L-Arginine L-Glutamate Enhances Gastric Motor Function in Rats and Dogs and Improves Delayed Gastric Emptying in Dogs

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ABSTRACT

Amino acids are not only constituents of proteins, but also have multiple physiologic functions. Recent findings have revealed that ingested amino acids either activate luminal receptors or are metabolized, causing physiologic reactions in the gastrointestinal (GI) tract. We examined the effect of oral L-arginine L-glutamate (ArgGlu), a pharmaceutical amino acid salt used i.v. for the treatment of hyperammonemia, on gastric motor function in rats and dogs. Gastric emptying was determined using phenol red and 13C-breath test methods, whereas gastric relaxation was determined using the barostat method. ArgGlu (10–30 mg/kg, p.o.) dose-dependently promoted gastric emptying in rats. This effect was dependent on vagus nerve activation and comparable to that of the prokinetic mosapride. Intragastric ArgGlu (3–30 mg/kg intragastrically) also dose-dependently enhanced adaptive relaxation of rat stomachs, which was negated not by vagotomy of gastric branches, but by pre-treatment with N omega-nitro-L-arginine methyl ester (20 mg/kg i.v.), a nitric oxide synthase inhibitor. Its relaxing effect on the stomach was also confirmed in dogs and was equally as efficacious as treatment with sumatriptan (1–3 mg/kg s.c.). ArgGlu (30 mg/kg p.o.) significantly reduced the half gastric emptying time in clonidine-induced delayed gastric emptying of solids in dogs, and its effect was comparable to that of cisapride (3 mg/kg p.o.). This study demonstrated that the pharmaceutical ingredient ArgGlu, currently used i.v., enhanced gastric motor function when administered orally, suggesting that it could be a new oral medicine indicated for treatment of upper GI hypofunction or dysfunction like functional dyspepsia.

Introduction

Lifestyle and dietary factors, including psychologic stress, overfatigue, lack of physical activity or sleep, overeating, and overdrinking, are recognized to cause symptoms of meal-related discomfort, such as a heavy feeling in the stomach or anorexia (Feinle-Bisset and Azpiroz, 2013). Aging also decreases gastrointestinal (GI) activity (Rayner et al., 2000), and psychologic stresses exacerbate GI functions through the imbalance of the autonomic nervous system (Mayer et al., 2001; Taché et al., 2001). The ingestion of food triggers a series of gastric functions (i.e., expansion of stomach volume, accommodation of the food, mixture of the gastric contents and gastric juices, and the emptying of digested contents into the duodenum). Any disturbance in these motor functions may cause a sensation of a heavy stomach or the feeling of being full.

Symptoms that are more serious are encountered in clinical practice and diagnosed as functional dyspepsia (FD). FD is a disease that causes chronic upper abdominal symptoms despite the absence of associated organic disease. FD has been classified into two categories, as follows: 1) postprandial distress syndrome (PDS), resulting in bothersome postprandial fullness and/or early satiation; and 2) epigastric pain syndrome, resulting in epigastric pain and/or epigastric burning (Tack et al., 2006). Epidemiologic studies have shown that 20–30% of the population have chronic or recurrent upper abdominal symptoms (Tack et al., 2006), and pathophysiological studies of subsets of FD patients have revealed the presence of delayed gastric emptying (23–59%), impaired gastric accommodation (40–50%), and hypersensitivity to gastric distension (34–66%). In these studies, gastric emptying was slower in patients with FD compared with healthy asymptomatic subjects, and impaired gastric emptying was associated with bothersome postprandial fullness. In a barostat study, approximately 40–50% of FD patients had reduced gastric accommodation after meals, and impaired gastric accommodation was associated with early satiation (Tack et al., 2006). Therefore, strategies for improving gastric motor dysfunction have been proposed to relieve sufferers from such symptoms of discomfort. Prokinetics are used as symptomatic treatments in clinical situations (Geeraerts and Tack, 2008), but these are not widely available (Camilleri and Stanghellini, 2013).

Amino acids are not only constituents of proteins, but also have multiple physiologic functions. Ingested amino acids activate luminal receptors or are metabolized to cause physiologic reactions in the GI tract. Glutamic acid is a dietary amino acid, and its sodium salt, monosodium glutamate, activates taste nerves and induces a unique sensation, umami.
through activation of taste receptors in the oral cavity (Chaudhari et al., 2000; Li et al., 2002; Nelson et al., 2002; San Gabriel et al., 2009a). Recent findings have revealed that the ingestion of glutamate can also be sensed in the GI tract (Uneyama et al., 2006), facilitating upper GI motor function (Zai et al., 2009; Toyomasu et al., 2010; Teramoto et al., 2014) and secretions (Akiba et al., 2009), and that arginine is the most important precursor for the inhibitory neurotransmitter nitric oxide (NO) that controls GI motility and blood circulation by relaxing smooth muscle (Sanders and Ward, 1992). These findings encouraged us to repurpose L-arginine L-glutamate as an oral formulation for treatment in those suffering from upper GI discomfort symptoms. L-arginine L-glutamate is the pharmaceutical ingredient approved in Japan as “Argimate® 10% solution for drip intravenous infusion” for the treatment of hyperammonemia. In this study, we examined the potential of L-arginine L-glutamate to modulate GI motor function and demonstrated that it facilitates gastric emptying and relaxation of the stomach in rodents and canines as well as improving clonidine-induced delayed gastric emptying in conscious dogs.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (Charles River Laboratories Japan, Yokohama, Japan) were housed in group cages under standard controlled environmental conditions at 23 ± 3°C with a 12-hour light/dark cycle and free access to tap water and rat chow. Forty adult, healthy female Nosan beagles were purchased from Nac (Chiba, Japan). Twenty dogs were used for gastric tone measurements, and the other 20 were used for the gastric emptying study. Animals were housed individually in experimental cages and acclimated for at least 1 week before entry into the study. Dog chow (DS-A; Oriental Yeast, Tokyo, Japan) was provided, and water was available ad libitum. Animals were housed under standard controlled environmental conditions at 23 ± 3°C with a 12-hour light/dark cycle.

