Leptin Counteracts Sodium Butyrate-Induced Apoptosis in Human Colon Cancer HT-29 Cells via NFkappa-B Signaling

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SUMMARY

This study shows that leptin induced a rapid phosphorylation of p42/44 mitogen-activated protein kinase, an enhancement of both NF-κB DNA binding and transcriptional activities, and a concentration-dependent increase of HT29 cell proliferation. These effects are consistent with the presence of leptin receptors on cell membranes. The leptin induction of cell growth was associated with an increase of cell population in S and G2/M phase compared to control cells found in G0/G1 phase of the cell cycle. Moreover, cyclin D1 immunoreactivity was enhanced in leptin-treated HT29 cells and this increase was essentially associated with cell population in G0/G1 phase. On the other hand, we observed that sodium butyrate inhibited cell proliferation by blocking the HT29 cells in G0/G1 phase of the cell cycle. Interestingly, at physiological concentration, leptin prevented sodium butyrate-induced morphological nucleus changes, DNA laddering and suppressed butyrate-induced cell cycle arrest. This anti-apoptotic effect of leptin was associated with HT-29 cell proliferation and activation NF-κB pathways. However, the phosphorylation of p42/44 MAPkinase in response to leptin was reduced in butyrate-treated cells. These data demonstrated that leptin is a potent mitogenic factor for intestinal epithelial cells through MAPkinase and NF-κB pathways. They further showed, for the first time, that leptin promotes colon cancer HT29 cell survival upon butyrate challenge by counteracting the apoptotic programs initiated by this short chain fatty acid probably through NF-κB pathways. Although further studies are required to unravel the precise mechanisms, these data may have
a significance in the pathogenesis of colorectal cancer and ulcerative colitis diseases.
Introduction

Leptin, the protein product encoded by ob gene, is a 16-kDa circulating hormone produced primarily by the adipose tissue and is a multifunctional hormone that regulates body weight homeostasis, neuroendocrine function, fertility, immune function, and angiogenesis (1-4). The biological actions of leptin on target tissues are carried out through interaction with its specific receptors, Ob-R. The leptin receptor (Ob-R) is a member of the gp130 family of cytokine receptors (5) which occurs in several receptor variants (Ob-Ra through Ob-Rf) that are generated by alternative splicing of the db leptin receptor gene. These isoforms share the same extracellular domain but differ in the length of the transmembrane/cytoplasmic (6,7). The long Ob-Rb subtype (Ob-R_L) appears as the functional, signal-transducing isoform, responsible for the action of leptin (8). The long isoform, Ob-Rb, can activate the signal transducers and activators of transcription (STAT) pathways, whereas both Ob-Rb and the short isoform (Ob-Ra) can transduce signals through IRS (insulin receptor substrates) and through mitogen-activated protein kinase (MAP kinase) dependent pathways (for review see (9)). Although, it is currently though that leptin action is largely mediated by central nervous systems, the expression of Ob-R_L in peripheral tissues indicate that they are direct targets of leptin. There is now an emerging role of leptin as a growth factor for several cell types (10-12).

In the gut, leptin has been reported to stimulate the proliferation of gastric and
intestinal epithelial cells through activation of the MAP kinase dependent pathways (13) (14).

Besides this leptin growth effect, we have previously demonstrated that leptin can enhance the activity of the brush border Pept-1 transporter (15) and the uptake of butyrate via the monocarboxylate carrier MCT-1 in the human intestinal Caco-2 cells (16).

Butyrate is a short-chain fatty acid (SCFA) that is produced in the colonic lumen by bacterial fermentation of carbohydrate and dietary fibres (17). In the proximal large bowel, butyrate represents the preferred respiratory fuels in the intestine through β-oxidation. Apart from the function of butyrate as a dominant energy source for colonocytes, its also inhibits cellular proliferation and induces apoptosis by regulating the key proteins controlling the cell cycle (18). In particular, NaB was shown to downregulate 25 genes including cyclin D1, a key regulator of G1/S phase and the proliferating cell nuclear antigen PCNA in colonic epithelial cells arguing for its pro-apoptotic property (19).

