Gastric motor effects of ghrelin and growth hormone releasing peptide 6 in diabetic mice with gastroparesis

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Abstract

AIM: To investigate the potential therapeutic significance of ghrelin and growth hormone releasing peptide 6 (GHRP-6) in diabetic mice with gastric motility disorders.

METHODS: A diabetic mouse model was established by intraperitoneal (ip) injection of alloxan. Diabetic mice were injected ip with ghrelin or GHRP-6 (20-200 μg/kg), and the effects on gastric emptying were measured after intragastric application of phenol red. The effect of atropine, N⁵-nitro-L-arginine methyl ester hydrochloride (L-NAME) or D-Lys²-GHRP-6 (a growth hormone secretagogue receptor (GHS-R) antagonist) on the gastroparalytic effect of ghrelin or GHRP-6 (100 μg/kg) was also investigated. The effects of ghrelin or GHRP-6 (0.01-10 μmol/L) on spontaneous or carbachol-induced contractile amplitude were also investigated in vitro, in gastric fundic circular strips taken from diabetic mice. The presence of growth hormone secretagogue receptor 1a transcripts in the fundic strips of diabetic mice was detected by reverse transcriptase polymerase chain reaction (RT-PCR).

RESULTS: We established a diabetic mouse model with delayed gastric emptying. Ghrelin and GHRP-6 accelerated gastric emptying in diabetic mice with gastroparesis. In the presence of atropine or L-NAME, which delayed gastric emptying, ghrelin and GHRP-6 (100 μg/kg) failed to accelerate gastric emptying. D-Lys²-GHRP-6 also delayed gastric emptying induced by the GHS-R agonist. Ghrelin and GHRP-6 increased the carbachol-induced contractile amplitude in gastric fundic strips taken from diabetic mice. RT-PCR confirmed the presence of GHS-R mRNA in the strip preparations.

CONCLUSION: Ghrelin and GHRP-6 increase gastric emptying in diabetic mice with gastroparesis, perhaps by activating peripheral cholinergic pathways in the enteric nervous system.

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Key words: Gastric emptying; Ghrelin; Growth hormone releasing peptide 6; Growth hormone secretagogue receptor; Diabetic mice

INTRODUCTION

Delayed gastric emptying occurs in more than 50% of patients with chronic diabetes mellitus (DM) and is always associated with impaired quality of life and diabetic control. While this delay is not always clinically apparent, the range of gastrointestinal symptoms may include nausea, vomiting, regurgitation, fullness, and bloating[3]. Diabetic patients with poor gastric emptying have a number of possible metabolic consequences, including poor glycemic control, increased risk of postprandial hypoglycemia and variable drug absorption. At its worst, gastroparesis can lead to intractable vomiting and an inability to feed, and carries a poor prognosis[3].

Present management of diabetic gastroparesis involves empirical use of prokinetic drugs such as domperidone, metoclopramide, cisapride[2,3] and erythromycin[4]. The effects of these drugs, however, are unpredictable. One possible explanation for this lack of sustained response to treatment is that gastroparesis may be originally associated with progressive autonomic neuropathy[5].

Ghrelin, a 28-amino acid peptide with an octanoyl moiety at Ser³, was discovered in 1999 as the endogenous
Mice were allowed free access to water 12 h before the experiment. DM mice were injected with either ghrelin (0, 20, 50, 100, or 200 μg/kg; ip) or GHRP-6 (0, 20, 50, 100, or 200 μg/kg; ip) in a random order. Modulation of the effects of the growth hormone secretagogue receptor (GHS-R) agonists by pharmacological blockers was tested by ip administration of atropine (1 mg/kg), L-NAME (50 mg/kg) or D-Lys3-GHRP-6 (5 μmol/kg) 15 min before administration of the GHS-R agonist (ghrelin 100 μg/kg or GHRP-6 100 μg/kg). Each drug treatment group consisted of at least six DM mice. An additional group consisting of at least 6 DM mice were injected with saline as normal controls.

