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

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Plaisancie, Pascale, Robert Ducroc, Mahmoud El Homsi, Annick Tsocas, Sandra Guilmeau, Sandra Zogbi, Olivier Thibaudeau, and Andre Bado. Luminal leptin activates mucin-secreting goblet cells in the large bowel. Am J Physiol Gastrointest Liver Physiol 290: G805–G812, 2006; doi:10.1152/ajpgi.00433.2005.—Leptin has been suggested to be involved in tissue injury and/or mucosal defence mechanisms. Here, we studied the effects of leptin on colonic mucus secretion and rat mucin 2 (rMuc2) expression. Wistar rats and db/db mice were used. Secretion of mucus was followed in vivo in the rat perfused colon model. Mucus secretion was quantified by ELISA, and rMuc2 mRNA levels were quantified by real-time RT PCR. The effects of leptin alone or in association with protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) inhibitors on mucus secretion by human mucus-secreting HT29-MTX cells were determined. Leptin was detected in the rat colonic lumen at substantial levels. Luminal perfusion of leptin stimulates mucus-secreting goblet cells in a dose-dependent manner in vivo in the rat. Leptin (10 nmol/l) increased mucus secretion by a factor of 3.5 and doubled rMuc2 mRNA levels in the colonic mucosa. There was no damage to mucosa 24 h after leptin, but the number of stained mucus cells significantly increased. Leptin-deficient db/db mice have abnormally dense mucus-filled goblet cells. In human colonic goblet-like HT29-MTX cells expressing leptin receptors, leptin increased mucus secretion by activating PKC- and PI3K-dependent pathways. This is the first demonstration that leptin, acting from the luminal side, controls the function of mucus-secreting goblet cells. Because the gel layer formed by mucus at the surface of the intestinal epithelium has a barrier function, our data may be relevant physiologically in defence mechanisms of the gastrointestinal tract.

rMucin 2; mucus; db/db; protein kinase C; phosphatidylinositol 3-kinase; leptin receptor; intestinal defence

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). Stomach-derived leptin is mostly secreted in gastric juice; it then enters the intestine, where it regulates intestinal biological functions (8, 9, 13, 18).

The biological effects of leptin are mediated by its receptor (Ob-R), which belongs to the gp130 family of cytokine receptors (25, 41). Several Ob-R isoforms generated by alternative splicing of the db Ob-R gene have been identified (25). Leptin has recently been shown to be involved in inflammation and immunomodulation. This is consistent with the structural and functional similarities observed between leptin and the interleukin (IL)-6 family of cytokines (44). Ob-R also has signaling capabilities similar to those of IL-6-type cytokine receptors (41), and there are indications that leptin may be linked to inflammatory conditions. Indeed, it has been suggested that leptin upregulates the inflammatory immune response (27). Leptin-deficient ob/ob mice have been reported to be resistant to colonic inflammation induced by oral dextran sulphate sodium or trinitrobenzene sulfonic acid, and leptin replacement in ob/ob mice renders these mice susceptible to such inflammation (34). Subsequent data from studies (7, 15, 29) using different experimental models of inflammation have indicated a proinflammatory role for leptin. This contrasts with the results of a study (39) suggesting the involvement of leptin in the protective mechanisms that allow an organism to cope with potentially autoaggressive effects of its immune system. In this line, leptin has been shown to exert gastroprotective effects by accelerating the healing of ethanol-induced gastric ulcers (14, 24). Furthermore, exogenous leptin has been shown to reduce the severity of acetic acid-induced colitis in rats (11). Thus it seems that, in certain conditions, leptin act as an anti-inflammatory and/or protective agent. However, it is unknown whether leptin directly modulates defence mechanisms within the gastrointestinal mucosa.

In this study, we investigated whether leptin in the colonic lumen modulated the expression of mucin and the secretion of mucus, a major component of physiological defence mechanisms that is produced by mucin-secreting cells. We assessed the effects of leptin on mucus secretion and on rat mucin 2 (rMuc2) expression in the in vivo rat colon model and in vitro in HT29-MTX human colonic goblet-like cells.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 220–240 g (Charles River Laboratories; L’Arbresle, France) and 6- to 8-wk-old female leptin-deficient (C57BL/6J ob/db) mice and their wild-type lean littermates (Elevage Janvier; Le Genest, France) were caged under standard laboratory conditions with tap water and regular food provided ad libitum on a 12:12-h light-dark cycle at a temperature of 21–23°C. Animals were treated in accordance with European Community guidelines concerning the care and use of laboratory animals.

Anesthetized perfused rat colons. Rats were fasted for 16 h in wire-mesh cages to avoid coprophagia with water ad libitum. Rats were anesthetized with ethylurethane (1.2 g/kg im, Prolabo; Paris, France). An inflow cannula was inserted 1 cm below the cecum, and the outflow cannula was set up at a distance of 1 cm above the rectum. The colonic segment was flushed with saline solution warmed 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).

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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 colonic 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-PAS 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, 55°C for 10 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'-ATTACCCCCAACAGTGAGCAA-3' and reverse 5'-GG-GATGTGCACCACAAAGTT-3' (designed with the assistance of Primer3 computer software); and cyclophylin A, forward 5'-CTTGTCCATGGCAAATGCTG-3' and reverse 5'-GTGATCTTCTTGCCTGGTCTTG-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 mucosa 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 mucus 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 µg/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'-GCCAACACCTGTTGCTCTCTC-3' and reverse primer 5'-AGAGAAGCATTGTTGACTG-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 (5.10⁵ cells/well) and were given fresh media every 2 days. Experiments were performed 21 days after cells reached confluency. 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 nM 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 cell culture media.

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 o-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 statistical 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
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.
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 mucus 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 mucosal damage but did increase mucin storage in goblet cells. However, this finding is not consistent with data showing that leptin enemas lead to colonic 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 intestinal functions. Moreover, it outlines a new function for leptin in the gastrointestinal tract.
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 colon 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 this study, the data in the rat have been extended to the human mucin-secreting goblet-like HT29-MTX cell line (26) in vitro. We found that leptin is able to stimulate the secretion of mucin glycoproteins from these cells expressing the functional Ob-R. Ob-R can activate various intracellular pathways, including the PKC and PI3K (40) pathways. This is consistent with previous data showing that leptin activates PI3K (20) and that the PI3K inhibitor wortmannin prevents leptin from overcoming the lipopolysaccharide-induced inhibition of mucin synthesis (37). Here, we observed that inhibition of conventional PKC isoforms prevented leptin-induced mucin, but this does not exclude the possible involvement of other isoforms (22). Similarly, pharmacological inhibition of PI3K activity prevents the leptin stimulation of mucin secretion. Further work is required to unravel the intracellular mechanisms involved, but our data suggest that leptin-mediated mucin exocytosis requires the activation of PI3K- and PKC-dependent pathways.

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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REFERENCES