In this study we investigate whether leptin could affect sodium butyrate-induced apoptosis in a human colon cancer cell line HT29 cells and examine the intracellular mechanisms involved. We demonstrated, for the first time, that leptin promotes intestinal epithelial HT29 cell survival upon butyrate challenge by counteracting the apoptotic programs initiated by this short chain fatty acid, probably through activation of NF-κB pathways.
EXPERIMENTAL PROCEDURES

Reagents. Human Leptin was purchased from R&D Systems (R&D Systems Inc., Minneapolis, USA) and stored as stock solution in phosphate buffered saline solution (PBS) at 20°C. Antibody sources. Rabbit polyclonal NF-κB p65 and IκB-α antibodies, goat polyclonal anti-Ob-R(C-20) were purchased from Santa Cruz Biotechnology, Inc (CA, USA) and mouse monoclonal cyclin D1 were from Inc and Cell Signaling Technology, Inc (Beverly, MA, USA). Fluorescein and horseradish peroxidase conjugated secondary antibodies were purchased from Sigma Chemicals (St Louis Mo, USA).

Cell culture. The human colon cancer cell line, HT29 were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere and in 5% CO₂ at 37°C. Subculture of HT29 cell line was performed by enzymatic digestion (trypsin / EDTA solution : 0.05/0.02%).

Cell growth and viability. Cells (3x10³/well) were seeded into 48-well dishes under the growth conditions described above. Three days after seeding, cells were serum deprived for 24 hours and then treated with recombinant human leptin (0 to 10 nM) for 24 hours. The time course effects (24, 48 and 72 hours) were in serum free culture cells using 1nM of rhLeptin, the medium being renewed every day. After 24, 48 and 72 hours, the cells were harvested by trypsin digestion and cell viability was determined by trypan blue exclusion with a hematocytometer.

RT-PCR of leptin receptor (Ob-Rb) in HT 29 cells. Briefly, total RNA (RNAₜ) were
extracted from HT29 cells, using Trizol® according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA then amplified with Ob-Rb primers. The primers of 5’-GCCAACAACTGTGGTCTCTC–3’ and 5’-AGAGAAGCACCTTGGTGACTG -3’ for Ob-Rb were designed on the basis of the previously published cDNA sequence for human Ob-Rb leptin receptor (GeneBank, accession number : CAA73211) which define an amplicon of 248 bp. PCR was performed under the thermocycling conditions as follows : 1-minute denaturation at 94°C, 1-minute annealing at 57°C and 1-minute extension at 72°C for 30 cycles. The last amplification was followed by final ten-minute elongation step at 72°C. PCR products were analyzed by electrophoresis in 2% agarose gel in the presence of ethidium bromide.

**Immunocytochemistry.** HT29 cells were grown on glass coverslips in DMEM supplemented with 10% of fetal bovine serum for 48 hrs. Cells were fixed at room temperature for 15 min with 4% paraformaldehyde solution, then rinsed three times with PBS. Non specific sites were blocked with 5% non-immune horse serum in PBS for 30 min. Coverslips were incubated overnight with a goat polyclonal anti- Ob-R (dilution 1:20); in humid atmosphere. After three washes for 5 min, they were labeled with a donkey anti-goat IgG conjugated to FITC (1:40) or with phalloidin-TRITC (1µg/ml) for 1 hr in darkness, followed by three rinses and were mounted with Vectashield and observed under a LSM 510 confocal microscope (Zeiss).

**Controls.** No immunostaining was observed in HT29 cells under the following conditions: a) normal goat serum used in place of immune rabbit serum; b) co-incubation of coverslips with Ob-Rb antiserum and the control immunogen peptide (sc-1832P).

**Cell cycle analysis and Flow cytometry acquisition.** Cells were seeded into flasks (3x10^5
cells/25cm²), and let to raise exponential growth. To ensure that cells progress through the cell cycle in synchronous manner, cells were maintained in serum-free media from 24-48 hours. Cells were treated with vehicle or leptin (1nM) for 24 hours. For flow cytometry analysis, cells were trypsinized, washed twice with PBS, and fixed in 80% ethanol for at least 18 hours at -20°C. Fixed cells were washed twice with PBS, and stained with mouse monoclonal FITC-conjugated cyclin D1 antibody (0.5 ng/mL) and/or with a staining solution containing RNAse A (0.2 mg/mL) and propidium iodide (10µg/mL) for 30 min at 37°C. The cell cycle was analyzed using a laser flow cytometer (Beckman Coulter). Data were acquired on a Coulter flow cytometer using Expo32 software. Ten thousand cells were acquired for analysis of HT29 cell population gated as double staining with anti-cyclinD1-FITC and propidium iodide.

