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Original Article

Lack of direct effects of acyl ghrelin, des-acyl ghrelin, and obestatin on rat lower esophageal sphincter motility *in vitro*

Chih-Yen Chen [a,b], Shih-Che Huang [c,*]

[a] *Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*
[b] *National Yang-Ming University School of Medicine, Taipei, Taiwan, ROC*
[c] *Department of Internal Medicine, E-Da Hospital and School of Chinese Medicine for Post-Baccalaureate, I-Shou University, Kaohsiung, Taiwan, ROC*

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Abstract

Background: Three ghrelin gene-derived peptides, acyl ghrelin, des-acyl ghrelin and obestatin, are implicated in the regulation of feeding, adipogenesis and glucose metabolism, as well as gut motility. The effects of ghrelin gene-derived peptides on lower esophageal sphincter motility, however, were unknown.
Methods: We investigated the influence of acyl ghrelin, des-acyl ghrelin and obestatin on lower esophageal sphincter motility using the *in vitro* isolated muscle strip method. Both resting and carbachol-stimulated rat lower esophageal sphincters were tested.
Results: Acyl ghrelin, des-acyl ghrelin and obestatin did not alter the resting tone of the lower esophageal sphincter, whereas carbachol increased the force of the muscle strips. Similarly, acyl ghrelin, des-acyl ghrelin and obestatin did not modify carbachol-induced stimulation of the lower esophageal sphincter, whereas papaverine significantly decreased the force of the muscle strips.
Conclusion: Since the contractile effects of carbachol and relaxant responses of papaverine on the lower esophageal sphincter were confirmed, our results demonstrate that acyl ghrelin, des-acyl ghrelin and obestatin have no direct effects on rat lower esophageal sphincter motility *in vitro*.

Keywords: Acyl ghrelin; des-acyl ghrelin; lower esophageal sphincter; motility; obestatin

1. Introduction

The novel gastric peptides acyl ghrelin, des-acyl ghrelin and obestatin all belong to the ghrelin gene products.[1] These three ghrelin gene-derived peptides are implicated in the regulation of feeding, adipogenesis and glucose metabolism, as well as gut motility.[2–4] Acyl ghrelin and des-acyl ghrelin are both 28-amino-acid peptides, whereas obestatin is a 23-amino-acid peptide. Although all are derived from ghrelin precursor proteins, des-acyl ghrelin[5–7] and obestatin[8,9] may biologically function against acyl ghrelin. In addition, acyl ghrelin,[10,11] des-acyl ghrelin,[5–7] and obestatin[8,9] have been demonstrated to be involved in the regulation of *in vivo* smooth muscle contractions in the rodent gastrointestinal tract. Furthermore, *in vitro* studies indicated that acyl ghrelin potentiated the cholinergic contraction in mouse fundic strips[12] and elicited a concentration-dependent contraction in rat jejunal strips.[10]

Gastroesophageal reflux disease (GERD), by Montreal definition, is a clinical condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications.[13] This disease is common, and its prevalence varies in different parts of the world. It is worthy of note that the prevalence of GERD is reported to be increasing across all geographic regions by approximately 4% per year,[14] including Asia.[15] Epidemiological data indicate that increasing longevity, rising obesity rate, greater consumption of medications affecting esophageal function, and changing (decreasing)

* Corresponding author. Dr. Shih-Che Huang, Department of Internal Medicine, E-Da Hospital, 1, E-Da Road, Yan-Chau, Kaohsiung 824, Taiwan, ROC.
E-mail addresses: shihchehuang@hotmail.com, huangshihche@gmail.com (S.-C. Huang).

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prevalence of *Helicobacter pylori* infection are the major reasons for this trend.[16,17] In addition to this, lifestyle factors link with the prevalence of GERD, even in people with the same genetic background.[18]

Acyl ghrelin might be associated with the development of obesity and metabolic diseases.[2,19] Plasma ghrelin was found to increase profoundly in asymptomatic subjects after the eradication of *H. pylori*.[20] This could lead to appetite increase and weight gain, and contribute to the increasing obesity seen in Western populations where *H. pylori* prevalence is low. Hence, this raises the plausible biological mechanism linking ghrelin and *H. pylori*, through increasing obesity, to the increase in the prevalence of GERD in the developed countries. Although the presence of *H. pylori* is protective against reflux symptoms, there is no evidence for the causative relationship between *H. pylori* and GERD.

