Transcytosis of gastric leptin through the rat duodenal mucosa

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Submitted 11 June 2007; accepted in final form 29 July 2007

Cammisotto PG, Gingras D, Bendayan M. Transcytosis of gastric leptin through the rat duodenal mucosa. Am J Physiol Gastrointest Liver Physiol 293: G773–G779, 2007. First published August 2, 2007; doi:10.1152/ajpgi.00260.2007.—Leptin is secreted into the gastric juice by epithelial Chief cells and reaches the duodenum in a biologically intact active form. We assessed the possibility that this gastric leptin crosses the intestinal mucosa by transcytosis through enterocytes to reach blood circulation. Endogenous gastric leptin secretion was triggered by cholinergic stimulation. In another set of experiments, recombinant leptin was inserted in vivo into the duodenal lumen. Plasma levels of leptin were assessed by enzyme immunoassay and Western blot, and duodenal tissue was processed for immunocytochemistry. We first observed that leptin was found inside duodenal enterocytes from fed rats but not inside those from fasted ones. Stimulation of gastric secretion by a cholinergic agent led to rapid increases in plasma leptin levels (202 ± 39%) except when the pylorus was clamped. Insertion of recombinant leptin into the duodenal lumen raised plasma leptin concentrations (558 ± 34%) quite rapidly, whereas carrier solution without leptin had no effect. The use of FITC-tagged leptin reinforced these results. Light and electron microscopy revealed the cellular compartments involved in its transcytosis, namely, the enterocyte microvilli, the endocytic vesicles, the Golgi complex, and the basolateral interdigitations. Leptin was also present in the lamina propria, in capillary endothelial cell plasmalemmal vesicles, and in capillary lumina. These results demonstrate that gastric exocrine leptin is internalized by duodenal enterocytes and delivered to the lamina propria and blood circulation. It may thus be able to play important paracrine and endocrine functions for the control of gastric emptying and nutrient absorption.

LEPTIN IS ONE OF THE MAJOR discoveries of the 1990s. This hormone, first discovered as a secretory product of white adipose tissue, brought an answer to the problem of lipostatic control of gastric emptying and nutrient absorption, mucosa renewal, and immunity, therefore participating in gut homeostasis (10, 23, 28, 29, 32).

Once gastric leptin has played its role on the enterocyte, one may expect degradation. However, two series of experiments led us to propose a challenging hypothesis. First, it was reported that refeeding fasted rats leads to rapid and significant increases in blood leptin levels that cannot be explained by adipose tissue secretion (4, 11). Second, as previously demonstrated by our group, large proteins such as orally administered insulin or endogenous pancreatic amylase and different lipases present in intestinal lumen are internalized by the intestinal epithelium, transcytosed, and delivered to the blood in their intact and biological active forms (7, 8, 9, 15, 36, 37). We therefore put forward the hypothesis that gastric leptin, once in the intestinal lumen, could cross the intestinal mucosa to reach the blood. Using FITC-tagged leptin, we unraveled the transcytotic pathway taken by leptin to reach the bloodstream on internalization by enterocytes.

MATERIALS AND METHODS

Antibodies. Polyclonal rabbit antibody against leptin (Ob A20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-FITC was from Dako (Glostrup, Denmark). Secondary antibodies anti-rabbit IgG linked to tetramethyl rhodamine isothiocyanate was from Miles-Yeda (Rehovot, Israel). Antibody anti-rabbit IgG bonded to FITC was from Chemicon International (Temecula, CA). Protein A-gold (10-nm gold particles) was prepared as previously described (24).

Animals. Five-week-old male Sprague-Dawley rats (mean body mass of 127 ± 8 g) were purchased from Charles River (St. Constant, Quebec, Canada). Before our experiments were conducted, all protocols were approved by the Comité de Déontologie de l’Expérimentation sur les Animaux de l’Université de Montréal (Deontology Committee on Animal Experimentation from the University of Montreal). Animal experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Animals were kept in individual cages at 24°C with a 12:12-h light-dark cycle. Standard Purina chow and water were provided ad libitum. Rats were fasted overnight.

