractility in vitro. Contractile activities of stomachs with treatment by motilin and ghrelin were monitored using an isometric force transducer (model UM-203; Iwashita Kishimoto Medical Industrial, Kyoto, Japan) and special software (PicoLog for Windows, Pico Technology). To normalize the data, a control contraction was measured using ACh (10⁻⁵ M) treatment in each tissue, and the contractions were expressed as a percentage of the control contractions. Concentration-response curves were made by cumulative addition of motilin and ghrelin at appropriate intervals to the organ bath.

**RESULTS**

In the fasting state, similar interdigestive contractions were observed in the gastric body of S. murinus as those reported in humans and dogs (29). In the present study, phase I, phase II, and phase III contractions were clearly recognized at regular intervals (Fig. 1A).

Effect of ghrelin administration in phase I of the MMC. At 10% (first half) and 70% (latter half) of the duration of phase I of the MMC, intravenous injections of ghrelin and saline were administered. Figure 1A is a schematic representation of the effect of ghrelin administration during the first half of phase I, and Fig. 1B shows the contractile response evoked by infusion of synthetic active human ghrelin (0.1, 0.5, 1, and 5 μg·kg⁻¹·min⁻¹) for 10 min. Although infusion of 5 μg·kg⁻¹·min⁻¹ ghrelin induced slight contractions, virtually no responses to other doses of ghrelin administration were observed in the first half of phase I. In the latter half of phase I, intravenous infusion of ghrelin showed gastric contractions (Fig. 2A), and Fig. 2B shows the contractions evoked by the infusion of synthetic ghrelin (0.1, 0.5, 1, and 5 μg·kg⁻¹·min⁻¹) for 10 min. Although ghrelin did not stimulate gastric contractions in the first half, MI of ghrelin administration in the latter half of phase I significantly increased in a dose-dependent manner (Fig. 3).

**Data and statistical analyses.** We repeated the recording experiments individually at least three times and obtained similar results. We also showed the numbers of animals used for statistical analyses in the figure legends. Values are mean ± SE. Statistical analyses were undertaken using ANOVA followed by the Student’s t-test. *P < 0.05 was considered significant.
Effect of ghrelin administration in phase II of the MMC. At 10–15 min after starting phase II contractions of the MMC, intravenous injections of saline or ghrelin were administered. Figure 4, A and B are the schematic representations of the effects of intravenous infusions of synthetic ghrelin; gastric contractions were observed at ghrelin doses of 0.1 and 0.2 μg·kg⁻¹·min⁻¹ for 10 min. Ghrelin administration increased the amplitude of spontaneous phase II contractions (Fig. 4C). Ghrelin significantly increased the MI of phase II contractions in a dose-dependent manner.

Effect of MA-2029 and D-Lys3-GHRP6 on gastric contractions in phase II and III of the MMC. The motilin receptor antagonist, MA-2029 (1 mg·kg⁻¹·h⁻¹) and saline (control) were infused intravenously for 120 min (Fig. 5A) in phase II. Administration of MA-2029 and saline showed no change in the MI in phase II (Fig. 5B), but the occurrence of phase III contraction was significantly delayed by MA-2029 administration (214 ± 28.3 vs. 77.4 ± 19.3 min, Fig. 5C). Conversely, a 120-min infusion of D-Lys3-GHRP6 (6 mg·kg⁻¹·h⁻¹ iv) in phase II suppressed spontaneous contractions (Fig. 5D and E), significantly delayed the occurrence of phase III contractions (270.2 ± 12.8 vs. 68.9 ± 7.8 min, Fig. 5F). Also, the coadministration of the motilin and ghrelin antagonists completely abolished spontaneous phase III contractions (Fig. 5G).

