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Motilin stimulates pepsinogen secretion in *Suncus murinus*

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ABSTRACT

Motilin and ghrelin are gastrointestinal hormones that stimulate the migrating motor complex (MMC) of gastrointestinal motility during the fasting state. In this study, we examined the effect of motilin and ghrelin on pepsinogen secretion in anesthetized suncus (house musk shrew, *Suncus murinus*), a ghrelin- and motilin-producing mammal. By using a gastric lumen-perfusion system, we found that the intravenous administration of carbachol and motilin stimulated pepsinogen secretion, the latter in a dose-dependent manner, whereas ghrelin had no effect. We then investigated the pathways of motilin-induced pepsinogen secretion using acetylcholine receptor antagonists. Treatment with atropine, a muscarinic acetylcholine receptor antagonist, completely inhibited both carbachol and motilin-induced pepsinogen secretion. Motilin-induced pepsinogen secretion was observed in the vagotomized suncus. This is the first report demonstrating that motilin stimulates pepsinogen secretion, and suggest that this effect occurs through a cholinergic pathway in suncus.

1. Introduction

Pepsinogen is a zymogen secreted by gastric chief cells that is activated by hydrochloric acid produced by parietal cells in the stomach mucosa to form the protein-digesting enzyme pepsin [1]. Pepsinogen is secreted in response to a meal but also occurs during the interdigestive state [2]. In vivo and in vitro studies on pepsinogen secretion have demonstrated that acetylcholine and cholinergic agents stimulate pepsinogen secretion and this response is inhibited by cholinergic antagonists [3,4], suggesting that muscarinic cholinergic receptors are directly and strongly associated with the stimulation of chief cells for pepsinogen secretion [1]. In addition, pepsinogen secretion appears to be regulated by several hormones. Some studies have shown that although histamine can stimulate pepsinogen secretion in vivo, it has no effect in isolated peptic cells from animal stomachs, suggesting that histamine acts as an indirect regulator of pepsinogen secretion by chief cells [1,5]. Similarly, gastrin and secretin stimulate pepsinogen secretion in vivo, but show weak effects in vitro [3]. Moreover, bombesin, glucagon, and somatostatin reportedly regulate pepsinogen release, while cholinergic agonists and cholecystokinin (CCK)-like peptides stimulate pepsinogen secretion by increasing intracellular calcium concentrations in gastric gland and chief cells [1]. Other studies have also reported the involvement of the vagus nerve in the stimulation of pepsinogen secretion [1].

Motilin, a gastrointestinal hormone consisting of 22 amino acids produced mainly in the duodenum [6], plays an important role in gastric contractions. Previous studies demonstrated that gastric contractions are strongly associated with plasma motilin levels and that gastric phase III-like contractions were induced by the intravenous administration of motilin in humans [7], dogs [8,9], and suncus [10,11].

Ghrelin, a well-known multifunctional hormone from the same peptide family as motilin, and originally isolated from rat and human stomachs [12], causes the initiation of food intake in many species after central or peripheral administration [13–15]. In addition, many studies revealed that ghrelin is an important regulator of the gastrointestinal tract [12,14,16] and its administration stimulates gastric acid secretion and gastric motility in the rat [17,18].

Although motilin was discovered decades ago, limited studies on its physiological functions, other than those on gastrointestinal

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motility, have been conducted. The lack of a suitable small laboratory animal model is the most important hindrance to study the biological actions of motilin, as most commonly used experimental animals, i.e., mice and rats, lack the genes for motilin and its receptors [19]. Therefore, we have recently been using suncus as a laboratory animal to study the physiological actions of motilin and the role of the motilin-ghrelin peptide family on gastrointestinal function. Using this animal model, we showed that the migrating motor complex (MMC) repeatedly occurred at 90–120 min intervals in the interdigestive state, and motilin stimulated gastric contractions in a dose-dependent manner, with a gastrointestinal motility pattern similar to that of humans and dogs [10,20]. The general appearance of the gastric mucosa in suncus is similar to that of humans, but differs from that of other widely used experimental animals, such as hamsters, rats, and mice [21]. For example, the suncus has no forestomach (stratified squamous epithelial region) such as that found in mouse and hamsters, and its gastric mucosa consists of glandular mucosa with well-developed luminal folds [21].