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Stimulation of gastric leptin secretion. Animals were anesthetized with a single intraperitoneal injection of urethane (1 g/kg body wt). Stimulation of gastric leptin secretion was carried out by an injection of carbachol (12 mg/kg carbachol; Sigma-Aldrich, Oakville, ON, Canada). Controls received saline. Blood was sampled at 0, 15, and 30 min and processed for plasma leptin measurements.

Preparation of leptin-FITC. Leptin (1 mg; Sigma-Aldrich) was incubated overnight with FITC (50 μg; Dakopatts) in 300 μl of NaHCO3 buffer (50 mM; pH 9) at 4°C as previously reported (8). The following day, nonbound FITC was removed by dialysis in PBS (pH 7.4) overnight at room temperature. Leptin-FITC was finally diluted to 1 mg/ml in a buffer containing sodium cholate (10 mg/ml), NaHCO3 (5 mM), and protease inhibitor cocktail Complete Mini (1 pill for 10 ml; Roche Diagnostics, Laval, Quebec, Canada).

Duodenal insertion of leptin. Leptin (1 mg) was dissolved in a buffer of the following composition: 10 mg/ml sodium cholate, 5 mM NaHCO3, and protease inhibitor cocktail Complete Mini. After urethane anesthesia, the duodenum was clamped below the pyloric sphincter and a duodenal portion upstream was removed for microscopy (time 0). In parallel, blood was sampled from the dorsal aorta. Leptin or leptin-FITC (400 μg) was then inserted inside the duodenal lumen. Control animals had insertion of carrier solution without leptin. Another clamp was installed downstream to the first one, while taking care to avoid swelling of the tissue. In between, the two clamps delineated a chamber of ~5 cm (2 in.) long. Blood and duodenal tissues were sampled at 15 and 30 min after the onset of insertion. Blood was collected in tubes containing EDTA, left 30 min at 4°C, and centrifuged. Serum was kept at ~80°C until use. Duodenal tissues were processed for light and electron microscopy.

Light microscopy. Duodenal tissues were fixed in Bouin fixative and embedded in paraffin tissue. Sections 5 μm thick were mounted on SuperFrost slides (Fisher Scientific, Montreal, Quebec, Canada) and processed for immunohistochemistry. After deparaffinization, sections were washed twice with PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4), incubated with pepsin (1 mg/ml in 0.01 M HCl) for 5 min, washed with PBS, and then covered with 1% ovalbumin. Incubation with antibodies (anti-leptin 1/50, anti-FITC 1/250 in PBS) was carried out overnight at 4°C. Sections were then washed, incubated with the secondary antibodies for 1 h (anti-rabbit 1/250 or anti-goat 1/200 in PBS), washed with PBS, and mounted with 1% 1,4-diazabicyclo[2.2.2]octane (DABCO) in 50% glycerol. Examination was performed with a LEITZ DMRB fluorescence microscope (Leica, St. Laurent, QC, Canada). Control of specificity was carried out by omitting the primary antibody. For immunoperoxidase, the protocol was similar except that samples were treated with H2O2 (1% in methanol) to quench endogenous peroxidase activity. Staining was delineated a chamber of

Western blots. Plasma samples were heated at 95°C for 5 min in loading buffer (Laemmli) and then resolved on a 10% polyacrylamide gel. After transfer at 4°C, nitrocellulose membrane were saturated in 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 containing 1% nonfat dry milk. Incubation with the primary antibody (anti-leptin 1/200, anti-FITC 1/1,000) was carried out overnight at 4°C with agitation. Proteins were revealed with a Lumi-light kit (Lumi-Light Plus, Roche Diagnostics).

Leptin measurement. Plasma leptin concentrations were determined with an enzyme immunoassay test available from SpiBio (Montigny le Bretonneux, France). This test is a double-antibody sandwich technique, using biotin-tagged polyclonal anti-rat leptin antibody and streptavidin-horseradish peroxidase tracer. 3,3’,5,5’-Tetramethylbenzidine (TMB) reagent forms a yellow compound, which emits at 450 nm. Results are reported to a standard curve drawn under the same conditions with purified rat leptin. Sensitivity of the assay is 50 pg leptin/ml (15-μl sample size).