All procedures were performed in accordance with the institutional Animal Care and Use Committee of the Research Center of Ajinomoto Pharmaceuticals (Kawasaki, Japan).

Compounds. L-Arginine L-glutamate hydrate was purchased from Ajinomoto (Tokyo, Japan), cisapride monohydrate from Spectrum Pharmaceuticals (Kawasaki, Japan) was provided, and water was purchased from Sigma-Aldrich (St. Louis, MO). Acotiamide hydrochloride, clonidine hydrochloride, N-omega-nitro-L-arginine methylester (L-NAME), and casein sodium salt (bovine milk) were purchased from Sigma-Aldrich (St. Louis, MO). Aciotiamide hydrochloride was synthesized and provided by the Research Center of Ajinomoto Pharmaceuticals.

Gastric Emptying in Rats. Gastric emptying of a meal was determined using the previously described phenol red method (Taché et al., 1987). The liquid meal consisted of 10% w/v casein NaCl, and purified water containing phenol red (50 mg/100 mL) was given intragastrically through a stainless steel gastric tube (1.5-mL volume) to freely fed rats. At 60 minutes after the administration of the meal, rats were euthanized by CO2 inhalation. The abdominal cavity was placed on a glass plate, and the stomach was isolated and rinsed in 0.9% saline. The stomach was placed into 30 mL of 0.1 N NaOH and homogenized (Polytron; Brinkman Instruments, Westbury, NY). The suspension was allowed to settle for 60 minutes at 23 ± 3°C, and then 0.5 mL supernatant was added to 1 mL acetonitrile and centrifuged at 3000 rpm at 4°C for 20 minutes. The absorbance of the supernatant was read at 560 nm by a microplate reader (Benchmark Plus; Bio-Rad Laboratories, Tokyo, Japan). The absorbance of the phenol red recovered from animals euthanized immediately after gavage of the liquid meal was taken as a standard 0% emptying. The percentage of emptying during the 15-minute period was calculated using the following formula:

\[
\text{percent emptying} = \left(1 - \frac{\text{absorbance of test sample}}{\text{absorbance of standard}}\right) \times 100.
\]

Experimental Protocols. L-Arginine L-glutamate was dissolved in liquid meal and administered together with it. Mosapride (3 mg/kg) was suspended in a 0.5% carboxymethyl cellulose solution and administered intragastrically (5 mL/rat) 1 hour before administering the liquid meal.

To test the involvement of vagus nerve activation, gastric branches of the ventral and dorsal vagal trunk were transected with the aid of a microscope in fasted 5-week-old rats under ketamine hydrochloride (75 mg/kg i.p.) and xylazine (5 mg/kg i.p.) anesthesia. Sham vagotomy consisted of a laparotomy and similar manipulation of the esophagus and stomach without any transection under anesthesia. Rats that underwent gastric vagotomy or sham vagotomy were maintained with tap water and rat chow for 10 days postsurgery before the gastric emptying measurements.

Measurement of Plasma Concentration of L-Glutamate and L-Arginine. ArgGlu (10 mg/kg) was dissolved in the test meal (0.5 mL/kg) and orally administered simultaneously with the meal. Blood was taken from the inferior vena cava under ether anesthesia immediately after (0 minute) as well as 15, 30, and 60 minutes after administration of the ArgGlu. Plasma concentrations of L-glutamate and L-arginine in the prepared samples were measured using liquid chromatography/electrospray ionization tandem mass spectrometry (Shimbo et al., 2009).

Vagus Nerve Recording from Anesthetized Rats. Male Sprague-Dawley rats weighing approximately 250–330 g were used for this experiment. Rats were fasted for 18 hours with free access to tap water prior to surgery. Surgical techniques and other experimental methods have been previously documented (Uneyama et al., 2006). Under urethane anesthesia (1 g/kg i.p.), a polyethylene catheter was inserted into the forestomach for intragastric infusion. Under a dissecting microscope (Olympus SXZ12, Tokyo, Japan), the nerve bundle of the left gastric branch was split with a sharp blade, leaving a length of approximately 3 mm. Fine vagal filaments were dissected from the main nerve trunk and placed on a silver hook-recording electrode. Perineural connective tissue was placed on a reference electrode. All recordings were made from the peripheral cut end of the vagal nerve still innervating the stomach. The abdominal wound was covered with a saline-moistened gauze, and rats were maintained at 37°C with heating pad (BWT-100; BRC, Nagoya, Japan).