A variety of receptors have been identified on abdominal vagal afferent neurons, which can either enhance or reduce activity, offering a range of potential therapeutic targets for the treatment of gastrointestinal dysfunction.[21] However, acyl ghrelin, des-acyl ghrelin and obestatin can exert their functions without the ghrelin receptor (growth hormone secretagogue receptor-1a, GHSR-1a), *in vitro*. Thus, acyl ghrelin, des-acyl ghrelin or obestatin could interact with the lower esophageal sphincter (LES), through direct local effect or vagal afferent pathway, and these three peptides might play some unknown, but important, role in GERD. The information regarding the effects of ghrelin family peptides in the LES is very therefore interesting. However, to date, there has been no study to investigate the direct effects of ghrelin gene products in the sphincter.

In this study, we investigated the *in vitro* effects of acyl ghrelin, des-acyl ghrelin and obestatin in resting and carbachol-stimulated rat lower esophageal sphincter.

2. Methods

2.1. Animals

All procedures were performed in compliance with relevant laws and institutional guidelines. The protocol for this work was approved by the Institutional Animal Care and Use Committee of E-Da Hospital and Buddhist Tzu Chi General Hospital, Hualien. Male Sprague-Dawley rats (eight weeks old, weighing 250–300 g) were obtained from BioLASCO Taiwan.

2.2. Measurement of contraction of resting muscle strips isolated from lower esophageal sphincters

Measurements of contraction in the isolated rat LESs were performed according to the procedure described previously for the guinea pig LES.[22–24] In brief, the rats (11–12 weeks old, weighing 350–400 g) were euthanized using CO_2. The stomach, including a portion of esophagus, was quickly removed and placed in the oxygenated standard incubation solution (see below). The esophagus and stomach were cut open in the longitudinal direction along the greater curvature and pinned flat with the mucosal side up. The mucosa was removed with microscissors. A transverse strip (2 mm wide and 10 mm long) was cut from the area of the LES, which was easily identified as a thickened region of muscle between the esophagus and the stomach. Isolated LES strips were placed in standard incubation solution, containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 14 mM glucose, 1.2 mM NaH_2PO_4 and 1.8 mM $CaCl_2$, continuously being gassed with 95% O_2 plus 5% CO_2. The final pH at 37 °C was 7.40 ± 0.05.

The LES strips were attached to organ baths using surgical silk sutures and incubated at 37 °C in the standard incubation solution, while continuously being gassed with 95% O_2 plus 5% CO_2. The strips were connected to isometric transducers (FT.03; Grass Technologies, West Warwick, RI, USA), which were connected to an amplifier (Gould Instrument Systems, Valley View, OH, USA) and a computer recording system (BIOPAC systems, Santa Barbara, CA, USA). The basal tension of the muscle strips was adjusted to 1.0 g.

Experiments were started after a 45 min equilibration period. For measurements of contraction of the LES, acyl ghrelin, des-acyl ghrelin and obestatin were added to resting muscle strips in a cumulative-dose administration. For contraction measurements, carbachol (1 μM)-induced contraction was used as a reference to express contractile response to peptides. Only one cumulative-dose response was constructed with each preparation in the experiments.

2.3. Measurement of relaxation of carbachol-contracted muscle strips isolated from lower esophageal sphincters

In separate experiments, peptides were added to carbachol-contracted muscle strips for measurements of relaxation of contracted LES. Acyl ghrelin, des-acyl ghrelin and obestatin were added to carbachol (1 μM)-contracted muscle strips 15 min after the addition of carbachol in a cumulative-dose administration. For relaxation measurements with carbachol-contracted muscle strips, the relaxation responses were represented as a percentage of the relaxation to 100 μM papaverine. Only one cumulative-dose response was constructed with each preparation in the experiments.

2.4. Preparation of drugs and reagents

Buffer reagents, carbachol and papaverine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rat *O-n*-octanoylated (acyl) ghrelin (96% pure), des-acyl ghrelin (98% pure) and obestatin (99% pure) were obtained from Peptides International, Inc. (Louisville, KY, USA), kept in powder form at −20 °C, and dissolved in 0.9% saline before use.