**Quantification of phosphorylated ERK1/2 proteins.** Fast activated cell-based ELISA (Active Motif Europe, Brussels, Belgium) was used to determine ERK phosphorylation relative to the total ERK protein found in cells following the manufacturer’s instructions. Briefly, HT29 cells were seeded on the 96-well plates, then serum starved for 24 hours. Then the cells were treated with leptin (1nM), NaB (5mM) either alone or in combination for various time-periods. Afterwards, the cells were fixed, one part of wells were used for determination of phospho-ERK using a specific antibody, while the other wells were used for determination of total-ERK with another antibody, followed by incubation with HRP-conjugated antibodies and developing reagent. A sensitive colorimetric readout was quantified by spectrophotometry.
at 450 nm with a reference wavelength of 630 nm. The relative number of cells in each well was then determined through the Crystal Violet reagent. The values of the phospo-ERK and total-ERK signals have been normalized for cells number. The data are expressed as a ratio phophor-ERK to total-ERK

**Western blot analysis.** Whole or fractionated (nuclear and cytoplasmic) cell extracts (20) were subjected to SDS-PAGE electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Membranes were first incubated with a specific antibody which recognizes specifically NF-κB p65 (dilution, 1:100) and further incubated with horseradish peroxidase-conjugated anti-IgG (at dilution, 1:3000) for 1 hr. The immune complexes were detected by chemiluminescence (ECL, Amersham Biosciences)

**Confocal microscopy analysis of NF-kappa B activation.** In order to determine whether the activation of transcription factor NF-κB occurred after addition of leptin, nuclear translocation of NF-κB was examined by confocal microscopy. Cells were cultured into labtek, and treated with or without 1 nM leptin for various times (0, 5, 15, 30 min, and 1, 3, 6, 9 and 24 hr). The cells were fixed as described above and were labeled with a rabbit polyclonal anti- NF-κB p65 (dilution : 1:100) followed by a FITC-conjugated anti-rabbit immunoglobulin G at dilution 1:200, then cells were stained by propidium iodide (10µg/ml). Cells were examined under a laser scanning confocal microscope (Zeiss, LSM 510). Co-localization of the antibody to NF-κB p65 and the intercalating agent propidium iodide was
analyzed using the co-localization LSM510 3.2 physiology software.

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared as described by Andrews and Faller (20) from serum free HT29 cells treated without or with 1 nM leptin. Five micrograms of proteins were incubated with 0.5 ng of 5-biotinylated NF-κB double-stranded oligonucleotides ((wild type NF-κB consensus oligonucleotide (WT) : 5-AGT TGA GGG GAC TTT CCC AGG C-3) or (NF-κB mutant oligonucleotide : 5-AGT TGA GGC GAC TTT CCC AGG C-3) in buffer containing 10mmol/L HEPES pH 7.5, 2mmol/L MgCl₂, 50 mmol/L KCl, 1 mmol/L dithiothreitol, 0.1 mmol/L EDTA, 20 % glycerol, and poly dI/dC (50 µg/ml) for 15 min at room temperature. The binding reaction was immediately resolved on native 7.5% polyacrylamide gel in Tris/glycine/EDTA electrophoretic buffer, then electrotransferred to nylon membrane (Biohylon Z+). After transfer, the membrane was exposed to UV light (1200 Joules) for 5min, then blocked for 30 min at room temperature in 5% bovine serum albumin in TBST (20 mM Tris-HCl (pH 7.5, 137mM NaCl, 0.1% Tween 20). The immune complexes were visualized using horseradish peroxidase-conjugated streptavidin (diluted at 1:100) and chemiluminescence (ECL, Amersham Biosciences). The specificity of the assay was confirmed in separate shift assays on the same nuclear extracts, demonstrating that activated NF-κB factor does not bind to NF-κB mutant oligonucleotide.

