Duodenal myotomy blocks reduction of meal size and prolongation of intermeal interval by cholecystokinin

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A B S T R A C T

We have shown that vagotomy (VGX) attenuates the reduction of meal size (MS) produced by cholecystokinin (CCK) -8 and -33 and that celiaco-mesenteric ganglionectomy (CMGX) attenuates the prolongation of the intermeal interval (IMI) produced by CCK-33. Here, we report the following novel data. First, by determining the distribution of CCK1 receptor messenger RNA, which mediates reduction of MS and prolongation of IMI by CCK, in seven regions of the gastrointestinal tract in the adult rat we found that the duodenum contains the highest concentration of this receptor in the gut. Second, based on the previous finding we performed a unique surgical technique known as duodenal myotomy (MYO), which severs all the nerves of the gut wall in the duodenum including vagus, splanchnic and enteric nerves. Third, we determined MS and IMI in duodenal MYO rats in responses to endogenous CCK-58 released by the non-nutrient, trypsin inhibitor, camostat and CCK-8 to test the possibility that the duodenum is the site of action for reduction of MS and prolongation of IMI. We found that, similar to the previous work reported by using CCK-8 and MS, duodenal MYO also blocked reduction of MS by camostat. Forth, duodenal MYO blocked prolongation of IMI by camostat. As such, our current results suggest that the duodenum is the gut site that communicates both feeding signals of endogenous CCK, MS and IMI, with the brain through vagal and splanchnic afferents.

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1. Introduction

Cholecystokinin (CCK) is a gut-brain peptide secreted by the enteroendocrine I cells of the small intestine [1–3] and evokes responses consistent with a role in the short-term control of food intake: satiation (reduction of meal size, MS) and satiety (prolongation of intermeal interval, IMI) [4,5]. Such responses by CCK are mediated by the CCK1 receptors [5–7] and through vagal and splanchnic afferents [7–10].

Reduction of cumulative food intake by CCK-8 and fatty acids, which releases endogenous CCK, was attenuated by selective [9,11–15] or total subdiaphragmatic vagotomy [8]. In addition, we have recently shown that reduction of MS by CCK-8 and -33 was attenuated by total subdiaphragmatic vagotomy [10], whereas prolongation of the IMI by CCK-33 was shortened by removing the celiaco-mesenteric ganglia [10]. These data provide evidence that the feeding responses evoked by exogenous CCK require differential neuronal pathways. However, they do not provide insight into the specific gut area that communicates these feeding signals, MS and IMI, by endogenous CCK to the central feeding control locations in the dorsal vagal complex (DVC) of the hindbrain.

The reasons are as follows: (1) The vagus and splanchnic nerves supply the majority of the gastrointestinal (GI) tract [16–18]; therefore, total subdiaphragmatic vagotomy (VGX) and celiaco-mesenteric ganglionectomy (CMGX) or splanchnicectomy (SPX), which we performed in our previous study [10], do not provide knowledge about the specific gut site for these neuronal pathways; (2) The enteroendocrine I cells that are the major source of endogenous CCK in the periphery are also distributed along the whole length of the small intestine [1–3]. As such, all regions of the small intestine that contain these cells (such as the duodenum, the jejunum and the ileum) are possible gut sites for the communication of the feeding signals of CCK with the DVC; (3) Cholecystokinin, receptor expression, which mediates reduction of food intake by CCK, can be found in the previous regions of the small intestine [19–22]. Thus, any of those regions that contain this receptor are likely gut sites for the communication of the satiation and satiety signals of CCK with the DVC; and finally (4), while all of the previous studies have focused on identifying the pathways by which CCK communicates the satiation signal to the brain [11,23–26], no report has investigated the site of the gut that communicates the satiety signal, or IMI, of CCK with the DVC.

There are multiple studies which have shown that the site of action for CCK-8 and fatty acids, which release endogenous CCK, to evoke reduction of cumulative food intake is the upper gastrointestinal tract, particularly the duodenum [11,23–27]. However, the gut...
site of action for endogenous CCK-58, the only endocrine form of CCK in the rat [28] which is secreted in response to the non-nutrient, trypsin inhibitor camostat [28,29], and evokes reduction of MS and prolongation of the IMI [5,30–32] is yet to be determined.