The electrode was connected to a head stage (JB-101J; Nihon-Koden, Tokyo, Japan), and the signal was differentially amplified 10,000 times before being filtered with a bandwidth of 150 Hz to 1 KHz (SEN-6000; Nihon-Koden). The neural signal output together with the signal from the pressure transducer was acquired by a PowerLab interface (PowerLab 4/30; ADInstruments, Nagoya, Japan) and viewed online with LabChart 7 software (ADInstruments). The nerve signal was digitally sampled at 4 KHz, which was sufficient to allow spike discrimination. Nerve activity was analyzed after the raw data were converted to standard pulses and counts (1-second bin width) by using an offline software spike histogram extension (ADInstruments).

After a 15-minute baseline recording for signal stabilization, a test substance was injected intragastrically or i.v. ArgGlu or distilled water (3 mL) was injected at a flow rate of 3 mL/min through the catheter inserted into the forestomach. As the ArgGlu solution is neutral (approximately pH 7.0), the pH was not adjusted for each intragastric solution. These solutions were kept at 37°C during gastric perfusion. Mosapride (1 mg/kg) dissolved in 20% Tween 80/saline or acotiamide (10 mg/kg) in 1% dimethylsulfoxide/saline was injected i.v. The mean baseline discharge was determined over a period of 15 minutes just prior to the start of injection. The effects of test substance injection on nerve activity were determined by comparing the mean number of spikes per 1 second over 15 minutes (i.e., the
mean value of 900 successive measured samples) before the injection as well as 10–50 minutes after.

In the second phase, the effect of a 10-minute exposure to ArgGlu on activation of the vagus nerve (afferent) was investigated. For the intragastric perfusion test, a solution of 6 mmol/L ArgGlu was perfused at a rate of 3 mL/min on the gastric lumen for 10 minutes, and then the perfusate was switched to 6 mmol/L sodium chloride solution, and was similarly perfused in the gastric lumen. As a control, 6 mmol/L sodium chloride solution was used.

Adaptive Relaxation of the Stomach in Anesthetized Rats. Male Sprague-Dawley rats (7–8 weeks old) were used for this experiment. Rats were fasted for 16–24 hours with free access to tap water before surgery. The surgical techniques and other experimental methods have been documented extensively elsewhere (Taniguchi et al., 2007). Under pentobarbital anesthesia (50 mg/kg i.p.), the rats were tracheally cannulated and maintained at 37°C using a heating pad. An intragastric balloon (RDDBG12; StarMedical, Tokyo, Japan; diameter, 2.5 cm; length, 2.5 cm; approximate capacity, 12 mL) and a cannula (inner diameter, 1.5 mm) were inserted through an incision in the proximal duodenum, passed through the pylorus, and positioned in the forearm of the laparotomized rat. Intragastric pressure and volume were measured with an electronic barostat (Distender Series II Dual Drive Barostat for Rats; G&J Electronics, Willowdale, Canada) connected to the balloon, and the pressure was increased stepwise by 2 mmHg to 10 mmHg for 2 minutes at each step.

Adaptive relaxation of the stomach was evaluated based on the intragastric volume corresponding to a stimulation pressure of 10 mmHg. Once stabilization was attained through three to five times of repeated distending stimulation at 20-minute intervals, a test substance, dissolved in 0.5% carboxymethyl cellulose solution (1.5 mL), was administered intragastrically through a transpyloric cannula. After a 60-minute treatment period, the intragastric solution was carefully drained through the cannula and the stepwise intragastric pressure was subsequently applied to trigger the adaptive relaxation responses. The effect of the intragastric test solution on adaptive relaxation was determined by comparing the intragastric volume immediately before and after the test solution treatment. In some experiments, gastric branches of the ventral and dorsal vagal trunk were transected immediately before the test solution treatment, as mentioned above. L-NNAME (20 mg/kg) was i.v. administered to the tail vein 50 minutes after the test solution treatment.

Measurement of Gastric Tone in Conscious Dogs. Gastric tone was measured using a barostat system in surgically prepared healthy female beagles (10–12 kg), as previously described (Xing and Chen, 2005). Briefly, after an overnight fast, the dogs were anesthetized with i.v. sodium thiopental (Ravonal; 25 mg/kg; Mitsubishi Tanabe Pharma, Tokyo, Japan) and maintained on halothane (Fluothane, 1.0%, inhalation anesthesia; Takeda Pharmaceutical, Osaka, Japan) in air delivered from a ventilator following endotracheal intubation. Laparotomy was performed with vital sign monitoring of mucosal color, pulse rate, and breath rate. A cannula was placed in the stomach 7 cm proximal to the pylorus for the assessment of gastric tone. After the placement of the cannula, the abdomen was closed and the anesthetics were stopped. Once consciousness was regained, the dogs were transferred to a recovery cage after receiving medication for postoperative pain control. The experiments described below were performed after dogs had completely recovered from surgery (14 days later).

A polyethylene bag (StarMedical, Tokyo, Japan) was inserted and positioned in the proximal stomach through the gastric cannula for the measurement of gastric tone for 30 minutes in the fasting state (overnight-fasted). Furthermore, the animal stood on an experimental table loosely restrained during the recording period and was acclimated to the experimental environment before the experiment.