Synergistic effect of motilin and ghrelin in gastric contractions in vitro and in vivo. Synthetic S. murinus motilin induced a phasic response in the prepared isolated stomachs at concentrations from 10⁻⁹-10⁻⁷ M in a dose-dependent manner (Fig. 6, A and B). Cumulative administration of active human ghrelin (10⁻¹¹-10⁻⁷ M) did not evoke gastric contractions in vitro (data not shown). Next, we studied ghrelin-induced gastric contractions in S. murinus in the presence of different concentrations of motilin. Pretreatment with 10⁻¹² M and 10⁻¹¹ M motilin had no effect on ghrelin treatment. However, after pretreatment with a low dose of motilin (10⁻¹⁰ M), ghrelin dramatically stimulated gastric contraction from 10⁻¹⁰ M in a dose-dependent manner (Fig. 6, C and D). Conversely, after pretreatment with 10⁻⁸ M ghrelin, motilin started to
evoke gastric contraction from low doses (10^{-10} M) (Fig. 6, E and F), and the dose-response curve shifted ~10-fold to the left, and the final mediators of this synergistic effect are cholinergic neurons (Fig. 6G). In the anesthetized S. murinus, although repeated injections of S. murinus motilin (10 ng/kg body wt given three times at 10-min intervals) and acylated human ghrelin (20, 40, and 80 ng/kg body wt given at 10-min intervals) did not provoke gastric contraction, coadministration of these two peptides induced synergistic gastric contraction in a dose-dependent manner (Fig. 7, A and B) as observed in the in vitro study. Moreover, the frequency (number/min) observed (mean ± SE) in anesthetized S. murinus (13.7 ± 1.9) was almost the same as the frequency of spontaneous phase III contractions (14.2 ± 0.4). The average amplitude (tension, gram wt) of the maximum contraction of synergistically induced gastric contractions was 4.6 ± 0.3, which was 75–85% of control contractions (5.5 ± 0.1). Taken together, these results suggest that the synergistic contractions observed with motilin and ghrelin treatment can be compared with phase III contractions.

**DISCUSSION**

To clarify the effect of motilin and/or ghrelin on phase I, II, and III contractions of the MMC, we studied the effect of the ghrelin/motilin family on gastric contraction in vivo and in vitro using S. murinus, as this produces ghrelin and motilin.
We found that continuous administration of ghrelin induced gastric contractions in the latter half of phases I and II. The ghrelin antagonist D-lys3-GHRP6, almost completely abolished spontaneous phase II contractions and delayed occurrence of the peak of phase III contractions. Intravenous infusion of the motilin antagonist MA-2029, did not inhibit phase II contractions but significantly delayed the phase III peak. Interestingly, coadministration of low doses of motilin and ghrelin synergistically induced gastric contraction in vitro and in vivo. This is the first report showing the coordination effect of the motilin/ghrelin family on the MMC as well as the synergistic effect of gastric contraction by motilin and ghrelin. S. murinus has the same responses and GI contractile properties as humans and dogs. Hence, results from the study of this animal model can be (cautiously) applied to human studies.

The relationship between plasma ghrelin levels and the MMC has been reported. For example, D-lys3-GHRP6 significantly attenuated spontaneous gastric phase III-like contractions in rats (1, 33) and changes in plasma ghrelin concentrations related to the occurrence of the MMC in rats (1) and dogs (46). In the present study, the stimulatory effect of ghrelin was in late phase I and phase II and in the initiation of phase III. Hence, it is likely that elevation of plasma ghrelin levels is important for continuation of the MMC in the gastric body of S. murinus. However, why ghrelin cannot stimulate gastric contractions in the early part of phase I remains unknown. One possibility is that strong inhibition by inhibitory neurons may occur at that stage and from the time onwards, inhibition gradually decreases. Hence, ghrelin can stimulate gastric contractions in the latter half of phase II. It has been shown that nitrite levels are increased by ghrelin administration in rats (3). Several studies also reported that transient inhibition of nitric oxide is associated with stimulation of phase III activity (21, 28, 30), as well as a reduction in quiescence after this phase III activity in humans (28). Therefore, endogenous elevated ghrelin levels in phase III may trigger nitric oxide release and this may cause strong inhibition in the early phase I period. Another possibility is related to secretion of gastric acid. Several authors have reported that ghrelin stimulates acid secretion (3, 20, 22), and a close relationship exists between interdigestive antroduodenal motility and secretion of gastric acid (5, 6, 9, 38). In dogs, the intragastric acidification blocks spontaneous phase III activity (23), and this effect has also been observed during exogenous infusion of motilin in humans (10) and dogs (40). Therefore, one may postulate that the endogenous increase of ghrelin in phase III stimulates acid secretion and may decrease the responses to ghrelin in the early phase I period.

