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Leptin Inhibits Gastric Emptying in Rats: Role of CCK Receptors and Vagal Afferent Fibers

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Summary

Leptin regulates energy homeostasis and body weight by balancing energy intake and expenditure. It was recently reported that leptin, released into the gut lumen during the cephalic phase of gastric secretion, is capable of initiating intestinal nutrient absorption. Vagal afferent neurons also express receptors for both CCK and leptin, which are believed to interact in controlling food intake. The present study was undertaken to investigate the central and peripheral effects of leptin on gastric emptying rate. Under anesthesia, male Sprague-Dawley rats (250-300 g) were fitted with gastric Gregory cannulas (n=12) and some had additional cerebroventricular cannulas inserted into their right lateral ventricles. Following recovery, the rate of gastric emptying of saline (300 mOsm/kg H_2O) was determined after instillation into the gastric fistula (3 ml, 37 °C, containing phenol red, 60 mg/l as a non-absorbable dilution marker). Gastric emptying rate was determined from the volume and phenol red concentrations recovered after 5 min. Leptin, injected intraperitoneally (ip; 10, 30, 60, 100 μg/kg) or intracerebroventricularly (icv; 5, 15 μg/rat) 15 min before the emptying, delayed gastric emptying rate of saline at the dose of 30 μg/kg or 15 μg/rat ($p<0.001$). When CCK_1 receptor blocker L-364,718 (1 mg/kg, ip), CCK_2 receptor blocker L-365,260 (1 mg/kg, ip) or adrenergic ganglion blocker bretylium tosylate (15 mg/kg, ip) was administered 15 min before ip leptin (30 μg/kg) injections, leptin-induced delay in gastric emptying was abolished only by the CCK_1 receptor blocker ($p<0.001$). However, the inhibitory effect of central leptin on gastric emptying was reversed by adrenergic blockade, but not by either CCK antagonists. Our results demonstrated that leptin delays gastric emptying. The peripheral effect of leptin on gastric motility appears to be mediated by CCK_1 receptors, suggesting the release of CCK and the involvement of vagal afferent fibers. On the other hand, the central effect of leptin on gastric emptying is likely to be mediated by adrenergic neurons. These results indicate the existence of a functional interaction between leptin and CCK receptors leading to inhibition of gastric emptying and short-term suppression of food intake, providing an additional feedback control in producing satiety.

Key words

Capsaicin-sensitive vagal fibers • CCK • Adrenergic • Gastric emptying • Leptin

Introduction

Leptin, the protein product of the *ob* gene, is an adipose tissue derived circulating hormone, which serves as a feedback signal for body weight regulation and energy balance (Zhang *et al.* 1994, Frederich *et al.* 1995,

Campfield *et al.* 1995) through its receptors (Ob-Rs) in the brain and in the peripheral tissues (Mercer *et al.* 1996). Although it is synthesized and secreted predominantly by the adipose tissue, leptin and leptin receptor isoforms have been reported in a wide variety of human and rodent tissues (Tartaglia *et al.* 1995, Hoggard *et al.* 1997, Mix *et al.* 1999, Hardwick *et al*. 2001), suggesting that leptin may have additional peripheral actions other than body fat mass regulation. Defects in the leptin signaling system results in obesity in rodents (Zhang *et al.* 1994), while the central or peripheral administration of leptin reduces food intake (Flynn *et al.* 1998, Holzer 2002). Since it is well known that alterations in food intake are associated with changes in gastric emptying (Moran and McHugh 1982), it is likely that leptin suppresses gastric emptying. However, despite previous observations, which demonstrated the inhibitory effect of leptin on gastric emptying (Smedh *et al.* 1998, Martinez *et al.* 1999), Barrachina *et al.* (1997a,b) have reported that the food intake-reducing effect of leptin was not always accompanied by delayed gastric emptying.