RESULTS

Presence of leptin in duodenum of fasted and fed rats. After food intake, leptin from Chief cells is discharged into the gastric juice and reaches the duodenum where it binds its membrane-bound receptors present on the microvilli (10). Indeed, duodenal villi from fasted rats present no significant staining for leptin (Fig. 2B), whereas those from fed rats show some staining at the surface and within some enterocytes (Fig. 1C). Omitting the antibody resulted in absence of staining (Fig. 1A).

Increases in plasma leptin after carbachol treatment. To determine whether gastric leptin may cross the intestinal wall, rats were separated into two groups of four animals each. For one of the groups, the pyloric sphincter was kept open, whereas the animals of the second group had a clamp closing it. Animals were treated with carbachol, a nonspecific secretagogue that triggers secretion of leptin by the gastric mucosa without affecting leptin secretion from white adipose tissue (3, 4). Blood was sampled before carbachol injection (time 0) and 30 min after. At time 0, plasma leptin levels were in the 2–3 ng/ml range, which are normal values for fasted rats (13). In animals with a closed pylorus, leptin levels remained stable at 30 min (90 ± 6% of value for time 0; Fig. 2A) and duodenal enterocytes yielded no cytochemical signal for leptin (Fig. 2B). On the other hand, when the pyloric sphincter was left open, carbachol treatment led to an increase in blood leptin (202 ± 39% of value for time 0; Fig. 2A) and to a leptin staining on villi (Fig. 2C). Controls with saline injection displayed no change in leptin blood levels and no leptin staining in enterocytes (not shown). This suggests that gastric leptin crosses the pylorus to get absorbed by the duodenal mucosa and reaches the blood.

Leptin blood levels increase after leptin duodenal insertion. To confirm this observation, recombinant rat leptin or carrier solution (control) was inserted inside a duodenal loop. For each animal (n = 4 for each group), blood was sampled before leptin or carrier solution insertion (time 0) and 30 min after leptin insertion. Plasma leptin concentrations (2–3 ng/ml) at time 0 were similar for all animals and for controls and are

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in accordance with previous reports (13). Insertion of leptin into the duodenal lumen led to a sixfold increase (558 ± 39% of time 0) in plasma leptin concentration (from 2.60 ± 0.57 to 17.8 ± 2.68 ng/ml) at 15 min, whereas controls with carrier solution alone displayed no changes (94.6 ± 1.8% of time 0) (Fig. 3). Results at 30 min were similar to those at 15 min (19.0 ± 4.75 ng/ml, 762 ± 53% of time 0) (Fig. 3).

Insertion of tagged leptin into the duodenum. To further confirm that increases in blood leptin arise from leptin inserted into the intestinal lumen, recombinant leptin was tagged with FITC. Leptin-FITC purity was qualitatively confirmed by Western blot using antibodies against leptin and FITC (Fig. 4A). Leptin-FITC was then inserted in the duodenal lumen. Blood was sampled at time 0 and at 15 and 30 min. By Western blot on blood samples, using the antibody against leptin, we had a single band at 16 kDa. This band was stronger at 15 and 30 min (Fig. 4B), confirming the results obtained with

Fig. 1. Leptin in duodenal tissues. Immunoperoxidase staining for leptin was carried out on duodenum from fasted and fed rats. No significant staining is shown in tissues of control animals (omission of the primary antibody; A) or in tissues of fasted rats (B). Dark staining is found in some enterocytes of the fed rat tissue (C). Bar = 50 μm.

Fig. 2. Effect of carbachol injection on blood leptin levels. Blood was sampled at time 0 (t0) and 30 min (t30) after carbachol injection. At 30 min, with closed pylorus, no increase in circulating leptin (90 ± 6%, n = 4; A) and no cytochemical positive staining (B) are observed in enterocytes. With the pylorus kept open, plasma leptin increases (202 ± 39%, n = 4; A) and leptin staining is present (C) inside duodenal enterocytes. Bars = 50 μm.
leptin insertion (Fig. 3). Incubation of the membrane with an antibody against FITC revealed the appearance of leptin-FITC in blood only 15 min after insertion (Fig. 4B). Intensities of the bands remained similar at 30 min. These results further indicate that the increase in plasma leptin concentration originates from intestinal absorption of luminal leptin. Furthermore, the molecular weight of the transcytosed leptin-FITC was the same as that of leptin, indicating that the complex was not degraded during its transit through enterocytes.