The barostat bag, used for the measurement of gastric tone, was noncompliant, had a maximal volume of 800 mL, and was attached to the distal end of a double-lumen catheter. The catheter was connected to a computer-controlled electrical barostat device (Distender Series IIR; G&J Electronics). After its placement in the fundus, the bag was briefly inflated with 150 mL air and then completely deflated. After a brief rest period, the minimal distending pressure was determined by inflating the bag in 1-mmHg increments until a pressure at which evident respiratory excursions were recorded and the bag volume was equal to or larger than 30 mL. Gastric volume, reflecting gastric tone, was recorded at an operating pressure of 2 mmHg higher than the minimal distending pressure during the entire experiment. Gastric volume was acquired by a PowerLab interface (PowerLab 4/30; ADInstruments) and averaged every 5 minutes with Chart 7. After 20 minutes of baseline acquisition, each test substance was administered. At a constant operating pressure, an increase in gastric volume reflects a decrease in gastric tone and vice versa.

Sumatriptan was dissolved in sterilized saline and administered s.c. (1 or 3 mg/kg). ArgGlu was dissolved in sterilized water and infused intragastrically at 2 mL/min for 25 minutes through a double-lumen catheter.

Clonidine-Induced Delayed Gastric Emptying in Dogs. Delayed gastric emptying in dogs was induced using clonidine, an α2-adrenergic receptor agonist. Gastric emptying was measured with a 13C-breath test using the BreathID System (Exalenz Bioscience, Modiin, Israel; http://www.exalenz.com) that analyzed the ratio of 13CO2 to 12CO2 in each breath sample, as has been previously described (Zai et al., 2009), with some modifications for animal use. Briefly, breath samples were automatically collected through nasal cannula from overnight-fasted dogs fed with 50 g solid dog food DS-A (Oriental Yeast) containing 20 mg L-2-aminoxyacetic acid (Cambridge Isotope Laboratories, Tewksbury, MA) and with saline 50 mL L-arginine L-glutamate (20 mM) dissolved in distilled water. This solution (50 mL) was administered orally at 20 minutes before the meal. Cisapride (3 mg/kg) was suspended in 0.1% methyl cellulose 400 and administered orally 30 minutes before the meal. Aclometamide (30 mg/kg) was dissolved in distilled water and administered orally 30 minutes before the meal. Clonidine (30 μg/kg) was administered s.c. 15 minutes before the meal. Gastric emptying was evaluated using half the gastric excretion (emptying) time (T1/2). The T1/2 time indicates the time at which half of the 13CO2 dose is excreted in relation to cumulative 13CO2 excretion when time is infinite.

Statistical Analyses. All results are expressed as means ± S.E.M. The statistical analyses described below were calculated using the statistical software SAS System version 8.2 (SAS Institute Japan, Tokyo, Japan). Parametric Dunnett’s multiple comparison test was used to analyze the dose-dependent effect of ArgGlu or mosapride on gastric emptying in the rat and that of ArgGlu or sumatriptan on gastric tone in dogs. Nonparametric Dunnett’s multiple comparison test was used to analyze the concentration-dependent effect of ArgGlu on vagus nerve activation. Steel’s test was used to analyze the dose dependency of the effect of ArgGlu on adaptive relaxation in rats. P values <0.05 were considered statistically significant after an adjustment for multiple comparisons. In the rat gastric emptying or adaptive relaxation experiments, an Aspin Welch’s test or a Student’s t test was used to compare the gastric emptying rate (%) or gastric adaptive relaxation between the vehicle-treated group and the ArgGlu-treated group. In the delayed gastric emptying model in dogs, a multiple analysis of variance was used to compare the T1/2 between the vehicle-treated group and ArgGlu-treated group.

Results

The Effects of ArgGlu on Gastric Emptying in Rats. The effect of ArgGlu on gastric emptying of a liquid meal was investigated using the phenol-red method in freely fed Sprague-Dawley rats aged 6–7 weeks. ArgGlu dissolved in test meal (a casein solution containing phenol red) was given to rats via oral administration at a dose of 0.5 mL/kg. ArgGlu dose-dependently promoted gastric emptying in a dose range of 0.3–30 mg/kg, and its promoting effect on gastric emptying...
at doses of 10 and 30 mg/kg was significantly higher compared with rats in the vehicle group (Fig. 1A). This effect of ArgGlu was comparable to that of orally administered mosapride, a prokinetic drug clinically used in Japan, in a dose range from 0.3 to 3 mg/kg (Fig. 1B).

**Plasma Concentrations of L-Glutamate and L-Arginine after Single Oral Administration of ArgGlu.** Concentrations of L-glutamate (Glu) and L-arginine (Arg) in the stomach and plasma were measured immediately after a single oral administration of 10 mg/5 ml/kg ArgGlu, the dose at which gastric emptying was significantly promoted. The calculated molar concentration of ArgGlu in this gavage solution is 6.2 mmol/L. Immediately following the intragastric administration of ArgGlu, the concentrations of Glu and Arg in the gastric juices of the experimental rats were 5.8 and 5.4 mmol/L, respectively (average, $N = 2$). However, there was no increase in the plasma concentrations of Glu and Arg following the administration of ArgGlu compared with those in the control group (Table 1), suggesting that ArgGlu acts locally in the stomach rather than systemically. To test this hypothesis, the effect of systemic exposure to ArgGlu was examined by the i.v. route. Intravenously administered ArgGlu at a dose of 10 mg/kg, the dose at which gastric emptying was significantly promoted by oral administration, had no effect on gastric emptying (Fig. 1C).