Recently, we found that motilin but not ghrelin stimulate gastric acid secretion in a dose dependent manner (manuscript preparation), and the intravenous administration of motilin causes gastric phase-III like contractions in suncus [22]. Gastric acid and pepsinogen secretion increase during the late phase II and phase III [23], and the higher luminal acid concentration leads to an increase in pepsinogen secretion in humans [24]. Considering these findings, we hypothesized that motilin may be involved in the regulation of pepsinogen secretion.

In this study, we established an experimental procedure for the measurement of pepsinogen secretion using a stomach perfusion system in anesthetized suncus to investigate the effect of ghrelin and motilin on pepsinogen secretion. In addition, we also examined the mechanism of motilin-induced pepsinogen secretion using receptor antagonists and vagotomized suncus.

2. Materials and methods

2.1. Animals

Both male (10–30 weeks of age) and female (5–30 weeks of age) adult suncus of an outbred KAT strain established from a wild population in Kathmandu, Nepal [25], with an average body weight of 50–100 g, were used to perform these experiments. Animals were housed individually in plastic cages equipped with an empty can for a nest box under controlled conditions (23 ± 2 °C, lights on from 8:00 to 20:00) with free access to water and commercial feeding pellets (No. 5P; Nippon Formula Feed Manufacturing, Yokohama, Japan). The pellets consisted of 54.1% protein, 30.1% carbohydrates, and 15.8% fat and had a metabolizable energy content of 344 kcal/100 g. All procedures were approved and performed in accordance with the Saitama University Committee on Animal Research (Saitama, Japan). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

2.2. Drugs

The following drugs were used: Urethane (Sigma Aldrich, St. Louis, MO), histamine dihydrochloride (Nakarai Chemicals Co., Ltd., Kyoto, Japan), carbachol (Tocris Bioscience, Ellisville, MO), suncus motilin (Scrum Inc., Tokyo, Japan), and human acylated ghrelin (Asubio Pharma Co., Ltd., Hyogo, Japan). Atropine sulfate (Mylan Pharma, Osaka, Japan) was used as a muscarinic cholinergic receptor antagonist. Motilin and ghrelin were dissolved in 0.1% BSA in PBS (phosphate-buffered saline), while histamine, carbachol, and atropine were dissolved in 0.9% saline. All solutions were prepared immediately before each experiment. Each agent was administered in a volume of 100 μL per 100 g body weight (BW).

2.3. Determination of pepsin output using an intragastric perfusion experimental system

Prior to pepsinogen secretion experiments, overnight-fasted animals were anesthetized intraperitoneally with an injection of 15% urethane solution at a dose of 1 mL/100 g body weight. After anesthesia, the animals' tracheas were exposed, cannulated, and exteriorized, and a catheter (polyethylene tube) was inserted through the mouth into the stomach, the tip was placed at approximately 3 mm from the cardia, and the tube was fixed to the esophagus with a suture. The stomach was exposed through an incision through the linea alba of the abdomen, and after exposing the pyloroduodenal junction, another polyethylene tube was introduced into the stomach via an incision in the duodenum. The inserted polythene tube was secured firmly with a ligature around the pylorus, and the abdominal cavity was closed with sutures. Reagents/drugs were administered via a cannula inserted into the jugular vein. The stomach lumen was washed with saline solution until the effluent was clear, perfused at 37 °C at a rate of 0.25 mL/min through the perfusion catheter using a peristaltic pump (Micro tube pump MP-3EYELA; Tokyo Rikakikai Co., Ltd., Tokyo), and the gastric output was collected through the liquid discharge tube. The body temperature of anesthetized suncus was monitored using a midi logger GL220 (Graphtec, Japan) and maintained at 35–38 °C. In order to stabilize the amount of pepsinogen secreted, we allowed 60 min from the start of the saline perfusion before commencing effluent tube collection continuously at 10-min intervals using a DF-2000 fraction collector (Tokyo Rikakikai Co., Ltd., Tokyo, JAPAN). For experiments using vagotomized suncus, a truncal subdiaphragmatic vagotomy was performed as described previously [22]. In brief, the lower part of the esophagus was exposed to isolate the dorsal and ventral vagus nerves. Both branches of the vagus nerves were cut, and segments of about 3 mm in length were resected. All the neural connections in the resected area were completely peeled using a wiping tissue (Kimwipes; Nippon Paper Crecia, Tokyo, Japan). A stereomicroscope was used to confirm the complete nerve disconnection. After performing the experiments, the suncus were anatomized and completness of vagotomy was confirmed.

