Leptin modulates the expression of secreted and membrane-associated mucins in colonic epithelial cells by targeting PKC, PI3K, and MAPK pathways

Mahmoud El Homsi,1 Robert Ducroc,2 Jean Claustre,1 Gérard Jourdan,1 Arieh Gertler,4 Monique Estienne,1,2 André Bado,3 Jean-Yves Scoazec,1 and Pascale Plaisancié1,2

1INSERM, UMR865, IFR62, Faculté R. Laennec, Université Claude Bernard-Lyon 1, Lyon; 2INRA, UMR Neuro-Gastroentérologie et Nutrition, Toulouse; 3INSERM, U773, CRB3 EQ2, Faculté de Médecine X. Bichat, Paris, France; and 4Institute of Biochemistry, Food Science, and Nutrition, Faculty of Agricultural, Food and Environmental, Jerusalem, Israel

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The intestinal mucosa represents an active defense barrier against harmful conditions present in the intestinal lumen. Protection against damaging agents is conferred by the epithelial layer itself and by factors such as peristalsis, intestinal proteolysis, intestinal microbiota, and mucus. The mucus coat plays a central role in the protection from mechanical insults, colonization by pathogenic bacteria and their toxins, luminal proteases arising from bacterial and mucosal cells, and potential carcinogens. The viscoelastic properties of mucus are mainly determined by the presence of mucins, which are high-molecular-weight proteins extensively O-glycosylated. To date, 20 different mucins (MUC) have been identified in humans and divided into two main classes: secreted and cell surface-associated mucins (34). Secreted gel-forming mucins include MUC2, MUC5AC, MUC5B, and MUC6, which are encoded by a cluster of genes on chromosome 11p15.5 in humans (34). Each of these mucins has a characteristic organ- and cell type-specific distribution. Thus colonic mucus is mainly composed of MUC2 that is produced by epithelial goblet cells, and alterations in expression of this mucin have been reported in patients with Crohn’s disease, ulcerative colitis, and colonic neoplasia (8, 35, 36). As in humans, the major mucins produced in rodent gut are Muc2, Muc3, and Muc4. Interestingly, it has been demonstrated that mice genetically deficient in Muc2 spontaneously develop colitis and colorectal cancer (45, 46), thus highlighting the importance of this secreted mucin in the maintenance of colonic integrity. Recently, the membrane-associated mucins also have received increasing attention for their role in the protection of epithelia (39). In the gut, prominent membrane-associated mucins are MUC1, MUC3, and MUC4 (22), which are detectable both in goblet cells and in enterocytes, providing a static external barrier that can limit direct access of pathogens. It is interesting to note that Muc3 is upregulated during intestinal infection (37), and several studies also provided a link between rare alleles of MUC3 and inflammatory bowel diseases (24, 25), thus suggesting that MUC3 is involved in the mucosal epithelial response to inflammation. Because secreted mucins in association with the membrane-bound mucins act as a physicochemical barrier for the protection of the epithelial cell surface, it may be speculated that any quantitative change in their production may modify the intestinal barrier function and have important physiological implications.

A new role in the defense of the intestine also emerges for enterocytes as sentinels against epithelial aggression and damage. Indeed, it has been demonstrated that intestinal epithelial cells secrete numerous regulating agents including cytokines such as IL-1β, IL-6, IL-7, IL-10, IL-15, IL-18, and interferons that will lead to the activation and recruitment of immune cells involved in defense mechanisms and functions (41). Interestingly, a recent study reported that, in patients with ulcerative colitis, colonic epithelial cells in the inflamed zone were leptin...
immunoreactive and the luminal leptin concentration during inflammation was over 15-fold higher than in normal colonic lumen (40). In some other aspects, the expression of leptin receptors, which have been demonstrated on human and rat colonic epithelial cell membranes (5, 18), suggests that leptin may play an important role in colonic epithelial functions. In line with the presence of leptin receptors in colonic cells, we recently demonstrated that luminally or systemically injected leptin induced a strong discharge of mucin in vivo in rat colon (33). However, it is unknown whether leptin may also directly modulate the expression of various colonic mucin genes.