2.5. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls *post hoc* test was used to

analyze the differences between the groups. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Effects in resting lower esophageal sphincter

To test the ability of acyl ghrelin, des-acyl ghrelin and obestatin to cause muscle contraction, muscle strips from the isolated rat LESs were prepared and their responses to acyl ghrelin, des-acyl ghrelin and obestatin studied. In resting rat LESs, carbachol (1 μM) increased the force of the muscle strips by 0.58 ± 0.14 g. Adding acyl ghrelin, des-acyl ghrelin or obestatin (at doses from 1 nM to 1 μM) to the resting muscle strips did not cause contraction or relaxation (Figs. 1 and 2).

3.2. Effects in carbachol-induced contraction of lower esophageal sphincter

Carbachol (1 μM) induced a fast contraction that had a long duration, reaching a plateau within 8 min (Fig. 3). Adding acyl ghrelin, des-acyl ghrelin or obestatin (at doses from 1 nM to 1 μM) to the 1 μM carbachol-contracted muscle strips at the plateau did not cause any relaxation, whereas papaverine (100 μM) decreased the force of the muscle strips by 0.96 ± 0.22 g (Figs. 3 and 4).

4. Discussion

Diagnosis of GERD is closely linked with higher socio-economic status, obesity and increasing public awareness.[16,17] Obese patients have been shown to have a higher risk of developing GERD after eradication of *H. pylori* infection,[25] though existing results are controversial.[26] Ghrelin gene-derived products have been found to be implicated in obesity and metabolic syndrome, including diabetes mellitus.[19] Thus, the relationship between ghrelin gene products and GERD is very interesting. The pivotal importance of transient LES relaxation has been established and confirmed in the pathogenesis of GERD.[27]

Many receptors have been cloned from LES, such as gamma-aminobutyric type B and metabotropic glutamate type 5 receptors.[28] Both the new gamma-aminobutyric type B receptor agonist, AZ3355, and the metabotropic glutamate type 5 receptor agonist, 2-methyl-6-(phenylethynyl)-pyridine, have been shown to be effective in inhibiting transient LES relaxation and reducing the number of reflux episodes.[29] These two new drugs have been developed for the treatment of GERD. Our study could be the first to investigate the *in vitro* effects of acyl ghrelin, des-acyl ghrelin and obestatin in the rat LES, because no previous literature was found.

Recent studies reveal that acyl ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells,[30] and promote the differentiation and fusion of C2C12 skeletal muscle cells[31] *in vitro*. Both are devoid of the GHSR-1a receptor. GHSR-1a expression has been demonstrated in the mucosal and muscle layers of human esophagus and stomach,[32] so it is likely that GHSR-1a could be expressed in the rat LES, a ring of thickened circular muscle at the gastroesophageal junction. Hence, it is worth investigating the potential effects of ghrelin gene products on the LES muscle. The results of this study did not show any direct effects of acyl ghrelin, des-acyl ghrelin and obestatin on the motility of this