**Effects of Glu and Arg on Gastric Emptying in Rats.** As substantial elevations in the concentration of Glu and Arg were detected in the stomach after the intragastric administration of ArgGlu, the effect of each constituent amino acid (Glu and Arg) was examined in the gastric emptying study. Glu promoted gastric emptying in a dose-dependent manner (from 1 to 10 mg/kg p.o.). The observed effect of Glu on gastric emptying at 10 mg/kg was significantly higher compared with rats in the vehicle group (Fig. 1E), although (Arg) had no effect on gastric emptying in the same dose range (Fig. 1F).

**Mechanisms of Action.** Orally administered ArgGlu promoted gastric emptying in a dose-dependent manner without any measured increases in plasma concentration, whereas i.v. administered ArgGlu had no effect on gastric emptying even at the same dose (10 mg/kg) as the aforementioned orally effective dose. To examine the contribution of the vagus nerve, the promoting effect of ArgGlu on gastric emptying was investigated.
in rats with a vagotomy of the gastric branches. ArgGlu (10 mg/kg) had no significant effect on gastric emptying in rats that received a vagotomy of the ventral and dorsal vagal gastric branches compared with rats in the vehicle group (Fig. 1D).

Activation of the afferent vagus nerve by ArgGlu was confirmed in anesthetized rats with urethane. The maximal effective dose of ArgGlu on gastric emptying was 10 mg/5 ml/kg, for a final molarity of 6 mmol/L according to the mol. wt. of ArgGlu (mol. wt. = 321.33) and average body weight of animals (b.wt. = ~300 g). Therefore, we chose 2, 6, and 20 mM, centering around 6 mM, as the experimental concentrations. Afferent vagus nerve activity, recorded from the nerve bundle of the left gastric branch, increased after the bolus administration of ArgGlu into the stomach in a concentration-dependent manner (2–20 mmol/L), and was significant at 6 and 20 mmol/L (Fig. 2A). The intragastric perfusion of 6 mM ArgGlu for 10 minutes

Table 1

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<th>Plasma L-Glutamate (µmol/L)</th>
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<th>Plasma L-Arginine (µmol/L)</th>
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<td>114 ± 13.5</td>
<td>116 ± 5.3</td>
<td>106 ± 6.8</td>
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<tr>
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<td>117 ± 6.3</td>
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Fig. 2. Effect of intragastric injection of L-arginine L-glutamate (ArgGlu) on neuronal activity from the gastric branches of the vagus nerve. Afferent neuronal activity was measured from the gastric branches of the vagus nerve in fasted rats under urethane anesthesia. ArgGlu was administered into the stomach as a 3 mL solution per rat. Water was administered as a control. (A) Concentration dependency of the increasing effect of ArgGlu (2–20 mM) on gastric emptying in rats. Relative firing rate (%) expresses the ratio between the mean firing rate from 10–60 minutes after and the mean basal firing rate from 10 minutes before a bolus injection of test substances into the stomach. N = 8; comparison with control group (nonparametric Dunnett’s test). (B) Persistent activation of the gastric branches of the vagus nerve after 10 minutes of intragastric perfusion of ArgGlu. The mean firing rates (%) relative to that of a 10-minute baseline increased gradually after intragastric infusion of 6 mM ArgGlu. The ArgGlu solution was perfused at a rate of 3 mL/min on gastric lumen for 10 minutes (solid line), and then the perfusate was switched to 6 mmol/L sodium chloride solution. As a control, 6 mmol/L sodium chloride solution was used throughout the experiments. The inserted bar graph indicates mean firing rates (%) relative to baseline. N = 5; comparison with control group (two-sided Wilcoxon). (C) Mosapride (1 mg/kg i.v.) surpassing its effective plasma level during the entire recording time in rats did not cause any significant change in vagus nerve activity. (D) Acotiamide (10 mg/kg i.v.) surpassing its effective plasma level during the entire recording time in rats did not cause any significant change in vagus nerve activity. In all cases, data represent the mean ± S.E. *P < 0.05; **P < 0.01.
also increased vagus nerve activity in the afferent gastric branch, and its effect persisted at least 50 minutes after the intragastric washout with 6 mM NaCl, whereas 6 mM NaCl did not cause any increase in vagus nerve activity (Fig. 2B).

Neither mosapride nor acotiamide, administered at a dose surpassing their effective plasma levels, caused any significant change in vagus nerve activity during the entire recording time in rats (mosapride, 1 mg/kg i.v.; acotiamide, 10 mg/kg i.v.) (Fig. 2, C and D).