2.4. Pepsin measurement

Pepsin (pepsinogen) content in the gastric output was measured according to a previously described method [26]. Briefly, 100 μL of gastric content were incubated with 1 mL of 2% hemoglobin (Sigma Aldrich, St. Louis, MO) for 10 min at 37 °C, and 5 mL of 5% trichloroacetic acid were added to stop the reaction. Following centrifugation (6000 g, 30 min), supernatants containing the soluble hydrolysis products were collected. The absorbance of the supernatants was measured at 280 nm, and the amount of pepsinogen secreted was calculated from a standard curve of authentic pepsin (Sigma Aldrich, St. Louis, MO), and expressed as μg/10 min. The magnitude of the change in gastric pepsinogen secretion was calculated by deducting the area under the curve (AUC) of gastric pepsin/pepsinogen secretion 50 min before and after the administration of each drug. The values were expressed as Δ μg/50 min.

2.5. Experimental protocols

All drugs/agents were administered by an intravenous (i.v.) bolus infusion. Carbachol was administered at a dose of 10 μg/kg of

BW, while human acylated ghrelin and suncus motilin were both administered at a dose of 0.1, 1, and 10 μg/kg BW to study their stimulatory effect on pepsinogen secretion. Based on the dose response obtained, a motilin dose of 10 μg/kg BW was selected for subsequent experiments. Atropine (100 μg/kg BW) was used to evaluate the role of muscarinic acetylcholine (mACh) receptors on motilin-induced pepsinogen secretion. After confirming that pre-treatment of atropine (100 μg/kg BW) completely inhibited carbachol-induced (10 μg/kg BW) pepsinogen secretion, pretreatment with vehicle or atropine was carried out 30 min before motilin administration (10 μg/kg BW).

2.6. Statistical analyses

Individual experiments were repeated and recorded at least three times. All data are shown as mean ± S.E.M. GraphPad Prism 5 software (GraphPad Software Inc., CA, USA) was used to analyze the data. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons or Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of a perfusion system for pepsinogen measurement in suncus

We first examined the effect of carbachol (10 μg/kg BW) on pepsinogen secretion to establish a perfusion system for pepsinogen measurement in suncus. We found that i.v. administration of carbachol to urethane-anesthetized suncus increased pepsinogen secretion (488.1 ± 96.3 μg/50 min), in a manner comparable to that observed in other experimental animals (Fig. 1A,C). Pretreatment with atropine (100 μg/kg BW), a muscarinic acetylcholine receptor antagonist, abolished carbachol (10 μg/kg BW)-induced pepsinogen secretion (Fig. 1B). Compared to vehicle pretreatment, the amount of carbachol-induced pepsinogen secreted in 50 min was significantly reduced in atropine pretreated animals (Fig. 1C).

3.2. Effect of motilin on pepsinogen secretion

Although i.v. administration of vehicle (0.1% BSA in PBS) did not change basal pepsinogen (68.5 ± 10.9 μg/10 min) secretion, motilin caused a gradual increase after 10 min that reached a maximum within 20 min (Fig. 2A), and a return to baseline levels 40 min after administration (Fig. 2A). Motilin administration caused a dose-dependent increase in the levels of pepsinogen secretion of 56.1 ± 22.9, 172.3 ± 24.0 and 320.3 ± 49.2 μg/50 min at the doses of 0.1, 1.0, and 10 μg/kg BW, respectively (Fig. 2B).