Immediately after the injection of the drug, 5 mg/kg body weight phenol red test meal (0.5 g/L in 0.9% NaCl with 1.5% methylcellulose) was administered intragastrically with an orogastric canula. The mice were sacrificed 20 min later. The stomach was clamped with a string above the lower esophageal sphincter and a string beneath the pylorus to prevent leakage of phenol red. The stomach was cut just beneath the strings and was frozen at -70 °C until measurement of gastric emptying. Gastric emptying was determined spectrophotometrically using a previously described method[8,9]. The stomach of each mouse was cut just above the lower esophageal sphincter and the pyloric sphincter. Phenol red remained largely in the lumen of the stomach, although some was trapped in the mucus layer of the stomach, and a very small amount of phenol red was reabsorbed in the mucosa after 20 min. The stomach and its contents were submerged in 5 mL of 0.1 mol/L NaOH. The stomach was minced, and these samples contained the total amount of phenol red present in the stomach. The samples were further diluted in 10 mL 0.1 mol/L NaOH and left at room temperature for 1 h. Five mL of the supernatant was then centrifuged at 800 × g for 20 min. The absorbance was read at a wavelength of 546 nm with a spectrophotometer (Shanghai Yixian Company, China), and the phenol red content present in the stomach was calculated. The percentage of gastric emptying of the mice was calculated as (infusion-remained/infusion) × 100%.

Contractility measurements in vitro

DM mice were sacrificed by cervical dislocation, and the stomach was removed and rinsed with ice cold saline. Circular muscle strips, freed from mucosa (length 8-10 mm, width 0.2 mm) were cut from the fundus and suspended vertically in an organ bath filled with Krebs solution (120.9 mmol/L NaCl, 2.0 mmol/L NaHCO3, 15.5 mmol/L NaH2PO4, 5.9 mmol/L KCl, 1.25 mmol/L CaCl2, 1.2 mmol/L MgCl2, and 11.5 mmol/L glucose) warmed at 37 °C and gassed with 95% O2/5% CO2. One end of the strip was fixed to a hook on the bottom of the chamber while the other end was connected by a thread to an external isometric force transducer (B. K. Company, USA) at the top. After 1 h of equilibration at optimal stretch (0.75 g), the reproducibility of the contractile response to carbachol (0.1 μmol/L) was assessed. Mechanical responses in the smooth muscle strips were measured using an isometric force transducer and stored on a computer for analysis using the SMUP-E biological signal processing system (Chengdu Equipment Factory, China). To investigate the modification
of neuro-effector transmission by GHS-R agonists, the response was studied in the presence and absence of carbachol (0.1 μmol/L), which, when used, was added to the tissue bath 0.5 min before application of the GHS-R agonists. The effect of GHS-R agonists on spontaneous or carbachol (0.1 μmol/L)-induced contractile activity in DM mouse fundic muscle strips was studied by measuring the mean contractile amplitude of the muscle strips.

**Measurement of the growth hormone secretagogue receptor by RT-PCR**

Total RNA was prepared from DM mouse fundic muscle strips using Trizol reagent (Invitrogen, Carlsbad, CA). Single-stranded cDNA was synthesized using an oligo (dT) anchor primer and Superscript™ II RNase H reverse transcriptase ( Gibco BRL, NY, USA). The obtained cDNA served as a template for polymerase chain reaction, consisting of 35 cycles of amplification (95°C for 10 min, 94°C for 50 s, 60°C for 30 s, 72°C for 30 s) with a final elongation of 10 min at 72°C using 0.5 U of Taq DNA polymerase ( Promega, Sweden) and 0.5 μmol/L primers (forward: 5′-CGACCTGCTCT GCAAACTCT-3′ and reverse: 5′-CAGGCCCCACGACGAGAAG-3′). PCR using intron-spanning mouse β-actin primers (forward: 5′-CCTGTATGCTCTGTCGTA-3′ and reverse: 5′-CCATCTCTGCTGCTGAAGT-3′), demonstrated that cDNA was present and devoid of genomic DNA contamination. The expected sizes of GHS-R and β-actin fragments were 217 bp and 260 bp, respectively. All primers were selected from conserved regions identified by the alignment of published sequences for GHS-R mRNA in Genbank. PCR products were separated by electrophoresis on 1.4% agarose gels and photos of the separated products were taken.

