Atropine-resistant secretion of a putative luminal CCK-releasing peptide in conscious rats

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1Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173; 2The First Department of Biochemistry, Fukuoka University, Fukuoka 814-01; 3The First Department of Pathology, Kurume University, Kurume 830; and 4Division of Gastroenterology, National Kyushu Cancer Center, Fukuoka 815, Japan

Miyasaka, Kyoko, Kayoko Tateishi, Masao Masuda, Atsuo Jimi, and Akihiro Funakoshi. Atropine-resistant secretion of a putative luminal CCK-releasing peptide in conscious rats. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G287–G292, 1999.—The changes in levels of the newly discovered luminal CCK-releasing factor (LCRF) in the small intestinal lumen before and after bile-pancreatic juice diversion in conscious rats were examined by a specific RIA. Moreover, we also examined whether LCRF secretion was under cholinergic control. Anti-LCRF antiserum was raised in rabbits, and a sensitive RIA was established. The localization of LCRF was examined by immunohistochemistry. The luminal content of LCRF was significantly increased by bile-pancreatic juice diversion, during which luminal trypsin activity was eliminated. The increase in luminal LCRF content was not inhibited by intravenous infusion of atropine. The changes in plasma levels of CCK and pancreatic secretions were similar to those in luminal LCRF contents. LCRF immunostaining was observed in villus tip enterocytes of the small intestine and was most prominent in the duodenal portion. These results support our original hypothesis that LCRF may be released spontaneously into the small intestinal lumen from the villus tip enterocytes and its intraluminal degradation by proteases regulates CCK release. Furthermore, LCRF release was not subject to cholinergic regulation.

PANCREATIC EXOCRINE secretion in conscious rats is mainly regulated by protease activities in the proximal intestine (luminal feedback regulation) (3); intraduodenal proteases are inhibitory, while ingestion of protease inhibitors or diversion of bile-pancreatic juice stimulates pancreatic enzyme secretion. This increase is mediated by release of CCK (1, 10, 20). Moreover, we reported (20) that exclusion of both bile and pancreatic juice is a strong stimulant of CCK release and pancreatic protein secretion in conscious rats.

To explain the mechanism by which intestinal trypsin inhibits pancreatic secretion and CCK release, we hypothesized the presence of a trypsin-sensitive polypeptide (CCK-releasing peptide (CCK-RP)), which is considered to be spontaneously secreted by the small intestine into the intestinal lumen. This CCK-RP was hypothesized to stimulate CCK release and pancreatic secretion subsequent to its secretion into the lumen (14). This hypothesis was supported by our subsequent study (16) and a report by Lu et al. (11). Recently, Spannagel et al. (22) purified CCK-RP from rat intestinal perfusate and named it luminal CCK-releasing factor (LCRF). This peptide is composed of 70- to 75-amino-acid residues and has a molecular mass of 8,136 Da, and the NH2-terminal 41 residues were determined.

We synthesized the LCRF NH2-terminal fragment-(1—35) and examined its stimulatory effect on pancreatic exocrine secretion in conscious rats (12). We confirmed that intraduodenal injection of this LCRF-fragment rapidly increased pancreatic fluid and protein secretion (12). In the present study, we established a specific RIA for LCRF to clarify its physiological role. Using this method, we examined whether LCRF was secreted into the lumen. LCRF immunoreactivity in the duodenal mucosa was also measured, and the localization of LCRF was determined by immunohistochemistry. Whether a cholinergic mechanism is involved in luminal feedback regulation is controversial. Guan et al. (4, 5) observed no contribution of a cholinergic mechanism to pancreatic hypersecretion produced by bile-pancreatic juice diversion, whereas Lu et al. (11) reported that the release of CCK-RP was inhibited by atropine. Therefore, we examined whether the secretion of LCRF into the lumen was inhibited by atropine.

MATERIALS AND METHODS

LCRF Antiserum

Synthetic rat LCRF-(1—35) was conjugated with keyhole-limpet hemocyanin using 3-maleimidobenzoic acid N-hydroxysuccinimide ester, according to the previously described procedure (12). Rabbits were immunized with conjugate (100 µg as peptide) emulsified in complete Freund's adjuvant (6, 25). Booster injections of the same dose of conjugate in incomplete Freund's adjuvant were administered at 2-wk intervals beginning 3 wk after the first immunization. The antiserum used for this study was obtained 10 days after the eighth immunization.

Animal Preparation

Male Wistar rats (330–346 g body wt) were obtained from Shizuoka Jikkenn Dobutsu (Shizuoka, Japan). Animals were fed commercial rat chow (CRF-1, Oriental, Tokyo, Japan) before surgery and during recovery.

