Luminal leptin activates mucin-secreting goblet cells in the large bowel

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LEPTIN, the hormone encoded by the ob gene (45), is now considered to be multifunctional, not just regulating food intake and energy expenditure (32) but also neuroendocrine function (16), nutrient absorption (8, 13, 30), cell proliferation (19), and angiogenesis (35). This multifunctional role of leptin is consistent with the production of this hormone by various tissues and organs (17, 28), including the stomach (3, 38). This multifunctional role of leptin is considered to be multifunctional, not just regulating food intake and energy expenditure (32) but also neuroendocrine function (16), nutrient absorption (8, 13, 30), cell proliferation (19), and angiogenesis (35).

The colonic segment was flushed with saline solution prewarmed to 37°C to remove residual intestinal contents. It was then continuously perfused at a rate of 1 ml/15 min (Minipuls 2, Gilson; Paris, France).
LEPTIN IS A COLON MUCOSECRETAGOGUE

with Krebs-Ringer buffer (pH 7.5) containing (in mM) 120 NaCl, 4.5 KCl, 0.5 MgCl₂, 0.7 NaH₂PO₄, 1.5 NaHCO₃, 15 NaHCO₃, 1.2 CaCl₂, and 10 glucose. After a 30-min stabilization period, vehicle (control) or recombinant murine leptin (100 nM) in Krebs-Ringer buffer was perfused for different time periods. At the end of the period, segments were removed, and the luminal content (fluid content + adherent mucus gel) was collected and frozen at −20°C for subsequent determination of mucinlike immunoreactivity and luminal DNA content.

Empty colonic loops were divided into small segments. One part of the colon tissue was stored at −80°C until the determination of DNA content. The other part of the colonic tissue was used for total RNA extraction and for quantification of rMuc2 mRNA by real-time PCR. Finally, some colonic segments were used for histological analysis. They were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, stained with alcian blue (AB; pH 2.5) followed by the periodic acid-Schiff (PAS) reaction, and then counterstained with hematoxylin. The AB-AS method yielded a blue color when mostly acidic mucins were present and a magenta color when mainly neutral mucins were present.

Real-time PCR. Total RNA was extracted from colon tissues using TRizol and reverse transcribed into cDNA as previously described (42). Real-time PCR was performed with the real-time fluorescence detection method (Roche Diagnostics; Meylan, France) using a LightCycler System with a FastStart DNA Master SYBR Green I kit. The reaction mixture contained MgCl₂ (80 nmol), forward and reverse primers (8.12 nmol), and LightCycler Fast Start DNA Master SYBR Green I Mix (2 μl) in a volume of 10 μl. The reaction mixture was distributed into precooled capillaries, and diluted (1/10) cDNAs or purified and quantified cloned plasmid DNA for mucin (construction of a standard curve) in a volume of 10 μl were added as PCR templates. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 10 s, a touchdown (0.5°C/Cycle) annealing from 68 to 60°C for 8 s, and 72°C for 6 s. Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase. After the cycling, melting curves of the PCR products were acquired by cooling and maintaining samples at 65°C for 15 s and then by a stepwise increase of the temperature from 65 to 98°C. Primers were as follows: rMuc2 forward 5'-ATTACCCCCACAGTGGACAA-3' and reverse 5'-GG-GATGTGCCAACAAGTT-3' (designed with the assistance of Primer3 computer software); and cyclophilin A, forward 5'-CTT-GTCCATGGAATGTC-3' and reverse 5'-GTGATCTTTCTT-GCTGTTCTTG-3' (12). The expression of rMuc2 was normalized to the cyclophilin A mRNA level in each sample. Results are presented as percentages of the untreated control (means ± SE).

Leptin determination from luminal contents from the colon. Normally fed rats and rats that had been deprived of food for 16 h were anesthetized, and blood was collected from the abdominal aorta. Blood was centrifuged, and the plasma was removed and stored at −20°C until a leptin RIA. Rats were killed, and the entire colon from the cecum to the rectum was removed. The colon lumen contents including the feces were collected and centrifuged at 3,000 rpm for 10 min, and the supernatants were used for a leptin determination by a RIA (RIA kits, Linco Research; St. Charles, MO). Pooled colonic juices from normally fed rats were submitted to size exclusion chromatography using Superdex 200 column (16/60, Pharmacia Biotech; Freiburg, Germany) as previously described. The collected fractions were stored at −20°C until a leptin RIA. The column was adjusted with commercially available calibration proteins kits (Pharmacia Biotech).