The present study was thus undertaken to evaluate the direct effect of leptin on the expression of secreted and membrane-associated mucins. For this purpose, we used rat and human intestinal mucus-secreting cell lines as models to avoid interference of the nervous system and immune resident cells. These cell lines exhibit the characteristics of mucin-producing cells and provide reliable tools for the study of regulation of gastrointestinal mucin expression and secretion (26, 33, 48).

To mimic the presence of luminal leptin, the endoluminal effect of leptin on mucin expression was studied in vivo in a rat perfused colon model.

MATERIAL AND METHODS

Materials

Media and reagents (DMEM, penicillin/streptomycin, trypsin). Trizol, RT-PCR reagents and enzymes, and distilled RNase-free water were obtained from Invitrogen (Invitrogen, Cergy Pontoise, France). Culture flasks and plates were from Becton Dickinson Labware (Franklin Lakes, NJ). The FastStart DNA Master SYBRgreen I kit was from Roche Diagnostics (Meylan, France). The leptin antagonist (human leptin mutein D39A/L40A/F41A) (30) was from A. Gertler (Jerusalem, Israel). The bovine interleukin 2, 10% fetal bovine serum, and 10% horse serum were from Sigma (St. Louis, MO).

Cell Culture

The DHE cell line, a previously described mucin-producing rat colon adenocarcinoma cell line (44), was a generous gift of Pr. F. Martin (INSERM U517, Dijon, France). HT29-MTX, a human colon carcinoma derived mucin-secreting goblet cell line, was provided by Dr. Thecla Lesuffleur (26). The two cell lines were grown in 25-cm² plastic culture flasks in DMEM supplemented with 10% FBS and 100 mg/ml penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere in a humidified incubator. Medium was replaced every 2 days.

To study the effect of leptin, cells were seeded in 12-well culture plates. Experiments were performed 3 days (DHE cells) or 21 days (HT29-MTX cells) after cells reached confluency. At 24 h before the studies, the culture medium was replaced by serum-free medium to eliminate any interference from extraneous proteins or hormones. The experimental protocol was then the following: the serum-free medium was removed and the monolayer cultures of DHE or HT29-MTX were washed twice with PBS (37°C). Serum-free medium with or without leptin was added to the cells and incubated at 37°C for 60 min in a humidified atmosphere. Cellular signaling pathway blockade was performed by preincubating the cells with a leptin antagonist or with inhibitors of the JAK/STAT, MAPK, PKC, and phosphatidylinositol 3-kinase (PI3K) pathways for 10 min before leptin addition. The supernatants were then collected, frozen, and stored at −20°C. Cells were processed with trypsin. The cell number per well was determined and total RNA was isolated. All experiments were performed at least three times in triplicate.

Anesthetized Rat Perfused Colon

Male Wistar rats weighing 220–240 g (Charles River Laboratories, L’Arbresle, France) were caged under standard laboratory conditions with tap water and regular food provided ad libitum, in a 12:12-h light-dark cycle at a temperature of 21–23°C. The animals were treated in accordance with European Community guidelines concerning the care and use of laboratory animals. The animal studies were performed under license from the veterinary department of Paris, France (to A. Bado and R. Ducroc, authorization no. 75-955 of September 22, 2004 and no. 75-174 of October 9, 2003, respectively; agreement no. B75-18-02; decision no. 05/12 established on July 12, 2005 by Préfecture de Police de Paris, France).

Before experiments, rats were fasted 16 h in mesh-wire cages to avoid coprophagia with water ad libitum and then anesthetized with ethylurethane (1.2 g/kg, intramuscular) (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 prewarmed to 37°C to remove residual intestinal contents. Then it was continuously perfused at a rate of 1 ml/15 min (Minipuls 2; Gilson, Paris, France) with a Krebs-Ringer buffer (pH 7.5) containing (in mmol/l): 120 NaCl, 4.5 KCl, 0.5 MgCl₂, 0.7 Na₂HPO₄, 1.5 NaH₂PO₄, 15 NaHCO₃, 1.2 CaCl₂, and 10 glucose. After a 30-min stabilization period, vehicle (control) or recombinant murine leptin (0.1–100 nmol/l) in Krebs-Ringer buffer was perfused for 60 min. At the end of the period, the empty colonic loops were divided into small segments. One part of each segment was stored at −80°C until determination of DNA content. The other part of the colonic tissues was used for total RNA extraction and for quantification of Muc1, Muc2, Muc3, and Muc4 mRNA by RT-PCR.

