Does ghrelin explain accelerated gastric emptying in the early stages of diabetes mellitus?

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Submitted 26 October 2007; accepted in final form 31 March 2008

Ariga H, Imai K, Chen C, Mantyh C, Pappas TN, Takahashi T. Does ghrelin explain accelerated gastric emptying in the early stages of diabetes mellitus? Am J Physiol Regul Integr Comp Physiol 294: R1807–R1812, 2008. First published April 2, 2008; doi:10.1152/ajpregu.00785.2007.—During the early stages of diabetes, gastric emptying is often accelerated, rather than delayed. The mechanism of accelerated gastric emptying in diabetes has not been fully studied. A recent study showed that plasma ghrelin levels were elevated in diabetes. As postprandial antropyloric coordination plays an important role in mediating solid gastric emptying, we hypothesize that the elevated plasma ghrelin levels increase postprandial antropyloric coordination to accelerate emptying in the early stages of diabetes. To test this hypothesis, rats were made diabetic by streptozotocin (STZ; 50 mg/kg) injection, and, 2 wk later, pre- and postprandial plasma ghrelin levels, antropyloric coordination, and solid gastric emptying were determined. In control rats, plasma ghrelin levels were immediately reduced after feeding. In contrast, plasma ghrelin levels remained within the fasted levels in STZ rats after feeding. In STZ rats, gastric emptying was significantly accelerated (77.4 ± 3.2%, n = 6), compared with that of control rats (58.8 ± 2.5%, n = 6, P < 0.05). Treatments with anti-ghrelin antibodies attenuated accelerated gastric emptying in STZ rats (50.1 ± 3.5%, n = 6, P < 0.05), while having little effect in vehicle control rats. The incidence of postprandial antropyloric coordination was significantly increased in STZ rats, compared with that of control rats (P < 0.05). Treatments with anti-ghrelin antibodies suppressed this enhanced antropyloric coordination in STZ rats. Our study suggests that elevated endogenous ghrelin enhances antropyloric coordination, which accelerates gastric emptying in the early stages of diabetes.

On the other hand, solid gastric emptying is regulated by the coordinated motor activity of the antrum, pylorus, and duodenum in humans (4, 41) and dogs (13, 31). Similarly, we have previously demonstrated that the coordinated motor pattern between the antrum and pylorus (antropyloric coordination) plays an important role in mediating solid gastric emptying in rats (22, 23, 44), as well as dogs (55).

In rats, both the antrum and pylorus randomly contract up to 10 min after feeding, and this period seems to reflect the grinding process of the gastric content. Forty minutes after feeding, contractile patterns become significantly altered to coordinated patterns with low frequency (<3 cycles/min) and high amplitude between the antrum and pylorus. The contraction of the pylorus occurred usually 2–6 s after the contraction of the antrum. The incidence of antropyloric coordination was significantly increased at 40–80 min after the feeding (22, 23, 44). The coordination between the antrum and pylorus is an important factor in the emptying of solid foods (22, 23, 44).

Ghrelin, an orexigenic peptide, was discovered as the endogenous ligand for the growth hormone secretagogue receptor from the rat stomach (30). Ghrelin is produced by X/A-like cells in the stomach (7, 11) and is released into circulation after posttranslational processing where n-octanoyl acid becomes attached at serine in position 3 (2), which is essential for its major bioactivity.

It is well established that ghrelin stimulates gastrointestinal motility (16, 29, 38, 51). Ghrelin accelerates solid gastric emptying in humans (34), rats (17), and mice (8). Gastric emptying of nonnutrient liquid is also accelerated by ghrelin in rats (17, 33, 52) and mice (29).

Circulating levels of ghrelin rise before and decrease after a meal in normal-weight subjects. In contrast, the postprandial reduction of ghrelin is not obvious in obese subjects (32) and patients with new onset childhood Type 1 diabetes (20). In STZ-induced diabetic rats, postprandial ghrelin concentrations are higher than in control rats (24) and return to fasted levels rapidly after feeding (18). Gastric ghrelin-immunoreactive cells are slightly decreased in STZ-induced diabetic rats, whereas pre-proghrelin mRNA levels are extremely higher in STZ rats than in control rats (37). The increased plasma ghrelin levels and the decreased gastric ghrelin cells in the diabetic rats (37) may be due to an increase in ghrelin release from the stomach into the bloodstream.