Pathway followed by leptin through enterocytes. To determine the pathway taken by leptin to cross the intestinal mucosa, duodenal tissue was sampled before and after insertion of leptin-FITC and processed for light and electron microscopy. At time 0, a faint signal was detected for endogenous leptin, but no signal was detected for FITC (Fig. 5). At 15 min, stainings for FITC and for leptin were strong in enterocytes (Fig. 5). It is worth indicating that significant stainings for FITC and for leptin were mainly found at the apical portion of intestinal villi but not deep in the crypt (results not shown). At 30 min, stainings for both leptin and FITC were clearly visible inside the enterocytes and in the lamina propria (Fig. 5). Immunogold labeling further revealed the cellular structures involved in leptin-FITC transport. Cytoskeletal controls consisting of samples with primary antibody omitted displayed no labeling (Fig. 6A). At 30 min, anti-leptin (Fig. 6, B and C) and anti-FITC (Fig. 6D–F) antibodies combined to protein A-gold revealed the presence of FITC-leptin on enterocyte microvilli (Fig. 6B), in apical small endocytotic vesicles (Fig. 6B), at the basolateral membrane interdigitations (Fig. 6, B–D), and within Golgi cisternae (Fig. 6E). Junctional complexes appear devoid of labeling (Fig. 6C). Leptin-FITC was also found at the level of the lamina propria, in endothelial cell plasmalemmal vesicles of blood capillaries (Fig. 6F), and in capillary lumina.

DISCUSSION

Leptin found in the lumen of the digestive tract originates from gastric epithelial Chief cells, from salivary glands, and (in children) from maternal milk (10, 18, 31). In gastric juice, gastric leptin resists the harsh conditions thanks to a binding protein that prevents proteolysis (12). It reaches the duodenum in an intact form and binds its receptors expressed on the apical membrane of enterocytes (10). Luminal leptin is involved in nutrient absorption and mucosa renewal and possibly in enteric immunity (10, 23, 28, 29, 32). Leptin is also present in blood, originating mainly from white adipose tissue (35). Of particular interest is the fact that plasma leptin levels rise significantly at the beginning of meals. This rise cannot originate from white adipose tissue (35). Observations led us to investigate an appealing hypothesis that exocrine-secreted gastric leptin may cross the intestinal barrier and account for rapid increases in blood leptin. We have previously reported several data on the transcytotic pathway...
across enterocytes that allows for large pancreatic proteins to cross the intestinal wall (9, 15, 36). We herein present evidence that leptin follows a similar transepithelial pathway. This is of prime importance for the significant role leptin plays in processing of food intake, nutrient absorption, and energy storage.

In a first experiment, we have shown that endogenous gastric leptin crosses the duodenal epithelial barrier. Indeed, stimulation of gastric leptin secretion by carbachol led to significant increases in plasma leptin levels only when the pylori sphincter was kept open. White adipose tissue is not affected by carbachol in the short term (3) and therefore could not contribute to such a rapid rise of plasma leptin. However, we do not know whether the few scattered leptin endocrine cells reported in the gastric mucosa (10) are sensitive to carbachol. Their participation in the quick rise of leptin levels appears however unlikely because closing the pylori prevented the rise in leptin triggered by carbachol.

In a second set of experiments, leptin was inserted in the duodenal loop; after 15 min, plasma leptin levels potently increased. Controls with carrier solution did not have such effect. Eventually, in a third set of experiments, FITC-tagged leptin was inserted into the duodenal lumen leading to the appearance of the intact leptin-FITC complex in the blood after 15 min. Similar to results previously reported for other peptides and proteins (8, 9, 15, 36, 37), leptin inserted in the intestinal lumen led to significant increases in blood levels and reached plateau values.