RT-PCR of Mucins, Leptin Receptors, and Cyclophilin

Briefly, total RNA were extracted from rat colon, DHE, or HT29-MTX cells by using Trizol according to the manufacturer’s instructions and were reverse-transcribed into cDNA as previously described (48). cDNAs were amplified by PCR with primer sequences previously published: Muc1–Muc5AC (20, 28, 43, 47, 48), MUC1–MUC5AC (16, 48), and leptin receptors (17, 18, 42). The housekeeping gene cyclophilin was amplified as a reference gene (48). PCR was performed under the thermocycling conditions as follows: 2-min initial denaturation at 94°C, 30-s denaturation at 94°C, 1-min annealing at 60°C and 1-min extension at 72°C. The last amplification was followed by a final 10-min elongation step at 72°C. PCR products were analyzed by electrophoresis in 2% agarose gel in the presence of ethidium bromide. The number of cycles was chosen to fall into the exponential phase of amplification. The identity of PCR products was confirmed by sequencing the amplicons (BIOFIDAL, Vaulx en Velin, France). For semiquantitative analysis of mucin mRNA expression, gels were visualized and pixelized with the “Image System” (Quantum Appligene, Pleasanton, CA) and densitometrically analyzed with Scion Image version 4.0.2.

Real-Time PCR

Real-time PCR measurements were performed with the real-time fluorescence-detection method (Roche Diagnostics, Meylan, France), using the LightCycler System with a FastStart DNA Master SYBRgreen I kit. Primer sequences were previously published (48). Reaction mixture contained MgCl₂ (80 nmol), forward and reverse primers (8.12 pmol), and 2 μl of LightCycler Fast Start DNA Master SYBRgreen I mix in a volume of 10 μl. The reaction mixture was distributed into precooled capillaries, and diluted (1/10) cDNA or
purified and quantified cloned plasmid DNA for mucin (construction of a standard curve) in volume of 10 μl was added as PCR template. 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 touch-down (0.5° per cycle) annealing from 68 to 60°C for 8 s, and elongation at 72°C for 6 s. Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase. After cycling, melting curves of the PCR products were acquired by cooling and maintaining samples at 65°C for 15 s and then by increasing the temperature stepwise from 65 to 98°C.

**ELLA**

An enzyme-linked lectin assay (ELLA) was used to measure mucin glycoprotein secretion as previously described (44). Briefly, wells of a microtiter plate were coated with sample diluted in sodium carbonate buffer (0.5 M, pH 9.6) and incubated overnight at 4°C. The plates were then washed with PBS containing 0.1% Tween (PBS-Tween, pH 7) and blocked with 2% BSA in PBS-Tween for 1 h at 37°C. After washing five times, biotinylated wheat germ agglutinin in PBS-Tween-BSA was added and the samples were incubated for 1 h at 37°C. Colorimetric determinations using avidin-peroxidase conjugate and o-phenylenediamine dihydrochloride solution were performed at 492 nm with a micro-ELISA plate reader. Mucin-like glycoprotein content of samples was determined from standard curves prepared from DHE or HT29-MTX mucins isolated from 75-cm² flasks and purified by ultracentrifugation as described previously (10). The amount of mucin glycoprotein secreted in the incubation medium was expressed as nanograms of mucin glycoprotein per 10⁶ cells, and the results were given as percent of controls.