Based on these evidences, we hypothesize that the alteration of postprandial antropyloric coordination induced by elevated plasma ghrelin contributes to rapid gastric emptying in the
early stages of diabetes. As it has been shown that diabetic autonomic neuropathy develops 6–8 wk after the STZ administration (39, 49), we utilized rats who received STZ injection 2 wk before the experiments. The present study examines whether 1) solid gastric emptying is accelerated, 2) postprandial antropyloric coordination is enhanced, and 3) postprandial ghrelin levels are higher in STZ-induced diabetic rats. Anti-ghrelin antibodies were used to determine the role of endogenous ghrelin in mediating accelerated gastric emptying in STZ-induced diabetic rats.

MATERIAL AND METHODS

Animals. Protocols describing the use of rats were approved by the Institutional Animal Care and Use Committee of Duke University and carried out in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” All efforts were made to minimize animal suffering and to reduce the number of animal in experiments.

Male Sprague-Dawley rats, weighing 260–290 g, were housed in group cages with daily illumination from 0700 to 1900 (12:12-h light-dark cycle) and controlled temperature (22–24°C) and humidity (30–35%) at least 7 days before experiments and given free access to laboratory chow and water ad libitum. Before experiments, rats were fasted for 24 h, but given free access to water.

Induction of diabetes. Rats received intraperitoneal injection of STZ (50 mg/kg) in 0.2 ml of 10 mM citrate buffer (pH 5.5). Control rats received intraperitoneal injection of citrate buffer. All animals were given access to water and suitable rat chow ad libitum. Diabetic rats showed a nonfasting glucose concentration of >200 mg/dl in tail vein blood. Body weight was monitored daily in both groups.

Measurement of blood glucose and plasma ghrelin and insulin before and after feeding. Ten days after STZ injection, an intravenous catheter was inserted into the right jugular vein and exteriorized to the neck before and after feeding. Body weight was monitored daily in both groups.

After the measurement of blood glucose level, the remaining blood was injected after each sampling procedure to avoid dehydration.

Measurement of solid gastric emptying. Two weeks after STZ injection, rats were fasted for 24 h and received 1.5 g of solid caloric meal (PMI Feeds, Richmond, IN; 3.04 kcal/g, protein 23.4%, fat 4.5%, fiber 5.3%), as previously described (23). Thirty minutes before the solid meal ingestion, a specific anti-ghrelin antibody [rabbit anti-ghrelin (rats/mice) IgG; designed for acyl ghrelin (0.5 ml) or non-immuno-specific antibody (0.5 ml)] was given and then stored at 80°C. Plasma level of acyl ghrelin was measured by radioimmunoassay (RIA) using a RIA kit (LINCO Research, St. Charles, MO). This kit is designed to measure the active form of ghrelin (acyl ghrelin) and does not cross react with des-acyl ghrelin. Plasma levels of insulin were also measured by RIA.

Measurement of gastric motility recording. After the administration of a specific anti-ghrelin antibody (10 μg/kg; 0.5 ml ip) or non-immuno-specific antibody (10 μg/kg, 0.5 ml ip), rats were given a preweighed solid meal (1.5 g). Postprandial gastric motility was recorded continuously for 120 min.

Evaluation of antropyloric coordination. After the measurement of solid gastric emptying, a specific anti-ghrelin antibody (10 μg/kg; 0.5 ml ip) or non-immuno-specific antibody (10 μg/kg, 0.5 ml ip), rats were given a preweighed solid meal (1.5 g). Postprandial gastric motility was recorded continuously for 120 min.

RESULTS

Body weight changes in control and STZ rats. Figure 1 shows the changes in body weight, 2 wk after STZ or vehicle injection. Diabetic rats gradually gained weight during the 2 wk after STZ (50 mg/kg) injection. The body weight increment was calculated according to the following formula, as previously described (23): gastric emptying (%) = [1 – (dried weight of food recovered from stomach/weight of food intake)] × 100.

Recording of postprandial antropyloric coordination. Different rats were used to investigate antropyloric coordination. One week after STZ or vehicle injection, rats were anesthetized with pentobarbital sodium (45 mg/kg ip). Through a midline laparotomy, two strain-gauge transducers were implanted on the serosal surface of the gastric antrum and the pylorus to monitor the contractions of circular muscle, as previously described (22, 23, 44).