In the current study, we investigated if the duodenum is the primary site in the gut that communicates these feeding responses. MS and IMI [5,10,33–35] in response to endogenous CCK released by an orogastric gavage of the non-nutrient trypsin inhibitor camostat (200 mg/kg [5,7,36]) and in response to CCK-8 (1 nmol/kg, i.p. [34,35,37]) in duodenally myotomized (MYO) rats. Several previous studies from our laboratory have shown that these doses reduce MS through camostat and CCK-8 and prolong the IMI through camostat [5,10,33–35].

Duodenal MYO (Fig. 1) is a unique and a challenging surgical procedure performed by introducing six adjacent and complete circumferential incisions in the duodenum immediately following the pyloric sphincter. These incisions sever all the layers of the duodenal wall except the mucosa and submucosa. In addition, this procedure severs all of the neuronal elements in the duodenal wall, including the enteric nervous system (ENS) [38], vagal and sympathetic/spinal afferents. The choice of the surgical site for our duodenal MYO was based on the distribution of CCK1 receptor gene expression in seven regions of the gut. In our study, this receptor, which mediates reduction of food intake by CCK, was mainly distributed in the upper duodenum at the level of and immediately following the pyloric sphincter [11,23–27].

2. Materials and methods

The Tuskegee University Animal Care and Use Committee approved the animal protocols for this study. Adult male Sprague Dawley rats weighing between 250 and 300 g and housed in clear cages, allowing for the complete visualization necessary for behavioral rating, were used in this study. The rats lived in a controlled environment (12-h dark/12-h light cycle; lights off at 1800 h, at 21.5 °C, with ad libitum water and pelleted rodent chow, Teklad, WI).

To habituate the rats to the laboratory environment and the experimental design, each rat was weighed and handled for 10 min every day at the same time each day and was then given an intraperitoneal (i.p.) injection of saline and gavaged with 3.5 ml double distilled water (ddH2O), followed by a 10% sucrose solution, for 120 min in addition to chow and water. All injections consisted of a volume of 0.5 ml, and all gavage solutions consisted of a volume of 3.5 ml and were given at 0700 h (at 1 h into the beginning of the light cycle) to allow for the necessary preparation of solutions and needed materials before the experiment.

2.1. Distribution of CCK1 receptor

Ten rats were sacrificed with an overdose of sodium pentobarbital (100 mg/kg, i.p.). Approximately 200-ml-long intestinal segments were collected from each rat from the following areas of the intestine: a 1 cm² piece of the antrum of the stomach, 0.5 cm aboral from the pyloric sphincter (duodenum), 20 cm aboral from the same point (jejunum), 5–6 cm oral from the cecum (ileum), 1 cm of the cecal body, 5 cm from the cecum aborally (colon) and 1 cm² from the terminal part of the gastrointestinal tract (rectum).

Total RNA was isolated using the TRIzol method (Invitrogen-Life Technologies, Inc., Carlsbad, CA) [19,22] according to the manufacturer’s protocol, and the total RNA concentration was determined by ultraviolet absorbance at 260 nm (DU640, Beckman Coulter, Fullerton, CA). RNA from each sample was assessed for purity by determining the A260/280 ratios (ratios of 1.5 to 2 were used), and the integrity of each sample was assessed by samples produced on 2% agarose gels stained with ethidium bromide. All RNA samples were treated with RNase-free DNase (Ambion, CA) to remove residual DNA. Samples were selected based on the bright staining of the 18 s and 28 s ribosomal bands, with the latter showing twice the concentration of the former. First-strand cDNA was synthesized from 2 μg total RNA using Reaction Ready™ First Strand cDNA Synthesis (Super Array Bioscience Corporation, Frederick, MD).

mRNA levels were measured using RT-PCR in a 25 μl reaction mixture containing 12.5 μl RT2 Real-Time SYBR/Fluorescein Green PCR master mix, 1 μl first strand cDNA, 1 μl RT2 validated PCR primer sets for CCK1 and CCK2 (Super Array Bioscience Corporation) and 10.5 μl PCR-grade water (Ambion Inc). Samples were run in 96-well PCR plates (Bio-Rad, Hercules, CA) in duplicate, and the results were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPD) housekeeping gene. The amplification protocol was set at 95 °C for 15 min, followed by 40 cycles each at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a melting curve determination between 55 °C and 95 °C to ensure the detection of a single PCR product. A complete account of this protocol has been described in our previous work [19,22].