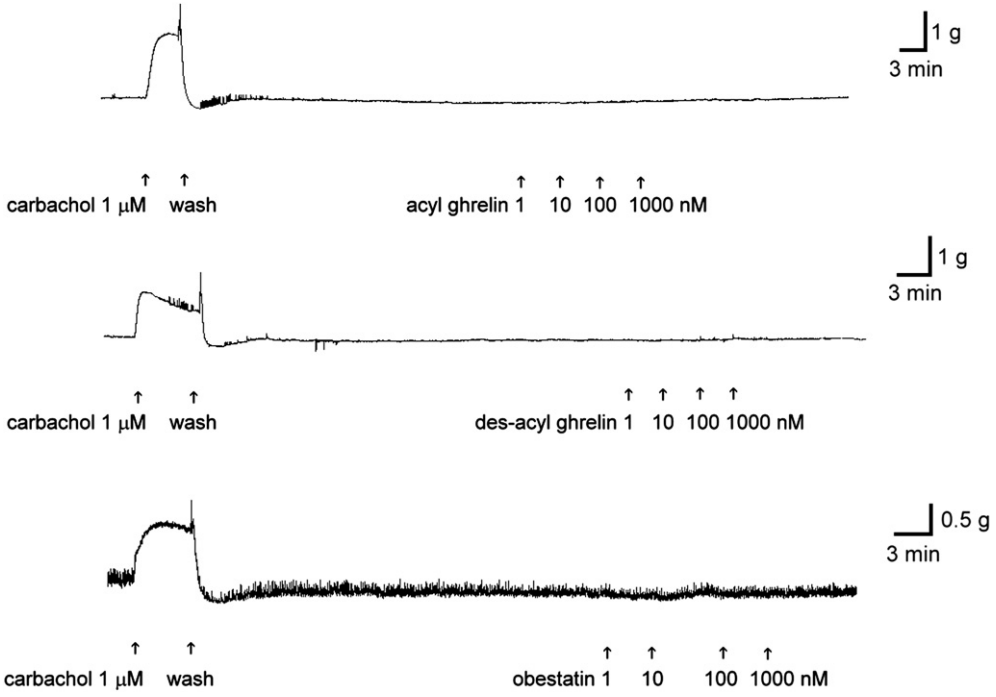

Fig. 1. Typical tracings showing no contractile responses of rat lower esophageal sphincters with the cumulative addition of acyl ghrelin, des-acyl ghrelin or obestatin at doses from 1 nM to 1 μM.

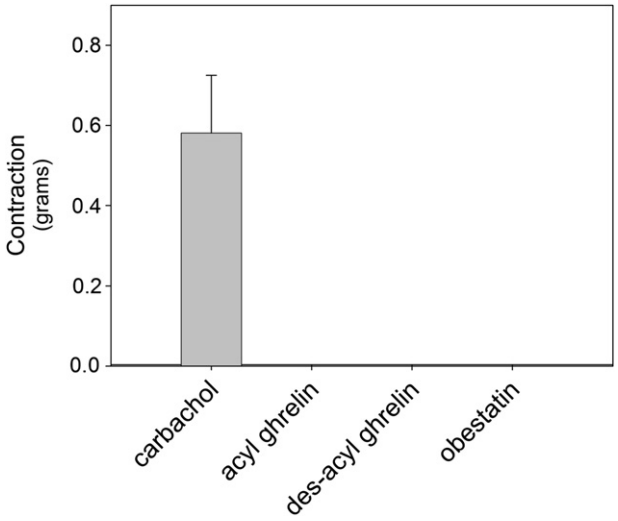

Fig. 2. No contraction of resting rat lower esophageal sphincters in response to acyl ghrelin, des-acyl ghrelin or obestatin (1 μM). In contrast, carbachol (1 μM) increased the force of the muscle strips by 0.58 ± 0.14 g. Results given are from at least three experiments. Vertical bars represent ± SEM.

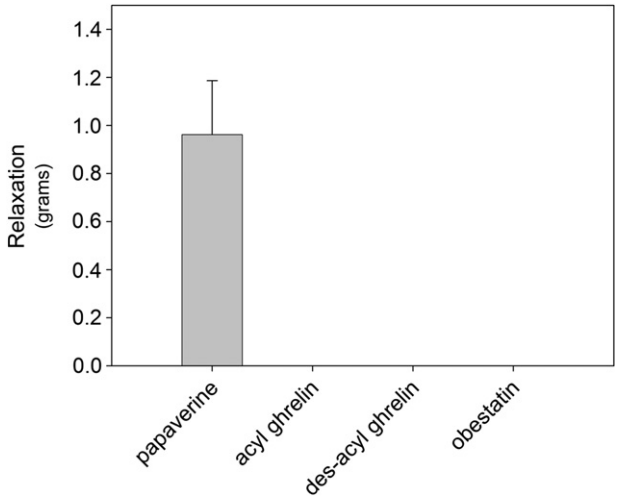

Fig. 4. No relaxation of carbachol-contracted rat lower esophageal sphincters in response to acyl ghrelin, des-acyl ghrelin or obestatin (1 μM). In contrast, papaverine (100 μM) decreased the force of the muscle strips by 0.96 ± 0.22 g. Results given are from at least three experiments. Vertical bars represent ± SEM.

muscle *in vitro*. If existent in the LES muscle, the ghrelin receptor GHSR-1a might mediate effects other than motility.[32]