The wires from transducers were exteriorized through the abdominal wall, placed under the skin, and ran up toward the back. Exposed wires were shielded in a protective jacket (Star Medical, Tokyo, Japan). After the surgery, rats were housed individually with standard diets and tap water. Rats were allowed to recover for 1 wk before motility recording studies.

After a 24-h fast, the wires from transducers were connected to a recording system (Power-Lab model MSP, ADI Instruments, Colorado Springs, CO), and contractions of the antrum and pylorus were monitored for 120 min in conscious and freely moving rats. Thirty minutes after administration of a specific anti-ghrelin antibody (10 μg/kg; 0.5 ml ip) or non-immuno-specific antibody (10 μg/kg, 0.5 ml ip), rats were given a preweighed solid meal (1.5 g). Postprandial gastric motility was recorded continuously for 120 min.

Statistical analysis. Statistical analysis was performed by one-way ANOVA, followed by the Tukey test or by Mann-Whitney U-test, depending on the dataset concern, by using StatMate III (ATMS, Tokyo, Japan). P values <0.05 were considered as significant. All results are expressed as means ± SE.
in STZ-injected rats was significantly less than the weight gain in control rats over the same time period (Fig. 1).

**Blood glucose levels before and after feeding in control and STZ rats.** Blood glucose levels were measured before and after feeding in control and STZ rats. The blood glucose levels in STZ rats at 60 min before feeding were 123.4 ± 19.2 mg/dl (n = 5), which was significantly higher than the levels in control rats (72.4 ± 2.6 mg/dl, P < 0.05, n = 5) (Fig. 2A).

Blood glucose levels increased immediately after feeding in STZ rats. Blood glucose levels at 30 min after feeding in STZ rats were 235.6 ± 23.3 mg/dl.

In STZ rats, blood glucose levels reached a maximum at 60 min after feeding (257.0 ± 30.8 mg/dl), which was significantly higher than glucose levels in control rats (118.2 ± 5.9 mg/dl, P < 0.01) (Fig. 2A).

**Plasma insulin levels before and after feeding in control and STZ rats.** Before feeding, plasma insulin levels were 0.45 ± 0.04 pg/ml (n = 6) in control rats. Sixty minutes after feeding, plasma insulin levels significantly increased to 1.43 ± 0.15 pg/ml (n = 6) in control rats.

Two weeks after STZ injection, preprandial insulin levels were 0.31 ± 0.02 pg/ml, which were not significantly different from the levels in control rats. However, postprandial plasma insulin levels in STZ rats (0.38 ± 0.03 pg/ml, n = 6) were significantly decreased from the levels in control rats (P < 0.01).

**Plasma ghrelin levels before and after feeding in control and STZ rats.** In a fasting state, plasma ghrelin levels were not significantly different between control and STZ rats (Fig. 2B).

In control rats, plasma ghrelin levels were significantly reduced from 220.7 ± 42.1 to 99.0 ± 21.6 pg/ml (n = 5) 30 min after feeding. Plasma ghrelin levels were further reduced to 54.1 ± 11.2 pg/ml (n = 5) 90 min after feeding.

In contrast, plasma ghrelin level remained within the levels of fasted animals even after feeding STZ rats. Postprandial plasma ghrelin levels were 268.4 ± 68.3 pg/ml at 30 min after feeding and 207.9 ± 37.3 pg/ml at 120 after feeding. Both were significantly higher than the levels in control rats (n = 5, P < 0.05) (Fig. 2B).

**Solid gastric emptying in control and STZ rats.** All rats consumed the entire 1.5 g of solid meal within 8–10 min (8.7 ± 0.5 min, n = 24). In control rats, solid gastric emptying at 90 min after feeding was 58.8 ± 2.5% (n = 6). Gastric emptying was significantly accelerated to 77.4 ± 3.2% (P < 0.05, n = 6) in STZ rats, compared with that in control rats (Fig. 3).

The treatment with the anti-ghrelin antibody did not affect gastric emptying in control rats. In contrast, accelerated gastric emptying was not observed in STZ rats that received anti-ghrelin antibody (n = 6, P < 0.01) (Fig. 3).