2.2. Myotomy

Two groups of rats (n = 7 per group) underwent duodenal myotomy (MYO) or sham surgery under general anesthesia through a ventral midline celiotomy incision. The anesthesia mixture (0.01 mg/kg intramuscularly; i.m.) prepared in our laboratory contained 5.0 ml of ketaset (100 mg/ml), 2.5 ml of Rompun® (xylazine 20 mg/ml), 1.0 ml of acepromazine maleate® (10 mg/ml) and 1.5 ml saline. The abdominal wall was prepared surgically by clipping and cleaning with betadine solution and alcohol swabs, and a ventral midline celiotomy was performed following the absence of a pedal reflex.

The stomach and small intestine were exposed, and using micro dissection scissors, six circumferential cuts (3–5 mm apart) were
performed under 40× magnification starting from the pyloric sphincter. The cuts were made through the serosa and the outer longitudinal and inner circular muscle layers. The submucosal layer of the intestine remained intact (Fig. 1). Sham surgery was performed through the manipulation of the intestine without performing any cuts.

The muscles of the abdominal wall in all rats were closed using polydioxanone II (4–0) absorbable suture material, and the skin was closed using surgical staples. Postoperative care included Metacam® [Meloxicam® (1.1 mg/kg)] subcutaneously for pain control and Baytril® [Enrofloxacin® (0.05 mg/kg)] intramuscularly as an anti-inflammatory. The drugs were given in the three days immediately following the surgeries, and all rats were allowed seven days for recovery before performing any food intake experiments. The criteria for complete recovery following surgery included the absence of clinical signs (e.g., pain and lethargy) and the return of food intake to baseline levels. During the recovery time, all rats received cookies, regular rat chow and liquid nutritional supplements (Ensure nutritional shake, vanilla and strawberry flavors, 30 ml/day).

The verification of MYO was performed immediately following the end of the experiment by staining the removed gut portion with H&E. A segment of the duodenum stained with hematoxylin and eosin showing the myotomy site (red line). As shown, the surgical site lacks the serosa, the two muscle layers and the two enteric plexuses, the myenteric and submucosal. Slide magnification is 200 ×, bar = 200 μm.

2.3. Food intake

Following a complete recovery, a baseline of food intake for each animal was determined as previously described [5,34,35,37]. Briefly, rats were deprived of food but not water at 1700 h. At 0700 h the following morning, 1 h into the light cycle, all rats received an orogastric gavage of 3.5 ml double distilled water (ddH2O) and an i.p. injection of saline. Immediately following the gavage and injection, rats were presented with a 10% sucrose solution in a separate container along with their drinking water. We chose a sucrose solution because it is a palatable test solution that has been used extensively in the literature.

Two trained examiners rated the min-to-min behaviors of the rats (as described in our early work [5,34,35,37]) and recorded the food intake of the rats immediately following the treatments. The behaviors observed included feeding (licking the sucrose bottle spout), licking (licking the water bottle), biting, grooming, locomotion (walking around the cage), rearing up (front paws on side of cage while hind paws on the floor of cage), standing (only hind paws touching floor of cage), sniffi ng and stretching and resting (no movement by the animal). The total test period lasted 120 min. Chow was returned immediately after the end of the second meal.