**Effect of ArgGlu on Adaptive Relaxation of the Stomach in Anesthetized Rats.** The effect of ArgGlu (1–30 mg/kg) on the adaptive relaxation of the stomach was investigated by the barostat method using pentobarbital in anesthetized rats. ArgGlu enhanced adaptive relaxation of the stomach in a dose-dependent manner, where this effect was significantly higher at doses of 3, 10, and 30 mg/kg compared with rats in the vehicle group (Fig. 3A). An acute vagotomy of gastric branches attenuated the adaptive relaxation of the stomach, but did not abolish the enhancing effect of ArgGlu (10 mg/kg) on gastric adaptive relaxation (Fig. 3B); however, a vagotomy of gastric branches did cancel the promoting effect of ArgGlu on gastric emptying (Fig. 1D). Pretreatment with L-NAME (20 mg/kg i.v.), a NO synthase inhibitor, eliminated the effect on gastric adaptive relaxation (Fig. 3C). To examine the contribution of each of the constituent amino acids of ArgGlu, the effect of each amino acid (Glu and Arg) was compared with ArgGlu at the same concentration (6 mM) on gastric adaptive relaxation. ArgGlu and Arg significantly enhanced gastric adaptive relaxation, whereas Glu had no effect (Fig. 3D).

**Effect of ArgGlu on Gastric Tone in Conscious Dogs.** The effect of the intragastric infusion of ArgGlu on gastric tone was investigated in conscious dogs using the barostat method. The intragastric infusion of ArgGlu (6 or 60 mM) into the stomach gradually increased the gastric volume at 2 mmHg plus minimal distending pressure (Fig. 4A). The effect was significantly higher at 6 and 60 mM compared with dogs in the vehicle group (Fig. 4B), and was comparable to the effect of sumatriptan (1 or 3 mg/kg s.c.), a 5-HT1B/D agonist (Fig. 4, C and D).

**Clonidine-Induced Delayed Gastric Emptying in Dogs.** Clonidine, an \( \alpha_2 \)-adrenergic agonist, pharmacologically induces gastroparesis and delayed gastric emptying in dogs. The clonidine-induced delayed gastric emptying model has been widely used to examine the effect of prokinetics in dogs (Tanaka et al., 1998; Kawachi et al., 2011). In the model in this study, ArgGlu (30 mg/kg p.o.) significantly reduced the half gastric emptying time (T1/2) (Table 2). Cisapride (3 mg/kg p.o.) also significantly reduced T1/2, although acotiamide (30 mg/kg p.o.) did not reduce it significantly, as one dog of the six used in this experiment showed an obvious increase in T1/2 after the treatment of acotiamide (N = 6, P = 0.331). The maximally effective oral dose for cisapride (3 mg/kg) and acotiamide (30 mg/kg) on canine gastric motility was chosen as a testing dose according to previous published studies (Mikami et al., 2008; Matsunaga et al., 2011).

![Fig. 3.](image-url) Effect of L-arginine L-glutamate (ArgGlu) on adaptive relaxation of the stomach in anesthetized rats. The adaptive relaxation of the stomach was measured using the barostat method in fasted rats under pentobarbital anesthesia. (A) Dose-dependent enhancement of the gastric adaptive relaxation by L-arginine L-glutamate. The adaptive relaxation of the stomach was evaluated 60 minutes after intragastric administration of L-arginine L-glutamate (1–30 mg/kg). N = 6; versus vehicle (Steel’s test). (B) The enhancing effect of ArgGlu (10 mg/kg intragastrically) on gastric adaptive relaxation was resistant to acute vagotomy of the gastric branches, which attenuated the adaptive relaxation of the stomach. N = 6, P = 0.844; versus vehicle (Steel’s test). (C) Pretreatment with a NOS inhibitor L-NAME (20 mg/kg i.v.) inhibited gastric adaptive relaxation and the enhancing effect of ArgGlu. N = 6, P = 0.844; versus vehicle (Aspin-Welch’s test). (D) ArgGlu and Arg enhanced gastric adaptive relaxation. Each test substance solution (1.5 mL) was intragastrically administered at 6 mM, which is comparable to 10 mg/kg for ArgGlu. N = 6; versus vehicle (Steel’s test). In all cases, data represent the mean ± S.E. *P < 0.05.
Gastric emptying was measured by a 13C-breath test. Delayed gastric emptying in adults at doses of 20 g/d. The safety of the oral administration of ArgGlu, which is used as an i.v. formulation for the treatment of hyperammonemia, enhanced gastric motor function when administered orally, suggesting it could be a new oral medicine for the treatment of upper GI dysfunction. ArgGlu facilitated gastric emptying through vagus nerve activation and enhanced gastric relaxation through NO production. This pharmacodynamic dual action could be effective for treating PDS symptoms in FD patients with delayed gastric emptying and impaired gastric accommodation. Indeed, ArgGlu improved clonidine-induced delayed gastric emptying in conscious dogs. These findings suggest that ArgGlu could be used to treat symptoms of upper dyspeptic disorders such as FD as a new prokinetic with a unique pharmacological profile: it accelerates gastric emptying and enhances gastric accommodation, distinguishing it from other prokinetics (5-HT4 and motilin agonists).