Effect of colonic leptin on mucosal architecture. In this set of experiments, 16-h food-deprived Wistar rats were slightly anesthetized with pentobarbital sodium (Sanofi Santé Animale; Libourne, France). Then, 3, 10, or 100 nM of recombinant murine leptin or vehicle were slowly administered (volume injected: 1 ml) through a catheter inserted rectally into the colon and positioned 10 cm proximal to the anus. Animals were killed 24 h after the injection, and food intake, body weight, length of the colon, and macroscopic changes were determined. Segment of the distal colon proximal to the rectum were removed and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and used for histological studies as described above.

A segment was also removed and stored at −20°C for the determination of tissue-associated myeloperoxidase (MPO) activity, which was determined as described previously (10). Protein concentration of the supernatant was determined by using a Bradford assay kit (Bio-Rad Laboratories; Hercules, CA) for calibration.

Cell culture. HT29-MTX cells, a human colon carcinoma-derived mucin-secreting goblet cell line (26), and parental HT29 cells were grown in DMEM (Invitrogen; Cergy Pontoise, France) supplemented with 10% FBS (Sigma; St. Louis, MO) and 100 mg/ml penicillin-streptomycin (Invitrogen). Cells were grown in plastic 25-cm² culture flasks and maintained at 37°C in a 5% CO₂ atmosphere within a humidified incubator. Media were replaced every 2 days.

RT-PCR of the leptin receptor. Briefly, total RNA was extracted from HT29 or HT29-MTX cells using TRizol according to the manufacturer’s instructions. Total RNA was reverse transcribed into cDNA and then amplified with Ob-Rb primers. Forward primer 5'-GCCAACACTGTTGCTCTTC-3' and reverse primer 5'-AGAGAAGCACCTTGTTGACTG-3' for huB219.1 were designed on the basis of the previously published cDNA sequence for the human Ob-Rb receptor (Accession No. U52914), which defines an amplicon of 246 bp. PCR was performed under the thermocycling conditions as previously described (8). PCR products were analyzed by electrophoresis in a 2% agarose gel in the presence of ethidium bromide.

Cellular model of mucin secretion. To study mucin secretion, HT29-MTX cells were seeded in 12-well culture plates (5.10³ cells/well) and were given fresh media every 2 days. Experiments were performed 21 days after cells reached confluence. Twenty-four hours before the secretory studies, cells were serum deprived. The monolayer cultures of HT29-MTX cells were washed twice with PBS and incubated in serum-free medium with or without leptin at 37°C for 15–60 min in a humidified atmosphere. Supernatants were then collected, frozen, and stored at −20°C. Cells were then processed with trypsin, and cell numbers per well were determined. All experiments were performed at least three times in triplicate.

ELISA for mucin secretion. Samples of luminal contents from colonic loops were incubated for 24 h with 100 mM 1,4-dithiothreitol at 4°C for reduction and were then assayed for rat colonic mucins by ELISA as previously described. The secretion of MUC5AC from HT29-MTX cells was measured by ELISA (42) using the primary monoclonal antibody 45M1 (Santa Cruz Biotechnology; Santa Cruz, CA). This monoclonal antibody recognizes the peptide core of gastric mucin M1 identified as MUC5AC (4). Porcine gastric mucin, which has been previously shown to react strongly with anti-human gastric monoclonal 45M1 antibody (23), was treated in the same way to obtain a mucin standard curve. The amount of mucin secreted from the colon was expressed as micrograms of mucin per milligram of tissue DNA. DNA content was determined according to the fluorometric method of Hinegardner (21). Samples of luminal stimulants were assayed to check for the absence of interference in the ELISA.

Enzyme-linked lectin assay for mucins from cell culture media. An enzyme-linked lectin assay (ELLA) was used to measure mucin-like glycoprotein secretion as previously described (42). Briefly, wells of a microtiter plate were coated with samples diluted in sodium carbonate buffer (0.5 M, pH 9.6) and incubated overnight at 4°C. Plates were then washed with PBS containing 0.1% Tween (pH 7) and blocked with 2% BSA in PBS-Tween for 1 h at 37°C. After samples were washed five times, biotinylated wheat germ agglutinin (Vector Laboratories; Burlingame, CA) in PBS-Tween-BSA was added, and samples were incubated for 1 h at 37°C. Colorimetric determinations using avidin-peroxidase conjugate and α-phenylenediamine dihydro-
chloride solution were performed at 492 nm with a micro-ELISA plate reader. The mucin-like glycoprotein content of samples was determined from standard curves prepared from HT29-MTX mucins isolated from 75-cm² flasks and purified by ultracentrifugation as described previously (33). The amount of glycoprotein secreted in the incubation medium was expressed as nanograms of mucin-like glycoprotein per 10⁶ cells, and results are given as percentages of controls.