In isolated rat forestomach circular muscle, acyl ghrelin showed no effect on basal smooth muscle tension, but dose-dependently facilitated the amplitude of contractions evoked by excitatory nerve stimulation.[33] Acyl ghrelin also induced chicken gastrointestinal longitudinal muscle contractions *in vitro* with regional specificity: greatest in the stomach and colon, moderate in the esophagus and weak in the small intestine.[34] In addition, an acyl ghrelin fragment (human ghrelin$_{1-5}$) decreased basal tension, electric field stimulation-elicited contractions and tension of the carbachol-induced contraction of the iris sphincter, whereas obestatin decreased the basal tension and increased the carbachol-induced

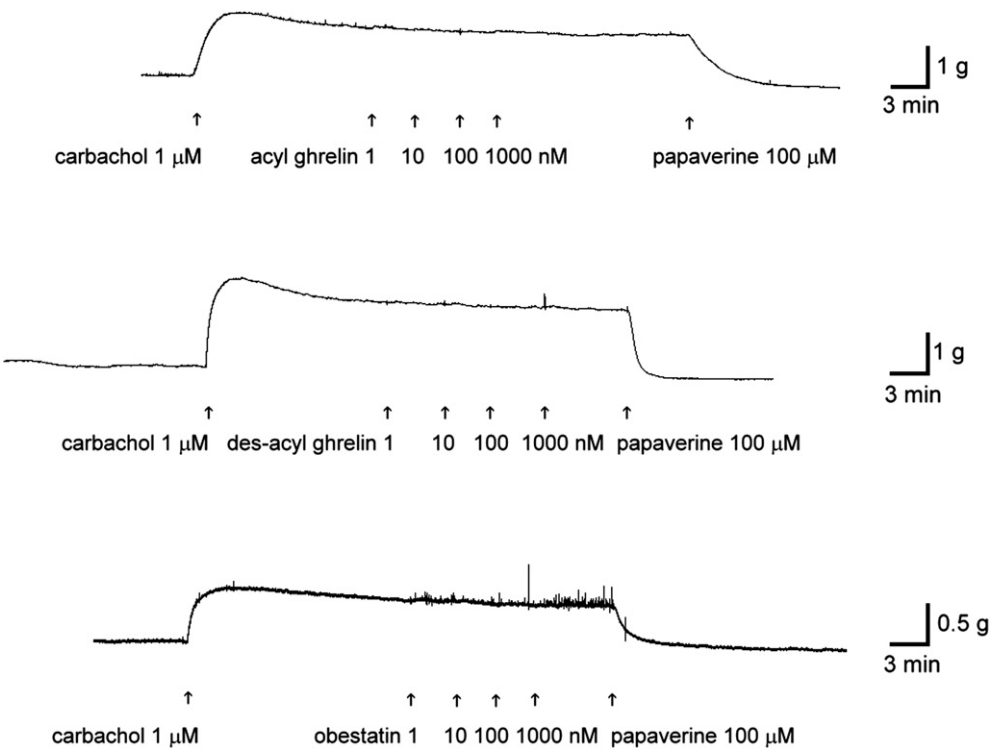

Fig. 3. Typical tracings showing no relaxant responses of carbachol (1 μM)-contracted rat lower esophageal sphincters with cumulative addition of acyl ghrelin, des-acyl ghrelin or obestatin, at doses from 1 nM to 1 μM.

contraction of the iris sphincter in rabbits.[35] Thus, investigating the effects of ghrelin gene-derived peptides in the LES is very interesting.

Previous studies showed that 1 μM acyl ghrelin potentiated the cholinergic contraction in mouse fundic strips[12] and elicited a moderate contraction in rat jejunal strips.[10] From our studies, acyl ghrelin, des-acyl ghrelin and obestatin, at 1 nM to 1 μM, had no direct effect in the resting LES because neither contraction nor relaxation was observed after the application of these peptides. The carbachol, however, induced a fast contraction of the muscle strips that was of a long duration. Acyl ghrelin, des-acyl ghrelin and obestatin did not affect carbachol-induced contraction of the LES, whereas papaverine effectively decreased the force of the muscle strips. Further studies of the effects of ghrelin gene products on the LES under electric field stimulation-elicited contractions may, however, be called for.

In conclusion, we found no direct influence of acyl ghrelin, des-acyl ghrelin or obestatin on rat LESs. Since the contractile effects of carbachol and relaxant responses of papaverine on the LES were confirmed, our results demonstrate that acyl ghrelin, des-acyl ghrelin and obestatin have no direct effects on resting and carbachol-stimulated LES motility *in vitro*.

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