**ELISA for Rat Mucins**

The secretion of Muc2 by the DHE cells was measured by an ELISA using the H-300 (sc-15334) primary polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (48). Samples were incubated for 24 h at 37°C in a 96-well plate. Plates were then washed three times with PBS containing 0.1% Tween and blocked with 2% BSA in PBS-Tween for 1 h. They were then washed again and incubated with 50 μl of the rabbit primary polyclonal antibody (1:100) for 1 h. The wells were then incubated with 100 μl of biotinylated goat anti-rabbit IgG conjugate (1:10,000) for 1 h. After three washes, 100 μl of avidin-peroxidase conjugate were added and plates were processed as described for the ELLA. Muc2 content of samples was determined from standard curves prepared from purified rat intestinal mucin. The results were given as percent of controls.

**Statistical Analysis**

Data were compared by repeated-measures ANOVA, followed by U-test of Mann-Whitney when appropriate or Mann-Whitney test alone for single comparisons. Differences with P < 0.05 were considered significant. Data were analyzed by using Statview 4.57 for Windows (Abacus Concepts, Berkeley, CA) and are presented as means ± SE.
RESULTS

Expression of Leptin Receptor and Effect of Leptin on the Secretion of Mucins in DHE Cells

To assess the direct effect of leptin on goblet cells and mucin gene expression, we used the rat intestinal DHE cell line, which synthesizes and secretes mucus. We first examined the expression of leptin receptors in DHE cells using RT-PCR. Predicted PCR products of Ob-Ra (short isoform) and Ob-Rb (long isoform) were obtained at 347 and 375 bp, respectively, using specific primers in both rat colon and DHE cells (Fig. 1A). The identities of the PCR products were confirmed by DNA sequence analysis. To further confirm the expression of these receptors, Western blot analysis was performed using the anti-Ob-R antibody (H-300, Santa Cruz Biotechnology). This antibody recognizes all isoforms (short and long; namely, Ob-Ra, b, c, d, and f). Analysis of immunoblots revealed a dominant 120-kDa protein band in DHE cells (Fig. 1A). This band corresponds to the long Ob-Rb isoform. In addition, another band at ~90-kDa consistent with the short Ob-Ra isoform of the receptor was observed. These two bands were also seen in parental HT29 colonic cells that are known to express both long and short isoforms and that were used as a positive control.

To investigate whether a direct effect of leptin on the induction of mucus secretion was in keeping with the presence of functional leptin receptor on rat DHE cells, we exposed cell cultures to leptin (0.01–10 nmol/l) for 60 min. As shown in Fig. 1B, leptin induced a dose-dependent secretion of mucin-like glycoprotein with a maximal response achieved with 10 nmol/l leptin (210 ± 3% of control, P < 0.05). Using an ELISA for Muc2, we also found that 10 nmol/l leptin induced a rise in Muc2 secretion (190 ± 8% of control, P < 0.05).

Leptin Stimulates the Expression of Muc2, Muc3, and Muc4 in DHE Cells

To analyze the effect of leptin on the expression of Muc1–Muc5AC, we cultured the mucin-secreting DHE cells without or with leptin (0.01–10 nmol/l) for 60 min. Total RNA was then isolated and mucin mRNA levels were analyzed by RT-PCR. As shown in Fig. 2, addition of leptin to the incubation medium induced a significant increase in Muc2, Muc3, and Muc4 mRNA levels compared with control cells. In contrast, leptin did not modify the expression of Muc1 and only induced a small increase in Muc5AC mRNA level (P > 0.05). RT-PCR products of cyclophilin A mRNA, used as internal control, were unaffected by leptin.

The dose-response effect of leptin (0.01–10 nmol/l) on the expression of Muc2, Muc3, and Muc4 was further evaluated after 60 min of treatment. RT-PCR analysis using Scion Image software. Each point represents mean ± SE of 3 experiments performed in triplicate. *P < 0.05 vs. control.