The operating procedures have been described in detail previously (17, 19). Briefly, a midline abdominal incision was made under enflurane anesthesia (Abbott, North Chicago, IL) delivered through a plastic face mask by means of a vaporizer. The cannulas used in this study were Silastic medical grade tubing (Dow-Corning, Midland, MI; ID, 0.025 in.; OD, 0.037 in.). Separate cannulas were introduced for draining bile and pancreatic juice along with a duodenal cannula and a right external jugular vein cannula. After the operation, the rats...
were placed in modified Bollman-type restraint cages and had free access to food and water in a room at 24°C with filtered air and light from 0500 through 1700. Bile and pancreatic juice were returned continuously to the intestine via the duodenal cannula.

**Experimental Design**

Bile and pancreatic juice were collected separately for a 30-min period, and the volume of pancreatic juice was measured with a Hamilton syringe. Samples of 20 µl of pancreatic juice were used to determine protein concentration, and the rest was mixed with the bile and infused into the duodenum with a syringe pump (compact infusion pump, Harvard Apparatus, Southnatick, MA) over the next 30 min. Bile and pancreatic juice were diverted from the intestine after a basal collection period of 90 min with return of bile and pancreatic juice, during which 0.05 M NaHCO₃ solution was infused at a rate of 1 ml/h for 4 h. The animals were killed before bile-pancreatic juice diversion and 2 or 4 h after the diversion. Samples of 5–6 ml of blood were withdrawn through the venous cannula, and the animals were killed. The proximal quarter of the small intestine was removed and washed with 10 ml of ice-chilled distilled water. The intestinal contents were immediately frozen and lyophilized for subsequent LCRF RIA. Blood samples were collected in ice-chilled EDTA-containing tubes and immediately centrifuged at 4°C at 3,000 rpm for 15 min. The plasma samples were stored at -70°C until subsequent LCRF and CCK assays.

To examine the effects of atropine, we started intravenous infusion of 100 µg·kg⁻¹·h⁻¹ of atropine 60 min before bile-pancreatic juice diversion, as previously reported (13). The changes in protein secretion, plasma CCK levels, and LCRF contents were examined.

Protein concentration in pancreatic juice was determined by measuring the optical density at 280 nm (9) of samples diluted 200-fold with 0.04 M Tris buffer, pH 7.8.

**Immunohistochemistry**

Three segments of small intestinal tissues (2 cm length) at 10, 45, and 70 cm from the pylorus were removed, fixed in 10% Formalin, and embedded in paraffin. Sections 5-µm thick were incubated with antiserum to LCRF-(1—35) at dilutions of 1:500 to 1:1,000 and stained by the avidin-biotin peroxidase complex method. Control sections were treated with specific antiserum preabsorbed with LCRF-(1—35) (final dilution of antiserum, 10 µg/ml).

**Assays**

RIA of LCRF. ¹²⁵I-labeled LCRF-(1—35) was prepared by the chloramine-T method and purified by Sephadex G-10 chromatography and diethyl aminoethyl-ion exchange chromatography (eluted with a gradient of 0–1 M NaCl in 0.01 M imidazole buffer, pH 7.5). Standard LCRF-(1—35) and samples were incubated with antiserum (R601; final dilution, 1:40,000) for 48 h at 4°C in a total volume of 500 µl of 0.01 M phosphate buffer (pH 7.4) containing 0.5% BSA, 0.025 M EDTA, 0.14 M NaCl, 0.05% (vol/vol) Tween 20, and 0.01% sodium azide. Next, 0.1 µl of tracer (−6,000 cpm) was added, and incubation was continued for 48 h at 4°C. The bound and unbound peptides were separated by adding goat-anti-rabbit IgG. Synthetic LCRF-(1—35) for use as a standard was prepared according to the net peptide content determined by amino acid analysis.

The antiserum used in this study reacted 100% with LCRF-(1—35) but not with rat monitor peptide (identical to rat pancreatic secretory trypsin inhibitor-61 (PSTI-61)) (8, 18), PSTI-56, human PSTI, or rat and porcine diazepam-binding inhibitor (DBI) (<0.001) (7). PSTI-61 and porcine DBI were reported to elicit CCK release (7, 8, 18). We could not check the cross-reactivity with native LCRF, since the full sequence of native LCRF is not available. The sensitivity of the assay was 15.6 fmol/ml, and both intra- and interassay coefficients of variation were within 10%. Dilution curves of plasma extract, tissue extract, and perfusate samples paralleled that of synthetic LCRF-(1—35) (Fig. 1).