Previous studies have shown that exogenous leptin synergistically interacts with cholecystokinin (CCK) to increase vagal afferent activities (Wang *et al.* 1997, Gaige *et al.* 2002) and leads to suppression of food intake (Wang *et al.* 1998, Matson *et al.* 2000), which is mediated by CCK_1 receptors and capsaicin-sensitive afferents (Barrachina *et al.* 1997a, b). There is a large body of evidence demonstrating that CCK potently reduces the size of the meal (Ritter *et al.* 1999) and contributes to the regulation of body weight (Matson and Ritter 1999, Matson *et al.* 2000). Moreover, rats lacking the CCK_1 receptors are hyperphagic and obese (Moran *et al.* 1998). In addition to their synergistic interaction in reducing food intake, it is also reported that both CCK and leptin afford similar gastroprotective (Konturek *et al.* 1998) and anti-inflammatory activities (Bozkurt *et al.* 2003). Plasma leptin levels were also shown to be elevated by exogenous CCK administration (Konturek *et al.* 1998) or *vice versa* (Gaige *et al.* 2002).

Factors that yield the changes in gastric emptying are commonly associated with the control of food intake. Although previous studies have shown the gastric inhibitory effect of leptin, which accompanies its anorexic effect, the mechanisms through which leptin affects gastric emptying remain to be determined. Thus, the present study was undertaken to assess the effect of centrally or peripherally administered leptin on gastric emptying rate. Since CCK and leptin have synergistic effects in several functions, our aim was also to identify the involvement of specific CCK receptors and vagal afferent fibers in the action of leptin on gastric emptying.

Methods

Animals

Adult male Sprague-Dawley rats weighing 250-300 g were housed individually in a light- and temperature-controlled room on a 12:12-h light-dark cycle, where the temperature (22±2 °C) and relative humidity (65-70 %) were kept constant. The animals were fed a standard pellet lab chow, which was withdrawn overnight before preparative surgery and emptying experiments, but access to water was allowed *ad libitum*. Experiments were approved by the Marmara University School of Medicine Animal Care and Use Committee.

Surgery

Rats were anesthetized by intraperitoneal (ip) injection of a mixture of ketamine (100 mg/kg) and chloropromazine (0.75 mg/kg) and aseptically prepared for abdominal surgery. A small stainless steel Gregory cannula was installed in the corpus as previously described (Dimaline *et al.* 1986). The cannula was exteriorized through a midline stab incision and the paramedian incision was closed in layers. Rats were housed individually and allowed to recover for 2-3 weeks before the experiments were commenced. During this time, rats were accustomed to light restraint in Bollman cages for 1-2 h every day.

Three weeks after the implantation of the gastric cannula, a group of rats were anesthetized (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine, ip) and placed on a stereotaxic instrument (Stoelting Lab standard stereotaxic instrument). The rats were fitted with stainless steel cerebroventricular guide cannulas (22-gauge; Plastic Products, Roanoke, VA) inserted into the right lateral cerebral ventricle (1.1 mm caudal and 1.5 mm lateral to the bregma, 3.2 mm ventral to the surface of the skull) according to the atlas of Paxinos and Watson (1986). The cannula was held in place by dental acrylic cement anchored around three stainless steel screws. Three days were allowed before starting the emptying experiments. After each experiment, correct placement of the cannula was verified by injection of methylene blue and brain section.

Measurement of gastric emptying

Prior to experiments, rats were fasted overnight and then placed in Bollman cages, the gastric cannulas were opened, the gastric contents were flushed gently with warm physiological saline (0.9 % NaCl, 37 °C) and the stomach was allowed to drain freely for 45 min. The rate of gastric emptying of saline (0.9 % NaCl, 300 mOsm/kg) was examined using methods described previously (Green *et al.* 1988). Physiological saline (3 ml) containing phenol red (PR; 60 mg/l) as a nonabsorbable marker was instilled into the gastric cannula, and the gastric emptying rate (ml/ 5 min) was determined from the volume and phenol red concentrations recovered from the cannula 5 min after instillation of saline. Phenol red concentration was determined spectrophotometrically from the absorbances read at 550 nm, as described by Debas *et al.* (1975). Gastric emptying (E; ml/5 min) is calculated from the absorbances (A1: absorbance of instilled solution; A2: absorbance of collected fluid) and volumes (V1: volume of instilled solution; V2: volume of collected fluid) according to the following formula:

$$E = [(V1 \times A1) - (V2 \times A2)] / (A1+A2)$$

Vagal afferent denervation with capsaicin

In order to study the involvement of vagal afferent fibers, on the day of the gastric cannula placement, a group of rats had local application of capsaicin on the vagal nerves. Rats were anesthetized and pretreated with atropine sulfate (2 mg/kg, ip) to decrease the acute effects of capsaicin on the respiratory and cardiovascular systems. A 1 % solution of capsaicin (Sigma) or vehicle (10 % Tween 80 in oil, sham-denervation) was applied on each vagus nerve in turn for 30 min. The total dose of capsaicin applied in each rat did not exceed 1 mg. Following the application, the area was rinsed with sterile saline. Animals were used in the emptying experiments 3 weeks after the capsaicin treatment. The efficacy of perivagal capsaicin treatment was assessed previously by the sulfated CCK satiety test (Mazelin *et al.* 1998).

Drugs

Leptin (Sigma, St. Louis, MO) was dissolved in 0.1 % (w/v) bovine serum albumin (BSA, Sigma) and aliquots were stored at –20 °C until use. Leptin or vehicle (BSA) was administered intraperitoneally (ip; 10, 30, 60, 100 μg/kg) or intracerebroventricularly (icv; 5, 15 μg/rat) 15 min before performing gastric emptying studies. CCK_1 receptor antagonist L-364,718 (1 mg/kg, a generous gift from ML Laboratories, PLC, London, UK) and CCK_2 receptor antagonist L-365,260 (1 mg/kg, ML Laboratories) were freshly prepared in 3.3 % dimethyl sulfoxide (DMSO, Sigma), while the sympathetic ganglion blocker bretylium tosylate (15 mg/kg; American Reagent Laboratories) was prepared in saline. CCK antagonists, sympathetic ganglion blocker bretylium tosylate or saline were given ip 15 min before leptin injections. Doses of the antagonists were chosen depending on the previous reports, in which they were found to be effective in reversing the gastric effects of the agonists. Previously, we have found that the gastric emptying rate in saline- or DMSO-treated rats does not differ.

Statistical analysis

The results are expressed as means ± S.E.M. with 6 rats per group. Instat statistical package (GraphPad Software, San Diego, CA, USA) was used. Following the assurance of normal distribution of data, one-way analysis of variance (ANOVA) was used for multiple comparisons and Student's t-test was used to evaluate the level of statistical significance between two groups. Differences were considered statistically significant if $p<0.05$.

Results

In control rats with a gastric fistula, the emptying of saline (3.05±0.04 ml/5 min) was rapid and similar to that described previously (Forster *et al.* 1991). Intraperitoneal administration of leptin resulted in significant ($p<0.001$) inhibition of saline emptying at only the 30 μg/kg dose (2.41±0.07 ml/ 5 min) (Fig. 1), whereas neither the lower (10 μg/kg) nor the higher doses (60 and 100 μg/kg) affected saline emptying. To investigate the participating mechanisms in leptin-induced delay in gastric emptying, the subsequent emptying experiments were continued with the effective dose (30 μg/kg). On the other hand, cerebroventricular administration of leptin at 15 μg/rat dose significantly (2.05±0.07 ml/ 5 min; $p<0.001$) delayed gastric emptying of saline.

Peripheral administration of CCK_1 receptor antagonist, CCK_2 receptor antagonist, sympathetic ganglion blocker or the respective vehicles did not influence the gastric emptying rate of saline (data not shown). CCK_2 receptor antagonist significantly reversed