Intracolonic Leptin Increases Muc2, Muc3, and Muc4 Mucin Gene Expression in Rat Vivo

To address the relevance of the findings obtained from rat cultured goblet-like cells, we investigated the effect of leptin on the expression of secreted and membrane-associated mucins in vivo in the rat perfused colon model. Luminal perfusion with 0.1–100 nmol/l leptin during 60 min induced a dose-dependant increase in Muc2, Muc3, and Muc4 mRNA levels, compared with control. The maximal response was observed with 10 nmol/l leptin for Muc2 and Muc4 (165 ± 3 and 187 ± 25% of controls, respectively, P < 0.05) and with 100 nmol/l leptin for Muc3 (194 ± 3% of controls, P < 0.05). In contrast, leptin did not modify the expression of Muc1. RT-PCR products of cyclophilin A mRNA, used as internal control, were unaffected by leptin.

Leptin Stimulates MUC2, MUC4, MUC5AC Expression in HT29-MTX Cells

Expression of leptin receptors in HT29-MTX cells. Our study was then extended to HT29-MTX cells, a human colonic cell line known to synthesize and secrete mucus. In a previous experiment (33), we showed by RT-PCR that HT29-MTX cells expressed Ob-Rb receptors, and that leptin increased mucin secretion in this cell line. Western blot analysis of immunoreactive Ob receptors revealed here a band at ~120 kDa and also another one at 90 kDa, as observed in DHE cells. The transcripts of the leptin receptors Ob-Ra and Ob-Rb were also evidenced by RT-PCR (Fig. 4).
Leptin increases the expression of mucin genes. RT-PCR analysis using Scion Image then showed that addition of leptin (10 nmol/l) to the incubation medium for 60 min induced an increase in MUC2, MUC4, and MUC5AC mRNA levels compared with controls (Fig. 5A). In contrast, leptin did not modify the expression of MUC1 and MUC3. RT-PCR products of cyclophilin A mRNA, used as internal control, were unaffected by leptin.

The stimulatory effect of leptin on the expression of MUC2, MUC4, and MUC5AC was dose dependent (Fig. 5B). The major secreted mucin produced by HT29-MTX cells is MUC5AC. To precisely quantify the effect of leptin on the expression of this mucin, we further performed quantitative RT-PCR. The level of MUC5AC mRNA was increased threefold following 60 min of treatment with leptin (10 nmol/l, P < 0.05) (Fig. 6A).

Mechanisms Involved in Leptin-Induced Mucin Expression in HT29-MTX Cells

The intracellular mechanisms involved in the effect of leptin were investigated on two mucins: MUC5AC, the main HT29-MTX secreted mucin, and MUC4, the membrane-associated mucin. To study whether the leptin receptor was involved in mucin expression and secretion, HT29-MTX cells were pretreated for 10 min at 37°C with the triple mutein of human leptin (L39A/D40A/F41A, 25 nmol/l) used as an antagonist, before addition of 1 nmol/l leptin for 60 min. A 25-molar excess of the mutein was used owing to the fact that both leptin and leptin mutein exhibit identical affinity toward leptin receptor (30). Such an excess is required to ensure 90–95% occupation of leptin receptor by the latter. Data obtained with quantitative RT-PCR showed that the effect of leptin on MUC5AC expression was inhibited by the L39A/D40A/F41A mutein, while the antagonist alone had no effect on MUC5AC mRNA level (Fig. 6A). L39A/D40A/F41A mutein also inhibited the rise in MUC4 mRNA level induced by leptin (data not shown).

As shown in Fig. 6B, the L39A/D40A/F41A mutein blocked leptin-induced mucinlike glycoprotein secretion. In contrast, L39A/D40A/F41A mutein alone had no effect on mucin secretion.

To identify the signaling pathways involved in leptin-mediated mucin upregulation, we first studied the effect of a MAPK kinase inhibitor (U0126, 10 μmol/l) on the mucin mRNA increase induced by leptin in HT29-MTX cells. The cells were pretreated for 10 min at 37°C with U0126 before addition of leptin (1 nmol/l, 60 min). Data obtained with quantitative RT-PCR showed that the effect of leptin on MUC5AC expression was completely inhibited by U0126 (Fig. 7). This blockade of leptin stimulation also occurred with the PKC inhibitor G6976 (1 μmol/l) and the PI3K inhibitor LY-294002 (10 μmol/l). U0126, G6976, and LY-294002 also inhibited the rise in the MUC4 mRNA level induced by leptin (data not shown). In contrast, the inhibitor of the JAK/STAT pathway (AG 490, 50 μmol/l) was without effect on the increased expression of MUC5AC and MUC4 induced by leptin. The inhibitors of PKC, PI3K, MAPK, and JAK/STAT pathway, used alone, had no effect on mucin expression.