**Transient Transfection and Luciferase activity assay.** Cells were seeded 1 day before transfection (2 ×10⁵ cells/well in 12-well dishes). Transfection was performed by jetPEI
transfection reagent (Polyplus-transfection) with 2 µg of (kappaB)3 IFN-luciferase plasmid (21). The reporter contained a single promoter for β interferon (IFNβ) gene along with three upstream κB sites. The κB site motif responds to NF-κB p65. Thus luciferase activity reflect the instantaneous concentration of its mRNA. After 24 hr transfection, cells were starved for 30 hr and then, stimulated with or without leptin (1nM) or with 10 % FBS for 30 min. Cell medium was removed, the cells were rinsed with PBS, and then lysed with 200µL of the Luciferase Assay System (Promega). 20µL of the extract was reacted with luciferase substrate (100µL) and the luminescence was quantified by a luminometer. The experiments were performed in triplicate and data are representative of three independent experiments. The results are expressed as “fold increase of luciferase activity” which was calculated relative to the basal level of (kappaB)3 reporter activity set to 1 unit and corrected for empty vector effects.

Multi-well colorimetric assay for active NF-κB (22). TransAm® assays (Active Motif Europe) was used to determine the NF-κBp65 DNA binding activity in the absence or in the presence of competitor oligonucleotide containing wild type NF-κBp65 consensus binding site, according to the manufacturer’s instructions. Briefly, a biotinylated double stranded oligonucleotide containing the NF-κB consensus site (5-GGGACTTTCC-3) have been immobilized on streptavidin plates, nuclear extracts containing active form of NFκB specifically binds to this consensus binding site in the oligonucleotide. The bound transcription factor is detected with a specific antibody to NF-κBp65 followed by a secondary HRP-conjugated antibodies and chromogenic substrate. A sensitive colorimetric
readout is quantified by spectrophotometry at 450 nm with a reference wavelength of 655 nm. Competitive assay of DNA binding activity was performed in the presence of an excess of oligonucleotide (20 pmoles) containing a wild type of consensus-binding site to prove that the transcription isoform detected is binding specifically to the immobilized probe. Activation of NFκB was expressed as unit of OD_{450nm}.

**DNA fragmentation.** Apoptosis was evaluated by examining the characteristic pattern of DNA laddering. To assay DNA fragmentation was assayed,. Briefly, after 48 hours of treatment with 1nM leptin or 5 mM NaB alone or both compounds, HT29 cells were harvested and washed in PBS. Then, a lysis buffer containing 0.6% of SDS, 10mM EDTA, and proteinase K (1 mg/mL) were added to the cells and incubated at 37°C overnight. Afterwards, NaCl was added to a concentration of 1M, mixed by inversion and centrifuged at 9000 rpm for 20min at 4°C. Supernatants were transferred and cold ethanol was added, mixed by inversion and the mixtures were left at -80°C for 1 hr, and centrifuged at 18000 rpm for 2 hrs at 4°C. DNA pellet was air dried and resuspended in 25 µL of 10 mM Tris-Hcl/ 1 mM EDTA pH 7.4. RNAse A was added to the suspension, incubated at 37°C for 1hr and electrophoresed in 2% agarose gel containing 0.5 mg/mL ethidium bromide.

**Statistics Analysis.**

The results are expressed as means ± SD. They were compared by one-way analysis of variance (ANOVA), followed by a Tukey-Kramer multiple comparisons test if significant results were obtained. Values were considered statistically significant when p was less than
0.05.
RESULTS

HT 29 cells express leptin receptor which mediates proliferation. By confocal microscopy (Fig 1A&B), leptin-receptor immunoreactivity was detected in HT-29 cells (Fig 1A) and was mainly localized at the periphery of the cell as shown by the double fluorescent labeling with actin labeled with Phalloidin-TRITC (Fig 1B). The immunostaining was specific since leptin-receptor immunoreactivity was no longer observed in the presence of the immunogen peptide (data not shown). Using specific Ob-Rb primers, an expected amplicon of 248 bp was obtained by RT-PCR (data not shown). Thus, HT-29 cells express functional leptin-receptors (Ob-Rb) on cell membrane.

Indeed, leptin induced a dose-dependent HT29 cell proliferation with an EC$_{50}$ value of 0.35 ± 0.02 nM (Fig 1C). In addition, HT29 cell viability was measured after 48 hours-exposure to leptin (1 nM) or sodium butyrate (5mM) or both combined compounds (Fig 1D). Leptin treatment significantly increased by 1.5 fold HT29 cell proliferation (P<0.01 vs control) whereas treatment of the cells with sodium butyrate (5 mM) reduced it. In the presence of sodium butyrate, HT29 cell growth was still maintained and significantly increased by 1.6 fold upon leptin challenge (P<0.001 vs NaB). These data show that leptin acts as potent mitogen on HT29 cells growth and inhibits sodium butyrate-anti-proliferative effects.