Although motilin is known to be important for gastric phase III contractions of the MMC, the contractile effect on phase II is not known. It has been reported that contractile patterns are different between phase II and phase III contractions in dog (i.e., irregular and regular phasic contractions in phase II and III, respectively). We found almost identical unique gastric patterns in the phase II and phase III period in the stomach of conscious S. murinus (data not shown). In the in vivo study in S. murinus, contractile properties induced by motilin were also almost identical to the spontaneous phase III contractions found in humans and dogs (29). However, motilin-induced gastric contractile properties in S. murinus were completely different from phase II contractions, suggesting that the stimulatory mechanisms of phase II and phase III contractions might be different. Accordingly, in the present study, we examined the relationship between motilin and phase II contractions using the motilin antagonist MA-2029. First, we studied the effect of MA-2029 on the gastric motility of S. murinus and found that intravenous infusion of MA-2029 completely abolished the phase III-like contractions evoked by exogenous bolus injection of S. murinus motilin even at high doses (data not shown) indicating that MA-2029 can antagonize S. murinus motilin in vivo. In the present study, MA-2029 administration did not change the MI in the spontaneous phase II contractions and significantly delayed initiation of the peak of the phase III contractions. Moreover, only higher doses of motilin (40 ng·kg⁻¹·min⁻¹ for 10 min) can enhance gastric phase II contractions (data not shown). These results suggest that endogenous motilin may not be essential for spontaneous phase II gastric contractions under physiological conditions, however, a certain level of plasma motilin is subsequently necessary in the phase II period for the initiation of phase III contractions. Taken together with the results of treatment with a ghrelin antagonist, the initiation mechanisms in phase II and phase III contractions seem to be different, and ghrelin (but not motilin) is important for phase II contractions.

In the present study, we showed that D-lys3-GHRP6 and MA-2029 significantly delayed the initiation of phase III contractions. These findings indicate that endogenous ghrelin and motilin are involved in the occurrence of phase III contractions of the MMC. We hypothesized that coordination of motilin and ghrelin stimulated this phase III contraction in the gastric body of S. murinus. Indeed, in the organ bath study, the synergistic
In consideration of these results, it is reasonable to effect of motilin and ghrelin in vitro and in the anesthetized stomach of S. murinus is mediated through the myenteric plexus. In consideration of these results, it is reasonable to hypothesize that the occurrence of phase III contractions in S. murinus is coordinated by motilin and ghrelin through a myenteric neural pathway.

Moreover, we recently observed that motilin-induced gastric contraction in S. murinus is mediated through the myenteric plexus. In consideration of these results, it is reasonable to hypothesize that the occurrence of phase III contractions in S. murinus is coordinated by motilin and ghrelin through a myenteric neural pathway.

The present study demonstrated that motilin and ghrelin are physiologically necessary for inducing the MMC in the stomach of S. murinus, and endogenous ghrelin is important for phase II contractions and also needed for the initiation of phase III contractions. Motilin may not be intrinsically involved in phase II contractions, but a significant level of motilin is important for the initiation of phase III contractions in the stomach of S. murinus. In consideration of the synergistic effect of motilin and ghrelin in vitro and in the anesthetized S. murinus in vivo, phase III contractions of the MMC seem to be regulated by this peptide family.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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