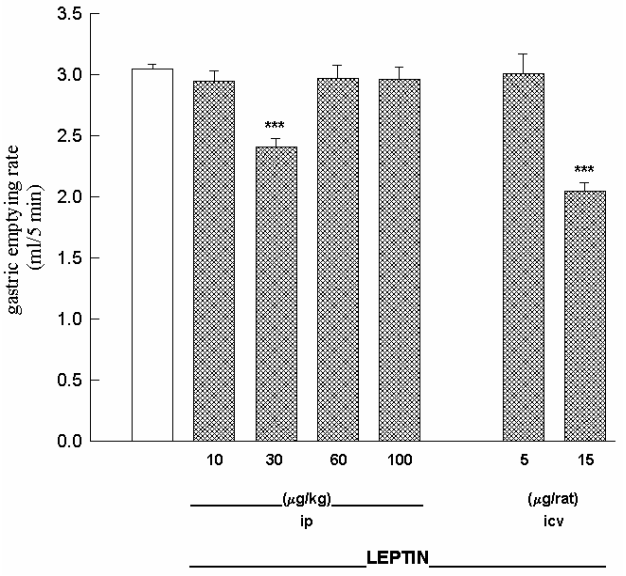

Fig. 1. Effects of intraperitoneal (ip) or intracerebroventricular (icv) administration of leptin on gastric emptying rate of saline (0.9 % NaCl, 300 mOsm/kg) in rats with gastric cannulas. ***p<0.001; compared to control group treated with vehicle (bovine serum albumin). Each group consists of 6 animals.

(2.71±0.09 ml/5 min; p<0.05), while CCK_1 receptor antagonist abolished (3.16±0.19 ml/5 min; p<0.001) the suppression of gastric emptying rate induced by intraperitoneal leptin administration (Fig. 2a). Topical capsaicin application, which was performed to clarify the role of vagal afferent fibers in the peripheral action of leptin, reversed the inhibitory effect of ip leptin significantly (2.75 ± 0.05 ml/5 min; p<0.01) (Fig. 2a). On the other hand, perivagal capsaicin had no significant effect on saline emptying in BSA-treated rats (data not shown).

Neither CCK_1- nor CCK_2-receptor antagonist influenced the delayed gastric emptying rate induced by icv leptin (Fig. 2b). Blockade of the sympathetic ganglia had no significant effect on the inhibitory role of peripherally administered leptin, while the delayed gastric emptying rate induced by icv leptin was significantly reversed (2.55±0.17 ml/5 min; p<0.01) by the sympathetic ganglion blocker.

Discussion

There is evidence of synergistic interaction between peripheral leptin and CCK in the short-term modulation of food intake, taking place at the level of vagal afferent terminals (Barrachina *et al.* 1997a, b). This short-term interaction was apparent following a low dose of peripheral leptin, which may act *via* leptin-sensitive