3.3. Effect of ghrelin on pepsinogen secretion

By contrast, i.v. administration of vehicle and ghrelin at doses of 0.1, 1.0, and 10 μg/kg BW caused an unremarkable increase in pepsinogen secretion (Fig. 3A). The levels of pepsinogen secreted in 50 min were 10.8 ± 13.8, 32.2 ± 9.8, and 49.5 ± 16.3 μg/50 min after administration of ghrelin at 0.1, 1.0, and 10 μg/kg BW, respectively. However, compared to vehicle, this increase was not statistically significant (Fig. 3B).

3.4. Effect of atropine and vagotomy on motilin-induced pepsinogen secretion

In order to examine whether the muscarinic acetylcholine receptor is the mediator of motilin-induced pepsinogen secretion, we next studied the effect of atropine on this response. Vehicle or atropine (100 μg/kg BW) were administered i.v. 30 min before the administration of motilin (10 μg/kg BW). Atropine treatment gradually decreased the baseline levels of pepsinogen secretion, which did not peak after motilin administration (Fig. 4B). The stimulatory effect of motilin on the 50-min cumulative pepsinogen secretion was completely inhibited (427.9 ± 61.5 μg/50 min vs 22.5 ± 26.1 μg/50 min) by atropine pretreatment, and this effect was significant compared to that of vehicle (Fig. 4C). To clarify the role of the vagus nerve on motilin-induced pepsinogen secretion, we administered motilin to vagotomized and anesthetized suncus. However, pepsinogen secretion did not differ between vagotomized and control animals (Fig. 4D,E). Moreover, the net change in the 50-min cumulative pepsin output between control and vagotomized suncus after motilin administration was not statistically significant (Fig. 4F).

4. Discussion

As common laboratory rodents, such as rats and mice, lack genes for motilin and its receptor (GPR38) [19], these animals unsuitable for motilin studies. Thus, in this study we used suncus, a motilin and ghrelin-producing mammal, to study the physiological roles of motilin. While suncus was initially established as an animal model for emesis and vomiting research [27], we found that it not only

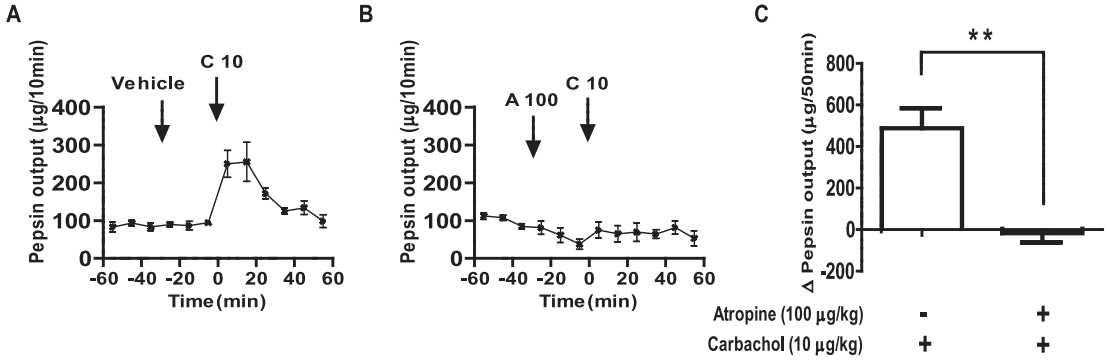

Fig. 1. Effect of carbachol on pepsinogen secretion. Pepsinogen secretion was measured after a 60-min basal period following the administration of carbachol to urethane-anaesthetized suncus (10 μg/kg BW) pretreated with vehicle (A) or atropine (B), a muscarinic cholinergic receptor antagonist. Changes in pepsin output were monitored at 10-min intervals throughout the experiment. The net change in the 50-min cumulative pepsin output was calculated for each treatment (C). Pre-treatment with atropine inhibited carbachol-induced pepsinogen secretion. Each value represents the mean ± SEM (n = 3). C: carbachol; A: atropine; figures after the abbreviations denote the concentration in μg/kg. $^{**}p < 0.01$.