ArgGlu, a natural amino acid salt of L-arginine and L-glutamate, is usually administered via i.v. drip infusion to adults at doses of 2–20 g/d. The safety of the oral administration of ArgGlu has been confirmed at doses up to at least 6 g/d, according to results from clinical studies conducted in healthy males, chronic alcoholic patients (Tobe, 1961), patients suffering from episodic encephalopathy (Eto et al., 1994), males with mild to moderate erectile dysfunction (Lebret et al., 2002; Kernohan et al., 2005), and postmenopausal females (Meston and Worcel, 2002). There have been no previously documented accounts using ArgGlu to treat GI symptoms.

ArgGlu (Fig. 1A) promoted gastric emptying in a dose-dependent manner in normal rats, as did orally administered mosapride, a prokinetic drug used clinically in Japan. ArgGlu also improved delayed gastric emptying in dogs, in which its positive effect was comparable to that of the preceding prokinetic, cisapride. Pathophysiological studies have revealed the presence of delayed gastric emptying in subsets of FD patients (23–59%) (Tack et al., 2006). A meta-analysis using the prokinetics domperidone and cisapride suggests the superiority of prokinetics over placebo for FD (Moayyedi et al., 2006). Cisapride has been withdrawn in many countries owing to concerns over cardiac safety (arrhythmia), and neither domperidone nor mosapride is widely available. Recently, a new prokinetic, acotiamide, was approved for the PDS symptoms of FD in Japan; however, the treatment of FD remains unsatisfactory (Camilleri and Stanghellini, 2013), and a safe and effective medicine is eagerly awaited.

The promoting effect of ArgGlu on gastric emptying was canceled by a vagotomy of the gastric branches, suggesting the involvement of vagus nerve activation. In fact, ArgGlu (2–20 mmol/L), administered intragastrically in the effective dose range for gastric emptying, activated the afferent gastric branches of the vagus nerve without any increase in blood concentrations of either L-arginine or L-glutamate. Intravenous ArgGlu did not show any effect of gastric emptying. Taken together, these findings indicate that ArgGlu exerts its prokinetic effect not through absorption into the bloodstream, but by activation of the vagus nerve, which most likely promotes gastric emptying after acting on local receptors in the gastric mucosa (Uneyama et al., 2006).

### Table 2

Effects of ArgGlu, cisapride, and acotiamide on clonidine-induced delayed gastric emptying of solids in conscious dogs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gastric Emptying $T_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + vehicle</td>
<td>136.4 ± 15.7</td>
</tr>
<tr>
<td>Clonidine + vehicle</td>
<td>305.1 ± 28.1</td>
</tr>
<tr>
<td>Clonidine + ArgGlu (30 mg/kg p.o.)</td>
<td>278.9 ± 26.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clonidine + cisapride (3 mg/kg, p.o.)</td>
<td>275.9 ± 18.0&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clonidine + vehicle</td>
<td>290.0 ± 31.1</td>
</tr>
<tr>
<td>Clonidine + acotiamide (30 mg/kg p.o.)</td>
<td>254.5 ± 13.9</td>
</tr>
</tbody>
</table>

<sup>*</sup>$ P < 0.05; versus vehicle (multiway analysis of variance).

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Effect of L-arginine L-glutamate (ArgGlu) on gastric tone in conscious dogs. (A) Time course of the increasing effect of ArgGlu on gastric volume changes from baseline ($\Delta$ gastric volume) at 2 mmHg + minimal distending pressure. ArgGlu (0.6–60 mM) was infused intragastrically at 2 mL/min for 25 minutes (solid line). (B) Concentration-dependent effect of ArgGlu on mean gastric volume change ($\Delta$ gastric volume) for 0–60 minutes. N = 5; versus vehicle (parametric Dunnett’s test). (C) Time course of the increasing effect of sumatriptan on $\Delta$ gastric volume at 2 mmHg + minimal distending pressure. Sumatriptan (1, 3 mg/kg) was administered s.c. at 0 minute. (D) Dose-dependent effect of sumatriptan on mean $\Delta$ gastric volume for 0–60 minutes. N = 5; versus vehicle (parametric Dunnett’s test). In all cases, the data represent the mean ± S.E. *$ P < 0.05; ** P < 0.01; *** P < 0.001.
In contrast, neither the 5-HT4 agonist mosapride nor the acetylcholinesterase inhibitor acotiamide (Kawachi et al., 2011; Yoshii et al., 2016), administered at doses surpassing their effective plasma levels, caused a significant change in afferent vagus nerve activity in rats. This suggests that ArgGlu could be acting via a different mechanism than these systemic prokinetics, modulating GI function through the activation of the afferent vagus nerve, leading to vagal-vagal reflection to control motor function (Lundgren, 1983) and the secretion of digestive juices and enzymes (Furness et al., 2014).

Glu resulted in a significant promoting effect of gastric emptying (Fig. 1E), whereas Arg had no effect on gastric emptying in the same dose range as Glu (Fig. 1F). This suggests that Glu is the main contributor to the promoting effect of ArgGlu on gastric emptying. Simultaneously, we confirmed that Arg has no inhibitory effect on gastric emptying despite enhancing stomach relaxation (Fig. 3D).