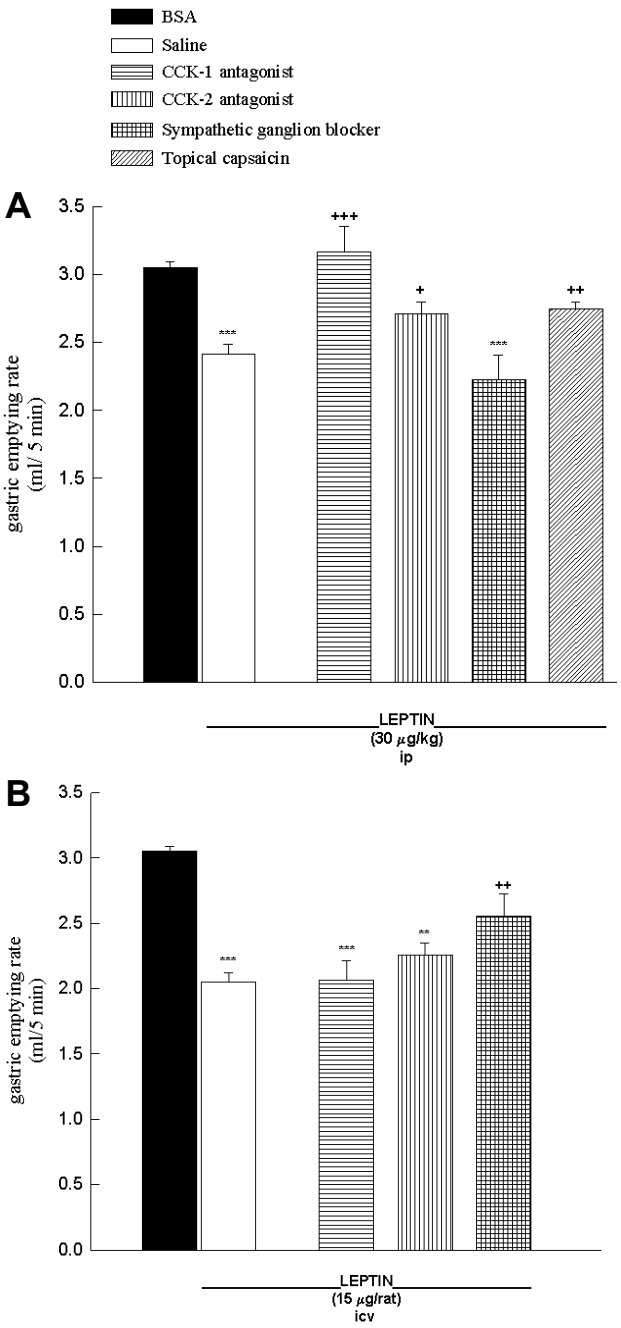

Fig. 2. Effects of **(A)** intraperitoneal (ip) or **(B)** intracerebroventricular (icv) administration of leptin in rats pretreated with CCK_1 receptor antagonist (L-364,718; 1 mg/kg) or CCK_2 receptor antagonist (L-365,260; 1 mg/kg) or sympathetic ganglion blocker (bretylium tosylate; 15 mg/kg) on gastric emptying rate of saline. Topical capsaicin was applied to clarify the role of vagal afferent fibers in the peripheral action of leptin (ip). **p<0.01, ***p<0.001; compared to control group which received bovine serum albumin (BSA). +p<0.05, ++p<0.01, +++p<0.001; compared to saline + leptin-treated group. Each group consisted of 6 animals.

vagal afferents to trigger enhanced neuronal activity in the hypothalamus (Wang *et al.* 1997). In accordance with these studies that characterize the role of CCK in the control of gastric emptying and food intake, current data

implicate that peripheral administration of leptin may activate the release of CCK from intestinal endocrine cells and initiate a vago-vagal inhibition of the gastric emptying through the CCK_1 receptors. On the contrary, the central administration of leptin delays gastric emptying *via* the activation of adrenergic receptors. However, immunohistochemical studies and tracing methods may be used to elucidate the potential neurohumoral interactions between leptin and CCK in the control of gastric functions.

Central or peripheral administration of leptin reverses the obesity syndrome found in *ob/ob* mice, stimulates the metabolic rate, and reduces food intake in lean mice and rats (Pelleymounter *et al.* 1995). Leptin could alter food intake by reducing the appetitive value of food or by inhibiting gastric emptying rate. In accordance with this hypothesis, several other peptides including CCK, calcitonin gene-related peptide and corticotropin-releasing peptide, which are known to decrease food intake, also inhibit gastric transit in rodents (Morley and Levine 1982, Morley *et al.* 1996, Sheldon *et al.* 1990). On the other hand, in the rats with ventromedial hypothalamic lesion, overeating and obesity were found to be associated with rapid gastric transit (Duggan and Booth 1986). Similarly, it was recently demonstrated that leptin-deficient obese mice have an increased proximal intestinal transit, but an overall decrease in small intestinal transit (Kiely *et al.* 2005b). However, Barrachina *et al.* (1997b) have reported that a single intraperitoneal injection of leptin (120 μg/kg) reduced food intake without a concomitant alteration in the gastric emptying of a solid nutrient meal. On the other hand, the results of the present study demonstrated that one-quarter of that systemic dose, as well as icv administration of leptin, inhibited the gastric emptying rate of a liquid, non-nutrient content, while the higher doses (60 and 100 μg/kg) were not effective on gastric emptying rate. As observed in the actions of many peptides, it appears that leptin has a bell-shaped effect on the gastric emptying rate. It may be suggested that the emptying mechanisms involved in nutrient-rich and non-nutrient gastric contents are different and leptin acting as a satiety signal, may be affected with the caloric content of the meal. However, the results suggest that leptin when given at a physiological dose is effective in the inhibition of gastric emptying rate, while the higher anorexogenic doses have no effect on the emptying of either caloric or non-caloric gastric contents.