Based on our previous reports [5,34,35,37], a meal was determined to have started immediately following gavage/injection and the presentation of sucrose, and was considered to be terminated following the recording of three consecutive one-minute observations without eating. The intermeal interval (IMI) was recorded immediately following the end of the first meal and was considered to begin at the end of the first minute, not the third minute (the third resting period), and was considered to persist until the beginning of food consumption in the second meal. The agreement rate between the examiners exceeded 98%. The examiners measured the sizes of the first two meals and the duration between them to determine the IMI. This feeding test was repeated 7–10 times during a two-week period until the intake of each rat was stable. The actual treatments with camostat and CCK-8 were begun following the determination of this baseline.

Again, all rats were fasted as described above. The next morning, the rats were given an orogastric gavage of either camostat mesilate (200 mg/kg) or ddH2O, or only an i.p. injection of either CCK-8 (1 nmol/kg) or saline on the odd-numbered days and a ddH2O gavage or saline injection on the even-numbered days. One day was reserved for the maintenance of the cages. Treatments were rotated between the animals until all the animals had received all of the treatments, ensuring that each animal served as a control for itself. Following the gavages/injections, the intake of 10% sucrose and behavioral ratings were recorded for a total of 120 min as described above. This procedure had also been used previously in other experiments [5,34,35,37].

2.4. Statistical analysis

The relative difference in receptor expression was compared between the groups using a modification of the delta-delta-CT method (ΔΔ CT) described previously [39]. Receptor gene expression was considered significant if the increase or decrease in expression was equal to or more than twice that of the expression of the housekeeping gene (the control genes in pancreas and the brain).

The meal data were analyzed by two-way repeated measures analyses of variance (ANOVA), with the two independent variables being procedure (MYO) and treatment (camostat, CCK-8, ddH2O and saline). Multiple comparisons were performed using a Holm–Sidak test (SigmaStat for Windows version 3.11, Systat system Software, Inc., 2004). All results were displayed as mean ± SEM, and data were considered significant if p < 0.05.

3. Results

3.1. Distribution of CCK1 receptor in the gut of adult rats

As expected, in adult rats the pancreas expressed CCK1 receptor. All sections of the gastrointestinal tract also contained CCK1 receptor (Fig. 3). The relative expression of CCK1 receptor compared to the baseline was duodenum > jejunum > stomach > Cecum > rectum > colon > ileum (Fig. 4).

Following analysis of the data for the 120 min period, which contained four meals and three intermeal intervals, and as we have shown in our previous work [5], we found that camostat and CCK-8 affected only the size of the first meal and the duration of the first IMI. Therefore, we focused our data presentation here on these two measurements.

3.2. Effect of MYO with camostat and cholecystokinin-8 on meal size

There was no significant effect produced by the myotomy (F (1, 4) = 0.521, p = 0.510). There was a significant effect produced by the treatment (F (3, 12) = 7.587, p = 0.004), and there was no interaction between the procedure and treatment (F (3, 12) = 2.844, p = 0.082). In sham rats, camostat and CCK-8 reduced MS relative to ddH2O (p = 0.001) and saline (p < 0.001), respectively (Fig. 5).
3.3. Effect of MYO with camostat and cholecystokinin-8 on length of IMI

There was no significant effect of the procedure (F (1, 4) = 2.507, p = 0.189). There was a significant effect of the treatment (F (3, 12) = 10.453, p = 0.001), and there was an interaction between procedure and treatment (F (3, 12) = 3.529, p = 0.049). In sham rats, camostat prolonged the length of IMI relative to ddH$_2$O (p<0.001) (Fig. 5), but CCK-8 failed to affect IMI relative to saline (p=0.935). In myotomized rats, camostat failed to affect the length of IMI relative to ddH$_2$O (p=0.298) (Fig. 6).