Subsequently, we examined the pathway taken by leptin to cross the duodenal mucosa to reach the bloodstream. Light microscopy revealed a time-dependent internalization of leptin-FITC through the duodenal epithelium. At the ultrastructural level, leptin was found to be associated with enterocyte microvilli, in endocytotic vesicles and the Golgi apparatus, and at the level of the basolateral membrane. Leptin-FITC was also found at the level of capillary endothelial cells associated to plasmalemmal vesicles as well as in capillary lumina. Such internalization appears to be quite rapid because all structures were significantly labeled already at 15 min. These results are similar to those described previously for pancreatic proteins (9, 15, 36). Paracellular entrance appears to be excluded or not significant, as we did not observe any labeling for leptin in the junctional complexes. The transport of leptin across the enterocytes appears to be quite specific since, with the use of radiolabeled albumin, no internalization by enterocytes was registered and no radiolabeled albumin was recovered in circulation (8). Thus we might further speculate that the internalization of leptin by enterocytes might be receptor mediated, although further studies are needed to establish this fact. Also, and as reported for the pancreatic proteins that were transcytosed across enterocytes (9, 15, 36), the leptin-FITC complex

Fig. 6. Immunogold localization of leptin-FITC in enterocytes. Duodenal tissues sampled at 15 min were processed for immunogold with anti-leptin (B and C) or anti-FITC antibody (D–F). Control without primary antibodies had no staining (A). Both antibodies reveal leptin-FITC antigenic sites; gold particles are present at the level of microvilli (mv) (B and C) and endocytotic vesicles (arrow) (B), in basolateral interdigitations (bli) (B–D), and in the Golgi (G) apparatus (E). Leptin-FITC labeling is also present in blood capillary plasmalemmal vesicles (arrows) (F). Junctional complexes are devoid of labeling (bracket) (C). end, Endothelium; RBC, red blood cells. Bars = 0.5 μm.
reached circulation in an intact form. No degradation occurred in the duodenal lumen or in its transit across the enterocyte. A point of interest is the occurrence of leptin in the Golgi apparatus. Leptin is not a glycosylated protein and is not synthesized by enterocytes (34, 35). However, the intestinal mucosa has been shown to express the soluble leptin receptor (26, 27). We suggest that leptin enters the Golgi apparatus to bind its soluble receptor before it is released as a complex in blood circulation, as is the case in adipocytes.

The object for gastric leptin in the digestive tract is in accordance with the presence of its intestinal membrane-bound receptors and its roles in energy management. Its increase after meal intake and its passage across the duodenal mucosa will allow leptin to act directly on enterocytes and in a paracrine way on neighboring intestinal cells. In support of this, leptin receptors are expressed on the basal membrane of enterocytes and on several entero-endocrine cells (2, 12, 19, 22). Leptin was reported to stimulate glucagon-like peptide 1 from L cells, cholecystokinin from G cells, and ghrelin from gastric mucosa (2, 19, 22), hormones that in turn control gastric emptying and pancreatic hormones secretion (14, 25, 33).

In summary, this in vivo study unravels a new observation for gastric leptin. Gastric leptin secreted in an exocrine way by the epithelial Chief cells reaches the duodenum and crosses the intestinal barrier by transcytosis, to be released in the interstitial space. This pathway may be highly relevant for the control of food intake and digestion. It further opens a new avenue on leptin treatment in certain patients (24, 30). Indeed, leptin injection is being clinically used on several pathologies. Our leptin treatment in certain patients (24, 30). Indeed, leptin was reported to stimulate glucagon-like peptide 1 from L cells, cholecystokinin from G cells, and ghrelin from gastric mucosa (2, 19, 22), hormones that in turn control gastric emptying and pancreatic hormones secretion (14, 25, 33).

ACKNOWLEDGMENTS
The authors thank Dr. Irène Londono for constructive involvement and Elizabeth Gervais for technical assistance.

GRANTS
This work was supported by Canadian Institutes of Health Research, Diabète Québec, and Fonds de la Recherche en Santé du Québec.

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