Uneyama et al. (2006) reported that L-glutamate is the only nutrient among amino acids, sugars, and electrolytes that activates rat gastric vagal afferents from the luminal side, suggesting the existence of L-glutamate receptors linked to vagus nerve activation in the gastric mucosa. It has been reported that receptors responding to L-glutamate, such as metabotropic glutamate receptor type 1 (San Gabriel et al., 2007), metabotropic glutamate receptor type 4 (Akiba et al., 2009), umami taste receptor T1R1/T1R3 (Bezençon et al., 2007; Akiba et al., 2009), and calcium-sensing receptor (Cheng et al., 1999; Conigrave et al., 2000), are expressed in the gastric mucosa as well as in the tongue (San Gabriel et al., 2009b).

Luminal L-glutamate activates the afferent vagus nerve through NO synthase (NOS) activation (Uneyama et al., 2006). NOS, once activated, produces a gaseous neurotransmitter, NO, activating the afferent vagus nerve. ArgGlu could supply L-arginine as a substrate to boost NO production. The expression of NOS in the gastric mucosa has also been confirmed (Price et al., 1996; Rajnakova et al., 1997), although the primary sites of action for the facilitation of gastric emptying by ArgGlu remain to be determined.

ArgGlu promoted gastric relaxation in anesthetized rats as well as in conscious dogs in a dose-dependent manner. This maximal effect on relaxation was comparable to that of sumatriptan, a 5-HT1B/D agonist primarily used to treat migraines. Pathophysiological studies have revealed the presence of impaired gastric accommodation in subsets of FD patients (40–50%). Sumatriptan (Tack et al., 2000, 2003), as well as buspirone (Chial et al., 2003; Van Oudenhove et al., 2008), induces relaxation of the gastric fundus and enhances accommodation in both humans and animals, allowing larger intragastric volumes before thresholds for perception or discomfort are reached. In patients with FD, both sumatriptan (Moro et al., 2004) and buspirone (Tack et al., 2012) improve gastric accommodation after food consumption and reduce the perception of gastric distension. These studies have established that impaired accommodation after food consumption is a major pathophysiological mechanism in FD, and that the restoration of accommodation is considered a potential therapeutic target (Camilleri and Stanghellini, 2013). As demonstrated in this study, ArgGlu reduced gastric tone as well, suggesting its potential to enhance gastric accommodation. ArgGlu enhanced gastric fundus relaxation at intragastric infusion concentrations of ≈6 mM in conscious dogs (Fig. 4B) and ≈2 mM in anesthetized rats (Fig. 3A). This similarity in effective concentration ranges between the two species suggests the presence of common mechanisms underlying the enhancement of gastric fundus relaxation.

In this study, acute vagotomy reduced the adaptive relaxation of the rat stomach (Fig. 3B), confirming that the vagus nerve mediates gastric accommodation in rats (Takahashi and Owyang, 1997). ArgGlu, however, recovered the impaired adaptive relaxation after acute vagotomy, suggesting that its recovery effect is independent of the vagus nerve reflex, whereas its promoting effect on gastric emptying depends on vagus nerve activation. Pretreatment with L-NAME, a NOS inhibitor, completely inhibited the enhancing effect of ArgGlu on adaptive relaxation, suggesting that NO production mediates the relaxation response.

L-Arginine is an important precursor for the inhibitory neurotransmitter NO, which controls GI motility and blood circulation by relaxing smooth muscle (Sanders and Ward, 1992). NO mediates the reflexive relaxation of the stomach to accommodate food or fluid (Desai et al., 1991), and its precursor, L-arginine, also causes gastric relaxation in humans at relatively high doses (Savoye et al., 2006). In this study, we confirmed that the effect of ArgGlu on gastric relaxation is attributed to L-arginine (Fig. 3D). ArgGlu could supply L-arginine as a NOS substrate to boost gastric relaxation through NO production. Tack et al. (2002) reported that NOS inhibition impairs accommodation and enhances meal-induced satiety in humans, and that the gastric accommodation reflex involves the activation of nitricergic neurons (Tack et al., 2002). The enhancing effect of ArgGlu on gastric relaxation, even under vagotomy, suggests that ArgGlu may have beneficial effects on gastric accommodation in patients with hypo- or dysfunction of the vagus nerve.

In the adaptive relaxation study in rats in vivo, all experiments were conducted under pyloric ligation to fix the cannula with a gastric balloon inside the stomach. Thus, pyloric ligation prevents gastric fluid from entering the duodenum, suggesting that intragastrically administered ArgGlu remained and acted luminally in the stomach. It has been previously confirmed that the rat gastric mucosa expresses the amino acid transporters System L, ASCT2, B0AT1, ATB(0 +-) (Kirchhoff et al., 2006), and NOS (Price et al., 1996; Rajnakova et al., 1997), suggesting that these transporters could mediate the absorption of ArgGlu to produce NO in the gastric mucosa.

In conclusion, this study demonstrated that ArgGlu enhanced both gastric emptying and relaxation in rats and dogs. It also improved clonidine-induced delayed gastric emptying in conscious dogs, in which its effect was comparable to those of the 5-HT4 agonist mosapride and the acetylcholinesterase inhibitor acotiamide, thus supporting a potential translation to humans. The dual actions of ArgGlu could enhance its efficacy in patients with FD. ArgGlu, with good safety profiles, could provide a new and promising option for the treatment of upper GI symptoms. Proof of its efficacy in clinical trials is awaited.

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