Flow cytometric data of cell cycle analysis of serum-free control cells (Fig 2A, left panel) indicated that 92 ± 1.3% of HT29 cells are in $G_0/G_1$ phase, 3.9 ± 0.5% in S phase and
2.8 ± 0.6% in G2/M phase. When serum-free HT29 cells were treated with 1 nM leptin for 24 hours, only 70.6 ± 1.6% of HT29 cells were found in G0/G1 phase, 8.2 ± 0.2% in S phase, and 21.1 ± 0.4% in G2/M phase (Fig 2A, right panel). These data indicate that leptin is a potent mitogenic factor for HT29 cells.

These results are consistent with dot plots of cyclin D1-FITC flow of cytometry data. As shown in Fig 2B, immunoreactive cyclin D1 cells were detectable both in serum-free control- and leptin-treated cells. Immunopositive cyclin D1 cells were counted in the quadrant D2 of the dot plot. As compared with serum-free control cells, the immunopositive cyclin D1 cells were significantly higher in leptin-stimulated HT29 cells (98.2 ± 0.4% vs. 61.9 ± 3.2%; P<0.01) indicating an increased expression of cyclin D1 upon stimulation of HT-29 cells by leptin.

Propidium iodide staining allowed to associate cyclin D1 positive cells with the cell cycle phase. In serum-free control HT29 cells, the major distribution of the cyclin D1 was found in cells in G0/G1 phase, but a very faint immunopositive cyclin D1 cells in S and G2/M phase has been detected (Fig 2B, left panel). 24 hours after leptin stimulation, immunoreactivity of cyclin D1 was significantly increased by +58% (P<0.01 vs. control) and this immunoreactivity was mainly associated with G0/G1 phase of cell cycle.

Leptin activates MAPkinase and NF-κB pathways in HT29 cells. Leptin (1nM) induced a significant 2-fold increase in the ratio of phosphorylated ERK to total ERK (P<0.001 vs
control) indicating that leptin activates p44/42 MAPK dependent pathways in HT-29 cells. NaB (5 mM) alone had no significant effect on ERK phosphorylation but significantly reduced leptin-induced ERK phosphorylation (P<0.05 vs leptin alone) (Fig 3A).

Because leptin promotes HT29 cell survival upon butyrate challenge, we investigated whether NF-κB could be involved in this effect. Leptin (1nM) induced a significant 4-fold increase in NF-κB DNA binding activity (P<0.001 vs control) in agreement with our data in Figure 4. On the other hand, NaB (5 mM) alone had no effect on NF-κB DNA binding activity and did not affect leptin-induced NF-κB DNA binding activity (Fig 3B).

**Leptin induces nuclear translocation and transcriptional activity of NF-κB in HT29 cells.**

Immunoblotting analysis of whole, cytoplasmic and nuclear extracts showed an increase of transcription factor NF-κB p65 immunoreactive protein in the nuclear extracts from leptintreated cells compared to control cells (Fig 4A). In addition, in cytoplasmic extracts from leptin-treated cells, one additional immunoreactive IκB-α protein was detected compared to control (Fig 4A) suggesting that IκB-α is ongoing to degradation process. This nuclear translocation of NF-κB was confirmed by confocal microscopy studies using the co-localization LSM510 3.2 physiology software. The transcription factor NF-κB was localized in the nucleus, 30 min after exposure of the cells to leptin (*data not shown*). Thus, leptin induces translocation of NF-κB p65 into the nucleus of HT-29 cells.

DNA binding activity of the transcription factor NF-κB was also determined by EMSA. As shown in Fig 4B, only nuclear extracts from leptin-treated cells (lane 3) bind to the wild NF-
κB oligonucleotide whereas nuclear extracts from control cells (lane 4) had no DNA binding activity. In the presence of the mutant NF-κB oligonucleotide, both nuclear extracts from control (lane 1) or leptin- treated (lane 2) cells showed any detectable DNA binding activity. Taken together these data indicate that leptin promotes the nuclear translocation NF-κB and enhances its DNA binding activity in HT-29 cells.