DISCUSSION

The colonic mucus layer is an important element of the protection systems developed at the colonic epithelial cell level. Recently, we showed that leptin had stimulatory effect on mucin secretion in vivo in rat colon (33). Here, we demonstrated that leptin increases the expression of secreted mucin gene as well as membrane-associated mucin gene in rat and human colonic gobletlike cells and in rat colon in vivo. This stimulation by leptin is dependent on activation of leptin receptors present on the goblet cells and on PKC, PI3K, and MAPK signaling pathways. Taken together, these data suggest
that leptin controls mucin gene expression and may promote protection during colonic injury.

Leptin, a 146-amino acid peptide hormone produced primarily by adipocytes, is considered to play a key role in the regulation of food intake, energy expenditure, and body weight homeostasis (15). Leptin expression has also been identified in other tissues, including the fundic epithelium of the stomach (27). Leptin is present in the gastric juice, bound to a protein corresponding to the extracellular domain of the leptin receptor (soluble receptor) that allows leptin to cope with the harsh conditions of the gastric juice (9). Thereafter, leptin enters the intestine, where it regulates nutrient absorption (7, 13). On the other hand, leptin is considered, both structurally and functionally, to act as a gut hormone, regulating the expression of various genes involved in the regulation of mucin production (14). This suggests a protective role of leptin in the intestine, which is in line with the finding that leptin enhances the expression of mucin genes in the HT29-MTX cell line (16, 17). The finding that leptin enhances the expression of mucin genes in the HT29-MTX cell line further supports the protective role of leptin in the intestine.

Fig. 5. Leptin specifically modulates MUC2, MUC4, and MUC5AC mRNA expression in the human colonic HT29-MTX cell line. A: effect of 10 nmol/l leptin (60 min) on the expression of MUC1-MUC5AC in HT29-MTX cells. Total RNA was isolated and mucin mRNA levels were analyzed by RT-PCR. The gels obtained from semiquantitative RT-PCR were pixelized and densitometrically analyzed with Scion Image software. Each point represents mean ± SE of 3 experiments performed in triplicate. *P < 0.05 vs. control. B: leptin dose dependently increases the level of MUC2, MUC4, and MUC5AC mRNA in HT29-MTX cells. HT29-MTX cells were exposed to different concentrations of leptin for 60 min. The gels obtained from semiquantitative RT-PCR were pixelized and densitometrically analyzed with Scion Image software. Each point represents mean ± SE of 3 experiments performed in triplicate. *P < 0.05 vs. control.