Previously, it was shown that intracerebroventricularly administered leptin inhibits feeding by reducing meal size, but it is not due to a depression of feeding behavior or due to an impairment in the rat's capacity to start a meal (Flynn *et al.* 1998). In the present study, central leptin administration inhibited gastric emptying rate, implicating a possible role of leptin in controlling meal size. Since leptin decreases appetite and increases basal metabolism (Halaas *et al.* 1995), the inhibitory effect on gastric emptying rate in this study would be consistent with those two functions in monitoring body weight and composition. Similarly, it was shown that leptin has a rapid inhibitory effect on sugar absorption (Lostao *et al.* 1998). In the human stomach, it was reported that vagal stimulation rapidly increases leptin secretion during the cephalic phase of gastric secretion and luminal leptin may be involved in nutrient absorption (Sobhani *et al.* 2002). This means that the inhibitory effect of leptin on food intake and body weight may involve decreased efficiency in the absorption of ingested nutrients with a concomitant reduction in the propogation of luminal contents to the absorptive surfaces.

Leptin-specific receptors have been found to exist in the sympathetic prevertebral ganglionic neurons and in afferent and efferent vagal neurons in rats (Miller *et al.* 1999, Buyse *et al.* 2001). When leptin was injected following CCK administration in cats, the small intestinal electromyographic activity was stimulated, while leptin had no effect on this activity in the absence of CCK (Gaige *et al.* 2003a,b). These findings strongly suggest that the mechanisms whereby leptin along with CCK enhances intestinal motility involve the vago-vagal reflex. Wang *et al.* (1997) have speculated that after a meal, type 2 gastric vagal afferent (GVA) terminals require CCK to be responsive to circulating leptin and to generate satiety signals. On the other hand, type 1 GVA terminals respond to circulating leptin without an interaction with CCK and provide a feedback signal for the maintenance of body weight, which appears to be a long-term effect. Therefore, the synergistic effects of CCK and leptin in the modulation of food intake and small intestinal motility are mediated by vagal afferent fibers. In accordance with these studies, the present data confirm that gastric emptying rate is governed by vagal afferent activity. Previous studies have shown the synergistic effects by administering these peptides exogenously. However, the results of the present study implicate that leptin, when administered in relatively lower doses than those used in studying synergistic interaction, acts *via* the

CCK receptors that involve the activation of capsaicin-sensitive vagal afferent fibers. Thus, leptin-induced delay in gastric emptying *via* the vagal CCK receptors may constitute a short-term regulatory mechanism that reduces the meal size. This short-term synergy may be mediated by an enhancement of CCK signaling *via* the vagus in the presence of increased extracellular leptin (Wang *et al.* 1997). Moreover, Kiely *et al.* (2005a) have reported a synergistic action of cholecystokinin and leptin in the regulation of small intestinal motility in leptin-deficient obese mice.

Since the leptin-CCK synergy is common in both body weight regulation and gastric emptying, it is likely that similar centers participate in the integration of these functions. It is possible that leptin-initiated vagal stimuli to the NTS may modify gastric motility and gastric emptying, while providing advance signals for body weight regulation. However, the peripherally administered leptin appears to act through a vago-vagal pathway to inhibit gastric emptying *via* CCK receptors, whereas the inhibitory effect of central leptin on gastric emptying is mediated by sympathetic fibers. Broberger *et al.* (1998) have reported that the parabrachial nucleus (PBN) in the pons is a potential site for the synergistic interaction between leptin and CCK for integration of satiety signals (Raybould *et al.* 1998, Tartaglia *et al.* 1995). Projections from NTS to the dorsal motor vagal nucleus (DMN), the nucleus ambiguus, and the inter-mediolateral column of the spinal cord form the basis for the vago-vagal and vago-spinal reflexes in the control of gastric fundus tone and antral motility (Rutecki 1990). However, the present data demonstrate that CCK signaling is not essential for the gastric inhibitory effect of centrally administered leptin, while projections from CCK-activated centers may control the gastric motility.

Our results further elucidate the potential interactions between leptin and CCK in the control of gastric functions through the activation of vagal afferent fibers that relay information to brain sites. These results indicate the existence of a functional interaction between leptin and CCK receptors leading to inhibition of gastric emptying and short-term suppression of food intake, providing additional feedback control in producing satiety.

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