Statistical analysis. All results are expressed as means ± SE. One-way ANOVA with Tukey-Kramer multiple-comparison post test and the Mann-Whitney U-test or Mann-Whitney test alone for single comparisons was performed using GraphPad Prism version 3.0 for Windows (Graphpad software; San Diego, CA). The level of significance was set at P < 0.05.

RESULTS

Intracolonic leptin increases mucus secretion. In the control rat colon, a spontaneous increase in the amount of mucus was observed after 30 min, with mucus levels remaining stable between 60 and 180 min (Fig. 1A). Luminal perfusion with 100 nM leptin induced a rapid increase in mucus secretion (over the course of 30 min) with mucus levels reaching four times those of the control (P < 0.05). This leptin stimulation of mucus remained large (2.5 and 3.3 times the basal values) after 180 min of leptin perfusion. In these conditions, 1 mM iv carbachol also induced an increase in colonic mucin secretion, but this difference was not statistically significant from that induced by luminal leptin (1,873 ± 408 vs. 1,207 ± 365 µg/mg DNA).

This effect of leptin was dose dependent (Fig. 1B) with a maximal response observed with 10 nM leptin (1,922 ± 317 vs. 545 ± 80 µg/mg DNA in controls) and an EC₅₀ value of 0.8 nmol/l. Intravenous leptin (5 nM) also increased mucus secretion compared with intracolonic administration (2,159 ± 211 vs. 1,144 ± 261 µg/mg DNA, P < 0.05).

Luminal leptin induces the discharge of mucus cells and increases rMuc2 mRNA levels. We carried out histological studies on colon mucosa sections from control- and leptin-treated colon mucosa of anesthetized rats. Goblet cells in the crypt epithelium were recognized by densely stained mucus granules that filled the apical cytoplasm. As shown in Fig. 2A, goblet cells of the rat colon 60 min after the intraluminal perfusion of 1 nM leptin have released their mucus granules. There were also large mucus materials in the crypt lumen. Under these conditions, we found that rMuc2 mRNA levels were twice those of the control after 60 min of treatment with 10 nM leptin compared with controls (Fig. 2B).

Abnormal high mucus-filled goblet cells in the colon of ob/ob mice. We also analyzed goblet cell morphology by histological studies of the colon of leptin-deficient ob/ob mice (Fig. 3). Far more mucus-filled goblet cells were observed in the mucosa of leptin-deficient ob/ob mice than in lean littermate mice. A very thick mucus gel also covered the epithelium of leptin-deficient ob/ob mice. Taken together, these data suggest that goblet cell function is critically dependent on active leptin.

Leptin-immunoreactive protein is detected in colon luminal content. Leptin-immunoreactive (IR) protein was detected in the colon lumen of untreated rats (Fig. 4). A trend toward a decrease was observed in leptin levels in the colon lumen of fasted compared with normally fed rats (Fig. 4, inset), but it did not reach statistical significance (2.1 ± 0.5 vs. 2.75 ± 0.6 ng leptin/ml, not significant). The levels of circulating leptin were significantly reduced by 40% in fasted compared with normally fed rats. The elution profile of the pooled samples (Fig. 4) obtained after size exclusion chromatography exhibited a leptin-IR peak that eluted at the expected molecular mass of 16-kDa leptin.

Intracolonic leptin increases goblet cell mucin content without damaging the mucosa. In another series of experiments, leptin was injected intrarectally into rats that were killed 24 h later. No significant changes were observed in food intake, body weight, or colon length (data not shown). Moreover, tissue-associated MPO activity, an index of neutrophil accumulation, did not change in leptin-treated compared with