4. Discussion

The current work provided four novel findings. First, among seven regions along the gastrointestinal tract of the adult rat, the duodenum contained the highest distribution of CCK$_1$ receptor, which mediates reduction of MS and prolongation of IMI by CCK. Second, duodenal MYO is a unique surgical procedure which can be utilized to sever the vagus, the splanchnic and the enteric neurons in any chosen region in the gastrointestinal tract. This procedure can be used to localize the site of action for any gut peptide, especially those involved in satiety and satiation. Third, duodenal MYO blocked reduction of MS by camostat, a potent releaser of endogenous CCK or CCK-58 [28-29], the only endocrine form of CCK in the rat [28]. Forth, duodenal MYO blocked prolongation of IMI by camostat. As such, our results support three conclusions. First, the upper duodenum immediately after the pyloric sphincter containing vagal, spinal and enteric neurons that individually or collectively communicate the feeding signals of CCK, MS and IMI, with the DVC. Second, duodenal MYO blocked reduction of MS by camostat, a unique surgical technique and endogenous CCK, our study confirms the MS finding demonstrated by others [11,23-26] that the duodenum is the potential site of action for CCK to reduce MS.

In addition, the current study shows that the duodenum not only communicates the satiation signal of CCK but also communicates the satiety signal of this peptide, the IMI. This is the study is the first in the literature that provides such a conclusion. Along with...
our previous data \[10\], which found that the IML is mediated by an intact splanchnic nerve, the current study suggests that the splanchnic afferents in the duodenum may be the main communicators of the prolongation of the CCK-produced IMI feeding signal with the DVC.

Other locations in the gut with CCK1 receptor, receptor and vagal and spinal afferents such as the jejunum and, to a lesser extent, the ileum may also be locations for CCK to communicate the MS and IML signals to the DVC. Single or multiple myotomy surgeries in these locations of the gut are required to examine this possibility.

The fact that only camostat, not CCK-8, prolonged IMI is predicted because the long forms of CCK, e.g., CCK-33, and −58 (which is released by camostat) stay in circulation longer due to their slower hepatic clearance rate than CCK-8 [57−59] and protect tertiary structure against circulating peptides. Therefore, it is expected that camostat will act for a longer duration than CCK-8 [5,7,34,35,60,61].

Duodenal MYO is a unique surgical procedure that indiscriminately disrupts three neuronal elements in the wall of the duodenum: vagus, splanchnic and enteric neurons (Fig. 1). An examination of the role of each of these nerves locally to evaluate their roles in the reduction of MS and the prolongation of IML by CCK (although potentially complicated) is therefore still required. Without such studies, our current data suggest only that all three nerves could be equally important in evoking the feeding responses by CCK. We have previously shown that total subdiaphragmatic VXG attenuates the reduction of MS by CCK-8, whereas CMGX/SPX attenuates the prolongation of IML by the longer form of CCK or CCK-33 [10]. The current work found that duodenal MYO blocked both reduction of MS by CCK-8 and by camostat, which releases endogenous CCK or CCK-58 [28] and produces the prolongation of IML by camostat.

The vagus and the splanchnic nerves are potential carriers of the satiation and the satiety signals of CCK from the gut to feeding areas in the DVC such as the area postrema (AP), the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMV). These nerves are located in close proximity to the endocrine I cells of the gut that secrete CCK. They have CCK receptors that mediate the reduction of food intake by CCK, and they can all be activated by CCK. In addition, because MYO severs all innervations in the wall of the duodenum, including vagal afferents, sympathetic/ spinal afferents and myenteric and submucosal plexuses (Fig 1), the results of the current study may also point at a possible role for the ENS in the reduction of MS and the prolongation of IML by CCK. This hypothesis, however, requires testing.

The present study provided anatomical data about the distribution of CCK receptor gene expression in seven regions of the gut in adult rats. We found that the CCK1 receptor is present mainly in the duodenum > jejunum > stomach. Such data, along with previously published activation data \[19,55,62\], indicate that the surgical site for duodenal MYO was appropriate. Furthermore, the CCK receptor distribution data will serve future studies for the purpose of examining the role of these receptors in reducing MS and prolongation of IML by CCK in various parts of the gut. Consistent with the role of CCK in the short-term control of food intake, the localization of the CCK1 receptor in the upper small intestine (duodenum > jejunum > ileum) follows the natural distribution of the endocrine I cells that secrete CCK and mirrors the high density of enteric [19,22], sympathetic and spinal vagal afferents [40] in the gut. These nerves may communicate the satiate and satiation signals of CCK from the gut to the DVC [63,64] individually or collectively.

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