Preparation and extraction of basal intestinal wash, intestinal mucosal tissues, and plasma samples. The samples of intestinal wash obtained before bile-pancreatic juice diversion (basal) contained high protease activities. As proteases digested antibody in the RIA system resulting in high values, proteases had to be excluded immediately. The intestinal wash (10 ml of ice cold water) was boiled at 96–100°C for 15 min, followed by overnight dialysis (using the membrane, cutoff level mol wt 1,000) at 4°C, centrifuged at 3,000 rpm at 4°C, and then lyophilized. We examined whether this extraction procedure affected the LCRF radioimmunoactivity itself by comparing the results of samples obtained after 2 h of bile-pancreatic juice diversion with or without extraction. This extraction procedure did not significantly affect LCRF radioimmunoactivity (t = 1.52, P > 0.15, n = 5 and 7 for with and without extraction, respectively). Therefore, samples obtained 2 and 4 h after bile-pancreatic juice diversion were directly assayed without extraction.

To measure tissue content of LCRF, we killed four rats by decapitation, and the proximal small intestine (20 cm) was removed, washed with 10 ml of cold distilled water, and opened longitudinally and the intestinal mucosa was removed by gently scraping the surface with a spatula. LCRF was extracted from the intestinal mucosa. Briefly, samples were homogenized with ice-cold distilled water for 1 min and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatants were used for LCRF measurement.

Plasma samples of 2–3 ml were obtained from each rat, and two 3-ml or three 2-ml plasma samples from different rats were combined into one 6-ml sample for LCRF measurement. LCRF was extracted from plasma samples by adsorption onto C-18 (Mega Bond Elut C18 Varian Sample Preparations Products). The recovery of LCRF-(1—35) added to pooled plasma was 90.6 ± 1.8% (n = 4).

These samples were stable and give similar LCRF immuno-reactivities with repeated RIA determinations.
RIA of CCK. Plasma CCK extracted by adsorption on Sep-Pak cartridges (Waters, MA) was measured by RIA as described previously (2, 6, 19, 24, 25), using antiserum OAL-656 and CCK-8 as a standard.

Statistics

Values are expressed as means ± SE. To analyze the results of pancreatic secretion, we performed multiple ANOVA followed by Newman-Keuls multiple comparison test with respect to time and treatment. Increases in protein secretion with or without atropine treatment were compared by Student’s t-test. P < 0.05 was considered to be significant.

RESULTS

Changes in Protein Secretion, Plasma LCRF and CCK Levels, and LCRF Contents in Rats Without Atropine

The exclusion of bile-pancreatic juice significantly increased pancreatic fluid and protein secretion as previously reported (19, 20). The results of protein secretion are shown in Fig. 2. The protein secretion peaked at 1–2 h after diversion and decreased thereafter, then remained at a level twofold higher than the basal value.

Plasma CCK levels also significantly increased and peaked 2 h after bile-pancreatic juice diversion, decreasing slightly thereafter (Fig. 3A). The luminal LCRF contents also increased significantly after bile-pancreatic juice diversion, and the changes were similar to those in CCK levels (Fig. 3B). The plasma LCRF levels were very low (4.0–5.4 fmol/ml) and were not affected by bile-pancreatic juice diversion.

Effects of Atropine

Intravenous infusion of atropine significantly decreased basal (bile-pancreatic juice return) protein secretion. However, the secretion in response to bile-pancreatic juice diversion was markedly increased, as previously reported (13) (Fig. 2). The integrated increase in protein secretion during the 4 h of bile-pancreatic juice diversion [(the value of protein secretion during 4 h of bile-pancreatic juice diversion minus protein secretion during 0.5 h before bile-pancreatic juice diversion) × 8] was 68.3 ± 7.5 mg (n = 8) in rats without atropine vs. 82.8 ± 17.5 mg (n = 5) in rats with atropine. The difference was not significant (t = 1.27, P > 0.2).

The plasma CCK levels and luminal LCRF contents were also increased significantly by bile-pancreatic juice diversion in rats treated with atropine (Fig. 3). There was no significant difference between rats with and without atropine treatment.
Immunohistochemistry and Tissue Contents of LCRF

LCRF immunoreactivity was strongest in enterocytes at the tips of villi of the proximal small intestine (Fig. 4A). Although luminal mucus strands contained LCRF immunoreactivity, proximal enterocytes, goblet cells, and enteroendocrine cells were negative for LCRF immunoreactivity (Fig. 4B).

Tissue contents of LCRF in the small intestine were 418.4 ± 78.8 fmol/g wet wt (n = 4).