![Fig. 1. Intracolonic leptin increases mucus secretion in the rat colon. A: time-course effect of leptin on mucus secretion. After a period of stabilization, Krebs-Ringer buffer (KRB) alone [control (CTRL)] or 100 nmol/l leptin in KRB was perfused intraluminally in the colon of anesthetized rats during different time periods. Mucus release was collected for each time period and quantified by ELISA as described. Each time period of mucus release was determined separately. As a positive control, the effect of an intravenous injection of 1 mmol/l carbachol on mucus secretion was determined over the 180-min period. *P < 0.05 and **P < 0.01 vs. the control. B: dose-response curve for luminal leptin stimulation of mucus secretion in the rat colon in vivo. Mucus secretion was determined as noncumulative responses to different concentrations of leptin after a 60-min intraluminal perfusion. Data are expressed as mucus secretion in micrograms per milligram of DNA and are means ± SE of 4–8 experiments for each dose of leptin.](http://ajpgi.physiology.org/DownloadedFrom)
control rats (1.79 ± 0.13 U·mg protein⁻¹·min⁻¹ for 100 nM leptin vs. 1.52 ± 0.73 U·mg protein⁻¹·min⁻¹ for controls). Furthermore, no change in the architecture of the colonic mucosa was observed for up to 100 nM leptin. Numbers of stained mucus cells were higher than those in controls, indicating that leptin replenishes cells by increasing mucin synthesis (Fig. 5). These data indicate that leptin at doses that do not damage the colonic mucosa increased mucin in goblet cells.

**Fig. 2.** Luminal leptin decreases goblet cell mucus granules and increases rat mucin 2 (rMuc2) mRNA in the rat colon. A: histology. Colonic loops were perfused with KRB alone or with leptin in KRB for 60 min. Sections (4 μm) of the colonic mucosa were stained with alcian blue followed by the periodic acid-Schiff reaction to reveal goblet cells. In the control colonic mucosa (left), goblet cells with densely stained granules can be observed along the length of the crypt. In the luminal leptin (1 nM)-treated colonic mucosa (right), the number of stained mucus cells was decreased, the crypt lumen was expanded, and the release of mucus can be seen from a goblet cell. B: rMuc2 mRNA levels in the rat colon after a 60-min luminal leptin perfusion. rMuc2 was analyzed by quantitative RT-PCR with cyclophilin A as an internal control. The expression of rMuc2 was normalized to the cyclophilin A mRNA level in each sample. Results are presented as fold increases of the untreated control (means ± SE). *P < 0.05 vs. the control.

**Fig. 3.** A deficiency in leptin alters the regulation of colonic mucin in mice. Leptin-deficient ob/ob mice had abnormal high mucus-filled goblet cells. Sections (4 μm) of the colonic mucosa were stained with the periodic acid-Schiff reaction to reveal goblet cells.

**Fig. 4.** Leptin-immunoreactive (IR) protein detected in the rat colon lumen. Shown is the size exclusion profile of pooled luminal content samples from the rat colon. The luminal juice content was fractionated on a Superdex 200 (16/60) column. Leptin was determined in each eluted fraction by a RIA. The peak corresponded to 16-kDa leptin. Inset: means ± SE of leptin-IR protein detected in collected luminal contents from the colon and in plasma samples of nonfasted (n = 7) and 16-h fasted (n = 8) rats. **P < 0.01 vs. nonfasted rats.
Mechanisms of action. We tried to identify the intracellular pathways involved in the action of leptin using human colonic HT29-MTX mucinlike glycoprotein-secreting cells (26). In these cells, a 246-bp amplicon was amplified by RT-PCR (Fig. 6A, inset). The cDNA was sequenced and found to be 100% identical to the human Ob-Rb cDNA sequence. We also investigated whether the induction of mucin discharge by leptin was consistent with the expression of Ob-R on HT29-MTX cells. Carbachol (1 mM) elicited a strong exocytotic response in HT29-MTX cells (Fig. 6A). Leptin also induced the dose-dependent secretion of a mucin-like glycoprotein (Fig. 6A) with a maximal response (+85% ± 18% above control, P < 0.05) being achieved with 1 nM leptin. No further increased was observed with 10 nM leptin. We also showed that 10 nmol/l leptin induced a specific increase in MUC5AC secretion (+60 ± 8% above control, P < 0.05).