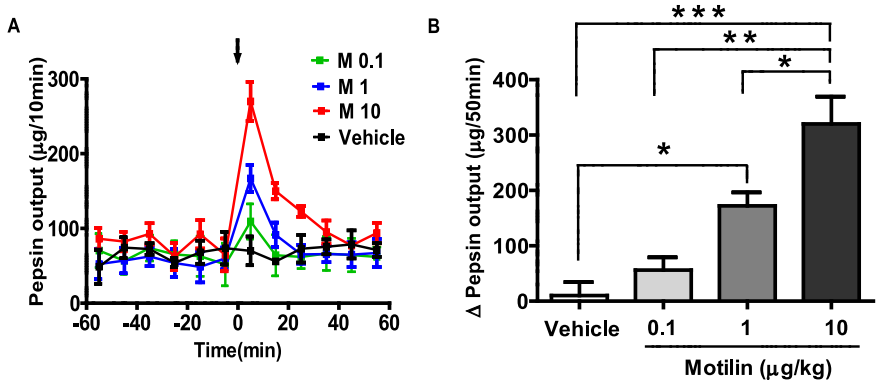

Fig. 2. Effects of motilin on pepsinogen secretion. Pepsinogen secretion was measured after vehicle and motilin administration (0.1, 1 and 10 μg/kg BW), and pepsin outputs were monitored at 10-min intervals throughout the experiment (A). The net changes in the 50-min cumulative pepsin output were calculated after administration of vehicle and different motilin doses (B). Motilin stimulated pepsinogen secretion in a dose dependent manner. Each value represents the mean ± SEM (n = 3; M 1, n = 4). M: motilin; figures after the abbreviations denote concentrations in μg/kg. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

produced motilin, but also expressed the motilin receptor [28]. In addition, the general appearance of the gastric mucosa and gastrointestinal motility, including motilin-induced gastric contractions during MMC in these animals appeared to be similar to those of humans and dogs [10,20,21]. Furthermore, the coordinating effect of suncus motilin and ghrelin on the regulation of the MMC of gastrointestinal motility was elucidated [11], and we found recently, that motilin stimulate gastric acid release via the histaminergic pathway (manuscript preparation). Considering the close relationship between acid and pepsinogen secretion, we hypothesized that motilin might be involved in the mechanism of pepsinogen secretion.

In this study, we found that intravenous administration of motilin but not ghrelin significantly stimulated pepsinogen secretion in a dose-dependent manner. Our results are consistent with a previous study which reported that exogenous administration of motilin increases pepsin output in dogs, with cyclical peaks in motilin plasma concentration coinciding with maximal pepsin secretory activity during the interdigestive state [2]. The cyclic release of motilin during the interdigestive period induces MMC phase III gastric contractions in humans [7] and dogs [8,9], and its exogenous administration induces gastric phase III-like contractions in dogs [8], humans [7], and suncus [10]. The physiological importance of phase III MMC activity in the gastrointestinal tract is related to the mechanical and chemical cleansing of the empty stomach in preparation for the next meal [29]. Previously, we found that motilin stimulates gastric acid secretion (Manuscript preparation) and this study showed that it can also stimulate pepsinogen secretion in a dose dependent manner. As previous study showed that increasing acid concentrations led to an increase in pepsin output [24], we also investigated whether motilin-induced gastric acid production stimulated pepsinogen secretion. However, intravenous administration of histamine (1 mg/kg BW) did not show any effect on pepsinogen secretion (Supplementary Fig. 1), despite causing a dramatic reduction in gastric pH. As pepsinogen is converted to active pepsin in the presence of gastric acid to enable protein digestion [1], our findings suggest that motilin-induced gastric acid and pepsinogen secretion may facilitate the complete digestion and food removal from the stomach. During the interdigestive MMC, motilin-induced gastric phase III activity and pepsinogen secretion lead to mechanical food removal and chemical gastric cleansing by breaking down proteins into smaller peptides, respectively, thus enhancing gastric emptying. Hence, impaired gastric phase III activity may cause the retention of gastric content for a longer period and bacterial overgrowth.