**Statistical analysis**

Data are expressed as mean ± SE. One-way ANOVA was used for statistical analyses of multiple comparisons, and a P value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Contractility in vivo**

Compared with the gastric emptying rate of the normal mice (28.10% ± 1.28%), the gastric emptying rate of the DM mice was significantly reduced (22.90% ± 1.42%, P < 0.05). In DM mice, ghrelin accelerated gastric emptying of the semi-liquid meal at doses of 50, 100 and 200 μg/kg; the emptying rate was significantly accelerated from 22.90% ± 1.42% to 27.80% ± 0.97%, 34.50% ± 1.20% and 32.90% ± 1.10% at doses of 50, 100 and 200 μg/kg, respectively (P < 0.05, compared to injection of saline) (Figure 1). Similarly, GHRP-6 increased gastric emptying dose-dependently with significant effects at 50, 100 and 200 μg/kg (P < 0.05) (Figure 1).

The effect of ghrelin or GHRP-6 on DM mouse gastric emptying was characterized pharmacologically. Ghrelin (100 μg/kg) or GHRP-6 (100 μg/kg) was unable to reverse the inhibition of gastric emptying due to pretreatment with atropine (1 mg/kg) or L-NAME (50 mg/kg) (P < 0.05). Pretreatment of DM mice with D-Lys³-GHRP-6 (5 μmol/kg) also delayed the accelerated gastric emptying induced by ghrelin or GHRP-6 (P < 0.05) (Figure 2).

**Contractility in vitro**

Fundic strips from the DM mice showed spontaneous contractile activity after 1 h of equilibration. Ghrelin (0.01-10 μmol/L) or GHRP-6 (0.01-10 μmol/L) did not significantly change spontaneous contractile responses in the strips (Table 1). However, in the presence of carbachol (0.1 μmol/L), ghrelin increased the carbachol-induced contractile amplitude at 0.1, 1 and 10 μmol/L. GHRP-6 also increased the carbachol-induced contractile amplitude at 0.1, 1 and 10 μmol/L (Table 2).

**Expression of the ghrelin receptor in mouse fundic strips**

The presence of GHS-R mRNA in the mouse fundic smooth muscle strips was verified by RT-PCR with
gene-specific primers. Analysis of the PCR products by electrophoresis revealed a band with the expected length of 217 bp (Figure 3).

**DISCUSSION**

We have demonstrated, for the first time, that ghrelin and the synthetic peptide GHRP-6 improve gastric emptying in diabetic mice with gastroparesis. This effect may be mediated through potentiation of peripheral cholinergic pathways in the enteric nervous system.