Fig. 6. Human mucus-secreting HT29-MTX cells are responsive to leptin through an Ob-R-dependent pathway. A: effect of the L39A/D40A/F41A mutein (leptin antagonist) on leptin-induced MUC5AC expression. MUC5AC mRNA from the 60-min-stimulated cells were analyzed by quantitative RT-PCR. The expression of MUC5AC was normalized to cyclophilin A mRNA level in each sample. In some experiments, HT29-MTX cells were preincubated for 10 min with the leptin antagonist L39A/D40A/F41A mutein leptin (25 nmol/l) before the addition of 1 nmol/l leptin for 60 min. The results are expressed as percent of associated controls (mean ± SE). *P < 0.05 vs. control. &P < 0.05 vs. leptin alone. Inset: gels obtained from semiquantitative RT-PCR of MUC5AC. Lane 1: DNA markers. Lane 2: leptin. Lane 3: leptin + mutein leptin. Lane 4: mutein leptin alone. Lane 5: control. B: leptin dose dependently increases the level of MUC2, MUC4, and MUC5AC mRNA in HT29-MTX cells. HT29-MTX cells were exposed to different concentrations of leptin for 60 min. The gels obtained from semiquantitative RT-PCR were pixelized and densitometrically analyzed with Scion Image software. Each point represents mean ± SE of 3 experiments performed in triplicate. *P < 0.05 vs. control (CT). &P < 0.05 vs. leptin alone.
ally, as a proinflammatory cytokine of the type I superfamily and has structural similarity with interleukin-6 (31). In keeping with this assumption, leptin significantly increased in acute infection or during inflammatory processes (31). Interestingly, leptin has been detected both in the lumen and in the colonic epithelial cells in inflamed zone of humans with ulcerative colitis but not in the normal colonic epithelial cells (40) and the expression of leptin receptor (Ob-R) has been reported on colonocytes (18). Here, we demonstrated that leptin increased the expression of the secreted mucin \textit{Muc2} and of the membrane-associated mucins \textit{Muc3} and \textit{Muc4} in rat colonic DHE cells. The concentrations of leptin used in our in vitro study were in the range of 0.1 to 10 nmol/l, the maximal effects on mucin expression being obtained at the concentration of 1 nmol/l. In our previous paper, we showed that leptin-immunoreactive protein was present in the luminal contents from healthy rat colon at a concentration of 0.3 nmol/l (33). However, it has been pointed out that the level of luminal leptin in inflamed colon was ~15-fold higher than normal (40). On the basis of these studies, it may be speculated that our data highlight an important direct effect of leptin on colonic epithelial cells that may be of major importance during colonic inflammation and injury.

It is worth mentioning that an increase in mucin production has been demonstrated as a genuine component of inflammatory responses of epithelial tissue (4, 37). Indeed, the expression of mucin genes appears to be controlled by several cytokine mediators that may regulate the maintenance of protective mucus gel during acute phase of injury. Recent data have thus demonstrated that \textit{Muc3} can be regulated by the cytokines II-4, II-6, TNF-\(\alpha\), and IFN-\(\gamma\) (38). \textit{MUC4} expression is also known to be enhanced by II-4, II-9, TNF-\(\alpha\), and IFN-\(\gamma\) (3, 11, 12, 32). Similarly, several studies showed that expression of the secreted mucin \textit{MUC2} is increased upon II-1, II-4, II-13 or TNF-\(\alpha\) stimulation (2, 14, 21, 23). Together, these data suggest that cytokines, and in particular members of the IL-6 cytokine family to which leptin belongs, are crucial factors of mucin gene activation during inflammation.

It is important to underline that leptin increased here the level of transcripts of \textit{Muc2} as well as those of \textit{Muc3} and \textit{Muc4} in both DHE cells and rat colon. The overall secretion of \textit{Muc2} was also observed in DHE cells after 1 h of treatment. The \textit{Muc2} mucin is the main secreted gel-forming mucin produced in colonic goblet cells and constitutes the framework for the intraluminal mucus gel. Thus this upregulation of \textit{Muc2} expression and secretion is particularly relevant and may be proposed as a protective mechanism to wash out noxious agents during mucosal injury. The effect of leptin on the membrane-associated mucins \textit{Muc3} and \textit{Muc4} may also play an essential role in intestinal mucosal defense because, beside their protective effect on epithelia, these mucins have been shown to be involved in growth, cell signaling, and modulation of immune system (4). Accordingly, a recent study demonstrated that \textit{Muc3} plays an active role in epithelial restitution and may represent a novel therapeutic agent for intestinal wound healing (19). The biological consequences of the increased expression of \textit{Muc2}, \textit{Muc3}, and \textit{Muc4} induced by leptin may thus be important in situations of colonic injury to promote mucosal healing and to restore epithelial protection. This hypothesis is supported by the observation that leptin