Acid and pepsinogen are considered the two major factors involved in the development of gastric ulcers, with pepsinogen being considered a pathogenic factor. Furthermore, duodenal ulcerogenesis in rats was found to be related to the combined effect of acid and endogenous rat pepsin [30]. Another study also showed that pepsin plays an important role in the pathogenesis of ischemia/reperfusion-induced gastric lesions [31]. Cyclical increases in gastric contractile activity and increased plasma immunoreactive motilin concentrations were associated with an

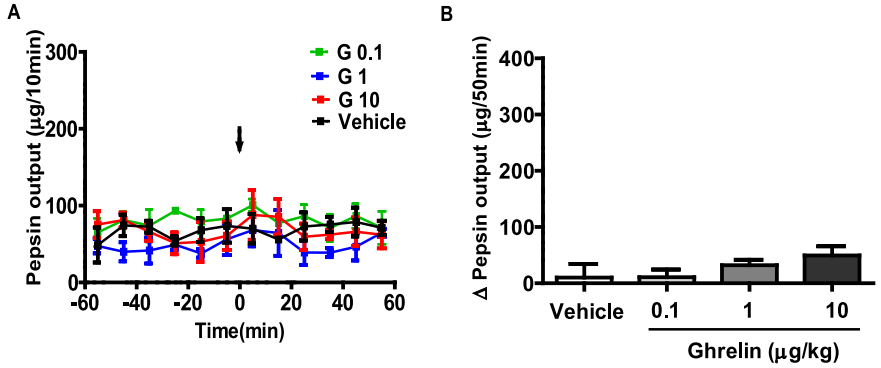

Fig. 3. Effects of ghrelin on pepsinogen secretion. Changes in pepsin output at 10-min intervals after intravenous administration of vehicle and ghrelin (0.1, 1 and 10 μg/kg BW) throughout the experiment (A). After administration of vehicle and different doses of ghrelin, changes in the 50-min cumulative pepsin output were estimated (B). Ghrelin had no significant effect on pepsinogen secretion. Each value represents the mean ± SEM (n = 3). G: ghrelin; figures after the abbreviations denote concentrations in μg/kg.