DISCUSSION

We raised antiserum specific to LCRF, established a specific RIA, and measured luminal LCRF content before and after bile-pancreatic juice diversion in rats. Furthermore, we examined the localization of LCRF in the small intestine by immunostaining and quantified its immunoreactivity in the small intestine. LCRF immunoreactivity in the small intestinal lumen increased after bile-pancreatic juice diversion in conscious rats. LCRF content peaked during the 2 h after bile-pancreatic juice diversion, and these changes paralleled those in plasma CCK levels. These changes also paralleled the protein output (19, 20), although the plasma LCRF levels were very low and did not change during bile-pancreatic juice diversion.

LCRF immunostaining was observed in the villus tips of the small intestinal mucosa but not in enteroendocrine cells, although we could not verify the origin of this LCRF. Moreover, the staining was stronger in the proximal than the distal intestine. This evidence is in accordance with the previous report (21) that luminal feedback regulation was only operative in the proximal small intestine. Taken together, these observations suggested that LCRF is secreted from the proximal small intestine to the lumen, but not to the circulation, and has a physiological role in CCK release and pancreatic protein secretion during bile-pancreatic juice diversion. Although immunohistochemical staining was recently observed in the myenteric plexus of the duodenum (23), neurons were not stained in our study. As our antiserum was raised against part of the NH2-terminal fragment, these observations might not be necessarily specific to LCRF, and further examinations are therefore necessary.

Intravenous infusion of atropine did not diminish luminal LCRF content, CCK release, or protein secre-

Fig. 4. Immunohistochemical localization of LCRF in duodenum. Representative section of duodenal villus cut longitudinally showing LCRF immunoreactivity (dark brown) in villus tip enterocytes (A) but not proximal enterocytes (B). Duodenal villus cut longitudinally where antiserum was preabsorbed with LCRF-(1—35). Note absence of specific staining in C compared with A. Original magnification: A and C, ×200; B, ×50.
tion produced by bile-pancreatic juice diversion but tended to enhance the responses to bile-pancreatic juice diversion. Thus we clearly showed that the release of LCRF is atropine resistant. Moreover, the changes in plasma CCK levels and the increases in protein output during 4 h of bile-pancreatic juice diversion were not different with or without atropine. The present results support those of the previous studies by Guan et al. (4, 5) in which the luminal feedback regulation in conscious rats was shown to be cholinergic independent. On the other hand, Lu et al. (11) reported that the CCK-releasing activity of intestinal perfusates obtained from donor rats treated with atropine was eliminated and that its secretion was cholinergic dependent. These different observations might be due to differences in experimental conditions between the studies. We (17) and Guan et al. (4, 5) analyzed animals after complete recovery from abdominal surgery, whereas Lu et al. (11) examined acutely anesthetized animals. Because bile-pancreatic juice diversion is a strong and long-lasting stimulator of CCK release and pancreatic protein secretion (20), anesthetized animals might be more susceptible to severe experimental conditions and/or to atropine treatment. Alternatively, because the CCK-RP proposed by Lu et al. (11) has not been purified, it is not known whether this peptide is the same as LCRF. As our RIA system did not show cross-reactivity with another candidate of CCK-RP, DBI (7), interaction of LCRF with this protein is unknown.

The maximal protein response was obtained by intraduodenal injection of 30 pmol of LCRF fragment (12). However, the luminal content of LCRF measured by RIA was <100 fmol. There are two possible explanations for this discrepancy: 1) LCRF fragment-(1—35) may be less potent than natural LCRF (22) or 2) when the bioactivity of LCRF was examined, a physiological dose of bile acid (taurocholate) was infused into the duodenum to reproduce physiological conditions, although pancreatic enzymes were eliminated (12). Thus, because luminal bile acid prevents CCK release directly and indirectly (19), larger doses of LCRF might have been required for the bioassay.

Feedback regulation of CCK release manifested by dietary protease inhibitors or intact protein was proposed to be mediated by both PSTI-61 (monitor peptide) in the pancreatic juice and LCRF (8, 12, 18, 22). The essential mechanism of action of these CCK-RPs is the same; when the luminal protease activity decreases below the threshold (15) by ingestion of trypsin inhibitors or bile-pancreatic juice diversion, CCK-RPs survive and elicit CCK release (Fig. 5). Exclusion of bile-pancreatic juice from the intestine is a strong stimulator of pancreatic secretion associated with a decrease in luminal trypsin activity in conscious rats (20). Nevertheless, PSTI-61 (monitor peptide) cannot be responsible for CCK release produced by bile-pancreatic juice diversion, because PSTI-61 was excluded from the intestinal lumen and PSTI-61 is not present in intestinal secretions (4). Therefore, LCRF may be more effective as a CCK-RP than PSTI-61.

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