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\caption{Effect of leptin on \textit{MUC5AC} gene expression is dependent on MAPK, PKC, and phosphatidylinositol 3-kinase (PI3K) pathways in human mucus-secreting HT29-MTX cells. HT29-MTX cells were preincubated for 10 min with inhibitors of MAPK kinase (U0126, 10 \(\mu\)mol/l), PKC (Go 6976, 1 \(\mu\)mol/l), PI3K (LY-294002, 10 \(\mu\)mol/l), or of JAK/STAT pathway (AG 490, 50 \(\mu\)mol/l) before the addition of 1 nmol/l leptin in the incubation medium. \textit{MUC5AC} mRNA level was analyzed by quantitative RT-PCR. The expression of \textit{MUC5AC} was normalized to cyclophilin A mRNA level in each sample. Results are presented as percent increase with respect to the untreated control (mean \pm SE). *\(P < 0.05\) vs. control. &\(P < 0.05\) vs. agonist alone.}
\end{figure}
exerts a potent protective activity against acetic acid-induced colonic inflammation (6) and also in normal wound healing (29).

In the course of characterizing the effects of leptin, we extended the data obtained in rat to the human colonic goblet-like HT29-MTX and demonstrated that leptin induced an increase of the expression of the major secreted mucin MUC5AC produced in this cell line, as well as the expression of MUC2 and MUC4, thus suggesting that these responses may take place in human as well. Since MUC4 is not restricted to goblet cells but is also expressed by colonicocytes, the possibility exists that the protective effect afforded by increased MUC4 through goblet cells could also be considered for the entire colonic epithelium. Such an hypothesis requires further investigation.

The leptin receptor is encoded by the diabetes gene (db) and is alternatively spliced, resulting in six isoforms that differ only in the length of their intracellular domains: one long form (Ob-Rb), four shorter forms (Ob-Ra, c, d, f), and a soluble one (Ob-Re) (1). The long-form receptor (Ob-Rb) is expressed mainly in the hypothalamus and plays an important role in the regulation of food intake, energy expenditure, and endocrine function. In contrast, the short-form receptor (Ob-Ra) is expressed fairly ubiquitously, representing the major isoform in many peripheral tissues. In the present study, we demonstrated the presence of Ob-Rb and Ob-Ra receptors on epithelial DHE and HT29-MTX gobletlike cells, in line with previous data showing the expression of leptin receptors on colonic epithelial cells in normal tissue (5, 18). We showed that pretreatment of the HT29-MTX cells with a specific human leptin antagonist inhibited the effect of leptin on mucin secretion and expression, confirming the functional relevance of these receptors and their involvement in the response observed. In addition, the finding that L39A/D40A/F41A mutein alone had no effect on mucin secretion compared with the control indicates that the basic expression and secretion of mucins are not dependent on endogenously secreted leptin.

Leptin binding to Ob-R may trigger off multiple signaling pathways including the JAK/STAT and the MAPK pathways but also the stimulation of the PI3K and PKC (31). In our study, we demonstrated that the leptin-induced MUC5AC and MUC4 expression was inhibited by a MAPK kinase-specific inhibitor, U0126, thus suggesting the involvement of this pathway in the HT29-MTX cell response. Similarly, studies performed in the colonic cell line LS174T showed that the expression of MUC2 was stimulated by IL-4, IL-13, and TNF-α through a process involving MAPK activation (21). In the human pulmonary carcinoma cell line, NCI-H292, the MAPK pathway was also found to play a role in the MUC5AC mRNA expression induced by TNF-α. Additionally, we found that pharmacological inhibition of PKC and PI3K activity prevented the leptin stimulation of MUC5AC and MUC4 mucin expression. These findings are in accordance with our previous study showing that leptin induced mucin secretion in HT29-MTX cells through the activation of a PI3K and a PKC-dependent pathway (33). Together, our data suggest that leptin-mediated mucin expression in colonic cells requires the activation of PI3K-, PKC-, and MAPK-dependent pathways.

In conclusion, our results show that human and rat goblet cell lines express the Ob-Rb receptor and Ob-Ra to a lesser extent. Through activation of these receptors, leptin triggers mucin secretion and expression of both secreted and membrane-bound mucins. Originating from the stomach and/or locally released during inflammation, leptin might thus be one of the actors of mucosal defense. These findings disclose what may be an important pathway in the defense mechanisms of the colonic mucosa.

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