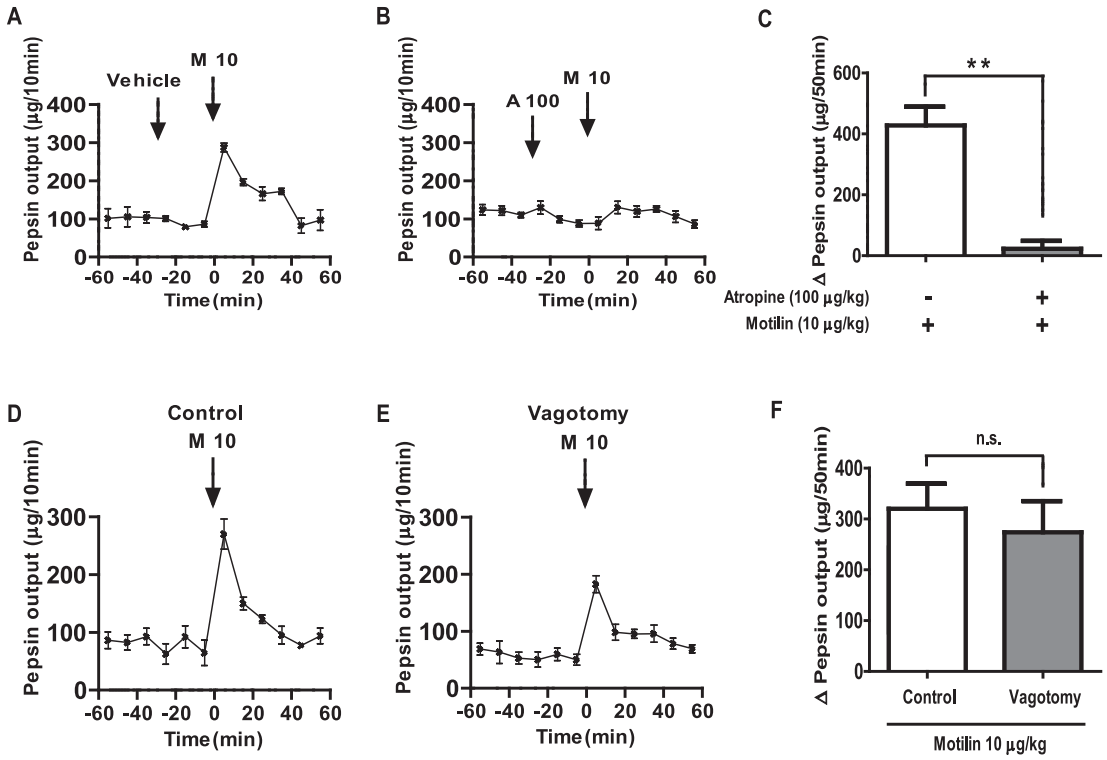

Fig. 4. Effect of atropine and vagotomy on motilin-induced pepsinogen secretion. Vehicle or atropine (100 μg/kg BW) were administered intravenously 30 min before motilin (10 μg/kg BW) treatment (A, B). Changes in pepsin output were monitored at 10-min intervals throughout the experiment. Motilin-induced pepsin output was observed in control (D) and vagotomized (E) suncus. The net change in the 50-min cumulative pepsin output after administration of motilin with or without atropine pretreatment (C) and vagotomy (F) were calculated. Atropine pretreatment, but not vagotomy, abolished motilin-induced pepsinogen secretion. Each value represents the mean ± SEM (n = 3; vagotomy, n = 5). A: atropine; M: motilin; figures after the abbreviations denote concentrations in μg/kg. $^{**}p < 0.01$, n.s. (not significant).

intermittent increase in pepsin output during the interdigestive state [2]. During fasting, cyclic release of gastric acid and pepsinogen for a long period may contribute to the development of gastric lesions. To the best of our knowledge, studies investigating the relationship between motilin and peptic ulcer have not been conducted. Thus, further studies are necessary to achieve a full understanding of the physiological role of motilin-induced pepsinogen secretion.

We observed that motilin-induced pepsinogen secretion was completely inhibited by atropine pretreatment (a muscarinic acetylcholine receptor antagonist), suggesting a cholinergic receptor-mediated response. In addition, results from a previous study also showed that atropine suppressed motilin-induced pepsin output, indicating that pepsinogen secretion was regulated by motilin through an intramural cholinergic pathway [2]. However, the specific pathway for motilin-induced cholinergic receptor activation and pepsinogen secretion is unknown to date. In this study, we did not observe a significant change in motilin-induced pepsinogen secretion in vagotomized suncus compared to control animals, indicating that this response was not mediated by the vagus nerve. Thus, there is a possibility that motilin may induce cholinergic stimulation of pepsinogen secretion through the enteric nervous system, as we found GPR38 mRNA expressed in both gastric mucosa and muscle layers in these animals. Further studies are required to elucidate whether motilin directly stimulates pepsinogen secretion by binding to GPR38 expressed in chief cells or through an indirect pathway.

In conclusion, this study demonstrated that unlike ghrelin, which had no effect on pepsinogen secretion, motilin stimulated pepsinogen secretion in a dose dependent manner through a cholinergic pathway in suncus. Detailed future studies on the mechanism of motilin-induced pepsinogen secretion are important to understand the pathophysiology of gastrointestinal diseases and facilitate drug development.

Conflict of interest

The authors have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.129.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.129.

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