**Antropyloric coordination in control and STZ rats.** Antropyloric coordination was not obvious within 40 min after feeding in control rats. The number of antropyloric coordination events was 1.25 ± 0.25 times/20 min within 40 min after eating STZ rats. Antropyloric coordination events were frequently observed and reached its peak (8.25 ± 0.85 times/20 min) 60–80 min after feeding in control rats. Eighty minutes after feeding, the number of antropyloric coordination events gradually decreased (Fig. 5).

In contrast, antropyloric coordination events were frequently observed even 0–20 min after feeding in STZ rats (4.25 ± 0.63 times/20 min, P < 0.05 vs. control rats; Fig. 4B and Fig. 5). The number of antropyloric coordination events reached a peak at 20–40 min after feeding (11.50 ± 3.52 times/20 min). The number of antropyloric coordination events in STZ rats was significantly higher than in control rats at 20–40, 80–100, and 10–120 min after feeding (Fig. 5).
Treatment with anti-ghrelin antibody did not affect the number of antropyloric coordination events in control rats (data not shown). However, the early manifestation of antropyloric coordination within 40 min was not observed after treatment with anti-ghrelin antibodies in STZ rats (Fig. 4C).

Anti-ghrelin antibody treatment also significantly reduced the number of antropyloric coordination events at 40–60, 80–100, and 10–120 min after feeding in STZ rats (Fig. 5).

**DISCUSSION**

It is widely accepted that gastric emptying is delayed in the late stages of diabetes (5, 36). However, rapid gastric emptying has been reported in other subgroups of patients (3, 28, 45, 47) and diabetic rats (19, 46, 56). Although a great deal of attention has been paid to diabetic gastroparesis, the mechanism of accelerated gastric emptying in the early stages of diabetes has not been fully studied.

In rats (53) and healthy subjects (6, 54), circulating plasma ghrelin levels decrease immediately after ingestion of a meal. In contrast, postprandial reduction of plasma ghrelin levels is impaired in obese subjects (14, 32) and patients with new onset of Type 1 diabetes (20). It has been shown that the postprandial ghrelin levels remain high (24) or quickly return to the fasting levels (18) in STZ-induced diabetic rats.

Our present study demonstrates that, in control rats, plasma ghrelin levels decrease immediately from 221 to 99 pg/ml within 30 min after feeding and plateau within 60 min at a slightly lower level of 68 pg/ml. In contrast, postprandial reductions in plasma ghrelin levels were significantly impaired in STZ rats. The postprandial ghrelin levels remained high, even at 120 min after feeding (208 pg/ml) in STZ rats. These results are consistent with the previous reports in diabetic patients (43).

Solid gastric emptying is enhanced by exogenously applied ghrelin in humans (34), rats (17), and mice (8). Ghrelin also can accelerate gastric emptying of nonnutrient liquids in rats (17, 33, 52) and mice (12).

Our laboratory has recently demonstrated that enhanced manifestation of antropyloric coordination plays an important role in ghrelin-induced acceleration of solid gastric emptying (1). Intrapерitoneal administration of ghrelin (4–8 μg/kg) accelerated gastric emptying. The number of antropyloric coordination events, which was not obvious within 20–40 min after feeding in saline-injected rats, was significantly increased in ghrelin-injected rats (1).

The number of antropyloric coordination events was not increased within 40 min after feeding in control rats. In STZ rats, however, the incidence of antropyloric coordination was significantly reduced. The Ghr-Ab also significantly reduced the incidence of antropyloric coordination 40–80 min after feeding in STZ rats: *$P < 0.05$ vs. control rats; †$P < 0.05$ vs. STZ + vehicle rats.
frequently observed within 20 min after feeding and reached a peak at 20–40 min after feeding. To verify that elevated ghrelin is involved in mediating accelerated gastric emptying and enhanced antropyloric coordination, STZ rats received anti-ghrelin antibodies 30 min before feeding.