Finally, the PKC inhibitors Gö-6976 or Ro-31-8220 significantly inhibited leptin-mediated mucin-like glycoprotein secretion in HT29-MTX cells (Fig. 6B). This blockade of leptin stimulation also occurred with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002. The concentrations of PKC inhibitors and PI3K inhibitor used had no effect on mucin secretion.
Gastrointestinal mucus is composed of secretory mucins of the mucin family. Four members of this family are generally thought to be able to form mucus gels: MUC2, MUC5AC, MUC5B, and MUC6. These mucins have distinct expression patterns along the human gastrointestinal tract. The normal stomach mucosa is characterized by the production of MUC5AC, primarily by surface epithelial mucus cells, and MUC6 by gastric glands. The epithelium of the small and large intestine contains characteristic goblet cells that produce MUC2. This mucin is the predominant secretory mucin in the healthy colon of humans, rats, and mice (43). Goblet cells secrete mucin via an unregulated constitutive pathway that is dependent on the continuous movement of mucin granules from the Golgi apparatus to the apex of the cell and by a regulated process dependent on the sudden release of mucin from granules. This regulatory process is controlled by a wide variety of stimuli, including nerve activation and inflammatory mediators (6, 31, 33). In this study, we clearly demonstrated that leptin is one of these stimuli. Indeed, luminal leptin at physiological concentrations markedly increased mucin secretion in the colon and rMuc2 mRNA levels. Thus leptin activates mucus secretion and helps to reconstitute the intracellular store of goblet cells by increasing mucin gene expression. Yet, this has to be clearly demonstrated. Circulating leptin, which is mostly produced by adipocytes in vivo (45), also increases the secretion of colonic mucus. The stimulation of mucus secretion in response to luminal or systemic leptin administration is consistent with the expression of the Ob-R on both apical and basolateral colonic cell membranes (2).

Interestingly, we found that the colon of leptin-deficient ob/ob mice contains an abnormally dense population of stained mucus cells, shown by comparison to lean littermates. This indicates that active leptin is essential for the maintenance of the balance of mucus secretion, synthesis, and storage by goblet cells. Only two reports have been concerned with the effect of leptin on mucus secretion. One study (37) has shown that leptin prevents the decrease in mucin synthesis induced by the lipopolysaccharide of P. gingivalis. The other study (1) has demonstrated that systemic administration of a high dose of leptin increases gastric mucus secretion and injury susceptibility. In this report, we demonstrated that exogenous administration of leptin (up to 100 nM) in the colon lumen did not induce colon mucosa damage but did increase mucin storage in goblet cells. However, this finding is not consistent with data showing that leptin enemas lead to colon mucosal injury (36). This discrepancy between our results and those published previously results (36) may be partly due to differences in the animal models and/or doses of leptin used. Indeed, the dose used in that study was 1,000 times higher than the one used here. We conclude that locally administered leptin, at a dose causing no damage to the mucosal wall, is a potent colon mucosecretagogue. Moreover, leptin-IR protein was detected in luminal contents from the colon at a concentration of 0.3 nmol/l, which is compatible with the activation of Ob-R. This finding, along with previous data reporting the expression of Ob-R on the apical side of colonocytes (2), argues for a luminal action of leptin on mucus production from colonic goblet cells.

What is the physiological relevance of these findings? Mucus is secreted into the lumen from intestinal goblet cells. Mucus forms a gel layer that covers the mucosal surface of the intestinal tract, acting as a semipermeable barrier between the...
lumen and epithelium. The stability of this mucus layer is essential for the preservation of the integrity of the intestinal epithelium, and any breakdown of this protection may lead to mucosal injury. It has been shown that leptin can reduce colonic injury caused by exposure to acetic acid (5, 11). This is close to those data showing that leptin has gastroprotective effects, accelerating the healing of ethanol-induced gastric ulcers (14, 24). Interestingly, one study (36) reported that, in humans with ulcerative colitis, colonic epithelial cells in the inflamed zone are leptin IR, whereas normal colon epithelial cells are not. In this context, our finding that leptin is present in significant amounts in the lumen and rapidly increases the production/secretion of the major secreted mucin in the intestine, rMuc2, might have physiological implications in the protection of the colonic mucosa. However, this remains to be clearly demonstrated by future studies. We also found that ob/ob mice had high mucin levels in their colonic mucosa. This is in line with the finding that ob/ob mice are resistant to the induction of experimental colonic inflammation and that the replacement of leptin renders ob/ob mice susceptible to disease (34). The mechanisms responsible for such an action largely involved the T cell activation capacity of leptin. Thus we speculate that the mucus layer may increase the ability of the colo of ob/ob mice to cope with aggressive factors, thereby contributing to the resistance of these mice to the induction of colitis. Such an involvement of leptin to pathophysiology mechanisms remains to be investigated.

In conclusion, this study demonstrates that leptin acting from the luminal side is a powerful colonic secretagogue, probably through the Ob-R coupled with the activation of PKC and PI3K. We also obtained evidence suggesting that leptin increases the production of the major mucin secreted in the intestine, rMuc2. Finally, the responsiveness of human mucus-secreting HT29-MTX cells to leptin suggests that our findings may be relevant to human intestinal physiology. Further studies are needed to define the relevance of these findings to the pathophysiology mechanisms of the gastrointestinal tract.

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