Ghrelin, a recently discovered peptide hormone, is primarily produced by endocrine cells in the oxyntic mucosa of the stomach in rats and humans. Ghrelin has also been found in the small intestine, testis, pituitary gland, ovary, liver, pancreas, kidney, placenta and hypothalamus, in both humans and rodents. Ghrelin is a natural ligand for GHS-R, and its receptor is found all over the body, including in the bowel, pancreas, stomach, heart, lungs and brain. In addition to its effect on growth hormone secretion by activating GHS-R in the pituitary gland, ghrelin enhances appetite, increases food intake, mediates energy balance, regulates glucose metabolism and insulin release, stimulates gastric acid secretion and promotes anxiety. It is well known that many gastrointestinal peptides participate in the regulation of gastrointestinal functions. Ghrelin is one of these candidate gastrointestinal peptides, because it is predominantly present in gastric endocrine cells and is secreted into the bloodstream. In fact, the potential of ghrelin and its synthetic peptide GHRP-6 as a prokinetic agent has been shown previously in *in vitro* and *in vivo* studies. Previous studies on the effect of ghrelin on gastric motility have demonstrated the involvement of vagal and central ghrelin receptors. Thus, the effect of ghrelin on gastric emptying is blocked by atropine and vagotomy in rats and mice. Peripheral ghrelin may stimulate fasted small intestinal motor activity through receptors on vagal afferents, which activate neuropeptide Y-containing neurons in the brain, as suggested by experiments in rats. In addition, expression of the ghrelin receptor in the rat nodose ganglion has been confirmed using RT-PCR. In addition to the known vagal pathways, ghrelin and GHRP-6 accelerate gastric emptying and small intestinal transit by activating cholinergic excitatory pathways in the enteric neuron system. Moreover, ghrelin has been shown to increase gastric emptying in patients with gastroparesis, and it has been proposed that ghrelin or its analogues may represent a new class of prokinetic agents for the treatment of gastroparesis. In our study, we investigated the effects of ghrelin and GHRP-6 on gastric motility in diabetic mice with gastroparesis. Our findings indicate the potential of ghrelin as a therapeutic approach for gastrointestinal motility disorders.

In our study, the gastric emptying rate in the DM mice was significantly reduced relative to the normal mice. Ghrelin and GHRP-6 accelerated gastric emptying of the diabetic mice with gastroparesis. In the presence of atropine or L-NAME, which delayed gastric emptying, ghrelin and GHRP-6 (100 μg/kg) failed to accelerate gastric emptying. D-Lys3-GHRP-6 also delayed gastric emptying induced by GHS-R agonists. Gastric emptying is a complex process involving excitatory and inhibitory nerves, which may contribute to both acceleration and retardation of the emptying process. L-NAME, which blocks inhibitory nitricergic nerves, delayed gastric emptying, probably by interfering with gastric accommodation and pyloric relaxation. Therefore, the effect of ghrelin may involve both excitatory and inhibitory pathways, as suggested by the inability of ghrelin to overcome the delay induced by L-NAME and atropine. Ghrelin has been shown to induce release of nitric oxide in the rat stomach, and in our study, a nitricergic pathway could be involved in the acceleration of gastric emptying because the prokinetic effect *in vivo* was lost in the presence of L-NAME. The GHS-R antagonist D-Lys3-GHRP-6, also blocked the effect of the GHS-R agonists, and this result indicates that the effect of GHS-R agonists on gastric motility...
occurs through GHS-R and likely does not involve cross interactions with other receptors. Ghrelin and GHRP-6 increased the carbachol-induced contractile amplitudes in fundic strips taken from DM mice, and this finding also indicates that GHS-R agonists accelerate gastric emptying of semi-liquid through the activation of GHS-R receptors, possibly located on local cholinergic enteric nerves. Moreover, the presence of GHS-R mRNA in the strip preparations was confirmed by RT-PCR.

It remains controversial whether ghrelin can exert a protective effect on gastric mucosa, although previous studies have suggested ghrelin might induce gastric mucosal lesion in rats by increasing acid secretion. It is unlikely the improvement in gastric emptying in DM mice induced by ghrelin or GHRP-6 could be explained by a protective effect of ghrelin and GHRP-6 on gastric mucosa. Acid may inhibit gastric emptying, but the effect of ghrelin on acid secretion remains a controversial issue itself.\(^{25,30}\)

In conclusion, ghrelin and its synthetic peptide, GHRP-6, increase gastric emptying in diabetic mice with gastroparesis, perhaps by activating peripheral cholinergic pathways in the enteric nervous system. Although further studies are needed to determine the underlying mechanisms, we propose that ghrelin or its analogues may represent a new class of prokinetic agents for the treatment of diabetic gastroparesis. Therefore, ghrelin and ghrelin agonists have the potential to become useful therapeutic agents for the treatment of diabetic gastroparesis. However, long-term animal experiments and clinical trials are needed.

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