Since available ghrelin receptor antagonists are limited and [d-Lys³]growth hormone-releasing peptide-6 (a ghrelin receptor antagonist) has been shown to interact with a 5-HT2B receptor (9), we used anti-ghrelin antibody in our present study. We showed that administration of anti-ghrelin antibodies significantly reduced the number of antropyloric coordination events and accelerated gastric emptying in STZ rats. This strongly suggests that elevated plasma ghrelin promotes antropyloric coordination and accelerates solid gastric emptying in STZ-induced diabetic rats. In contrast, anti-ghrelin antibodies did not affect solid gastric emptying in normal rats. This suggests that endogenous ghrelin levels may have a minor role in mediating solid gastric emptying under normal conditions.

As shown in Fig. 1, body weight in STZ rats was significantly less than in control rats. This may raise the possibility that the observed differences in gastric emptying and antropyloric coordinations between controls and STZ rats are secondary to different body weight. However, our preliminary study showed that there were no significant differences of gastric emptying between the rats of 250 g (59.6 ± 6.7%, n = 6) and 300 g (61.3 ± 6.5%, n = 6).

As it has been demonstrated that both fat weight and plasma leptin levels were reduced in STZ rats (24), we cannot exclude the possibility that reduced levels of leptin may affect plasma ghrelin levels.

The mechanism of elevated ghrelin levels in diabetes remains to be investigated. Ghrelin increases adiposity and mediates energy balance (53). Negative energy balance in diabetic rats might induce a compensatory signal to upregulate ghrelin mRNA expression and to increase ghrelin generation and secretion in the stomach (37).

Glucose and/or insulin have been suggested as important elements in the mechanisms regulating plasma ghrelin levels. Hyperinsulenic-euglycemic clamp studies demonstrated that insulin suppresses endogenous ghrelin release in humans (15, 48) and rats (40). This suggests that insulin, rather than glucose, is essential for the suppression of ghrelin levels.

Postprandial ghrelin remains increased in Type 1 diabetes when the patients do not receive insulin therapy (43). Ex vivo studies, using isolated, perfused rat stomach, showed that ghrelin secretion from the stomach was suppressed by insulin infusion (27, 35), indicating that insulin regulates circulating ghrelin levels. STZ destroys β-cells of the pancreas and reduces insulin secretion (25). Plasma insulin concentration is significantly reduced 2 wk after STZ injection, compared with that of vehicle-injected rats (24). Ghrelin is also known to inhibit glucose-induced insulin release (10). It is conceivable that the hyperghrelinemia observed in STZ rats may further reduce insulin secretion.

It is unclear whether high plasma levels of ghrelin and accelerated gastric emptying observed in the early stages of diabetes are due to hypoinsulinemia. Nowak et al. (46) previously showed that delayed gastric emptying in STZ-induced diabetic rats was improved by insulin treatments. Further studies are required to determine whether insulin replacement in STZ rats is sufficient to reverse accelerated gastric emptying, increase antropyloric activity, and elevate plasma ghrelin levels.

It has been shown that fasting and postprandial blood glucose levels were 178 and 314 mg/dl, respectively, when 65 mg/kg of STZ were administered in rats (42). Similarly, others demonstrated that fasting blood glucose levels were 210 mg/dl after STZ (65 mg/kg) injection (26). In our study, we used STZ at 50 mg/kg to induce a mild form of diabetes. As shown in Fig. 2, the fasting and postprandial blood glucose levels were 112 and 257 mg/dl, respectively, in rats treated with STZ (50 mg/kg). Body weight was not significantly increased 2 wk after STZ (65 mg/kg) injection (24, 26). In contrast, when STZ was applied at a dosage of 50 mg/kg, rats could gain their body weight (40 g) 2 wk after STZ injection in our experiment (Fig. 1).

As far as we know, this is the first study to demonstrate that accelerated gastric emptying occurs in an early stage of diabetes in rats. Use of this animal model may contribute to a better understanding of the mechanism by which diabetic hyperphagia, which is frequently observed in patients in the early stages of diabetes, contributes to accelerated gastric emptying.

In the early stages of diabetes, accelerated solid gastric emptying could make it easier to overeat, which is well recognized for exacerbating diabetes. If we could successfully control the hyperphagia and accelerated gastric emptying in the early stages of diabetes, this could possibly prevent and/or delay the development of gastroparesis and/or neuropathy, two complications that develop later in diabetes.

GRANTS
This study was supported in part by Veterans Affairs Merit Review (T. Takahashi).

REFERENCES