The *in vivo* transcriptional activity of NF-κB was measured by the luciferase activity in HT29 cells transiently transfected with the (kappaB)3 IFN-luciferase plasmid. Luciferase activity was 1.5 fold increase in leptin-stimulated cells as compared to control (Fig 4D). Leptin-induced transcriptional NF-κB p65 level is similar to that obtained with serum stimulation. Therefore, leptin activates the transcription factor NF-κB via leptin receptor in HT29 cells.

**Leptin counteracts butyrate-induced apoptosis in HT-29 cells.** Morphological aspects of propidium iodide-stained HT29 cells were illustrated in Fig 5. Serum-starved HT29 cells appeared as a monolayer (Fig 5A) whereas leptin–treated HT29 cells showed some cell superposition probably due to their proliferation (Fig 5B). Treatment of the cells with sodium butyrate (5 mM) reduced HT29 cell proliferation and induced morphological changes of the nuclei characterized by chromatin condensation (Fig 5C). Flow cytometric analysis showed that sodium butyrate caused cell cycle arrest by blocking the cells in G0/G1 phase of the cell cycle (Fig 5G). Interestingly, treatment of HT29 cells with 1nM of leptin prevented sodium butyrate-induced morphological nucleus changes (Fig 5D) and suppressed butyrate-induced
cell cycle arrest in the HT29 cells (Fig 5H).

In addition, leptin prevented the butyrate induction of DNA laddering (Fig 6, lane 4) whereas leptin alone had no effect on genomic DNA (Fig 6, lane 3). This anti-apoptotic effect of leptin was also associated with HT-29 cell proliferation observed 24 hours \( \text{(data not shown)} \) and even 48 hours (Fig 1D) after butyrate exposure. Moreover butyrate-treated cells exhibited an internucleosomal degradation of genomic DNA resulting in DNA laddering (Fig 6, lane 2) in agreement with the well-known pro-apoptotic property of sodium butyrate (23,24).
DISCUSSION

In this report, we demonstrate that human colonic HT-29 cells expressing leptin-receptor can sense and respond to physiological concentration of leptin by increasing their proliferation. In addition, we demonstrate that leptin promotes HT29 cell survival from butyrate apoptosis. The leptin stimulation of HT29 cell proliferation confirms earlier results suggesting that leptin is a growth factor for colonic epithelial cells (13). These data are also closed to the in vivo results, demonstrating that leptin stimulated epithelial cell proliferation in the colon mucosa (13,25) and those reporting an enhanced DNA content in the small intestine after chronic treatment with leptin (25). In this study, we clearly demonstrate that the leptin induction of cell growth is associated with an increase of HT29 cell population in S and G2/M phase of the cell cycle. Entry into the cell cycle requires fine coordination of events from the membrane to the nucleus. Mitotic cellular division requires the cell to leave the resting state (G0) and proceed through phases of DNA synthesis (S) and mitosis (M). Two gap phase G1 and G2 lie before and after S phase, are restriction checkpoints where the activities of cyclins promote the passages through these checkpoint. Among these cyclins, cyclin D1 is a rate-limiting for cell cycle progression (26). We showed that cyclin D1 immunoreactivity was enhanced in leptin-treated HT29 cells and this increase was essentially associated with cells in G0/G1 phase of the cell cycle. This effect together with our observation that leptin-treated cells undergo to mitosis with an increase cell population in S and G2/M phase of the cell cycle, argue for a mitogenic role of leptin. The leptin stimulation of HT29 cell proliferation was
preceded by a rapid phosphorylation of ERK-1/2 proteins suggesting that MAPkinase cascade is likely to mediate this effect. This is in consistent with several reports where activation MAPkinase mediate growth in response to leptin in various cell types including epithelial cells (13), non-epithelial such as rat pancreatic β RINm5F cells (27); C2C12 muscle cells (28), immune cells (10,11) and hematopoietic progenitors (29). Moreover, we have clearly identified for the first time, the transcription factor NF-κB as a downstream target of OB-Rb signaling in HT-29 cells. The NF-κB/Rel family of transcription factors which include NF-κB1 (p50), Rel A (p65), c-Rel, and Rel-B, have been involved in the regulation of cell cycle, differentiation and protection from apoptosis through regulation of gene transcription (reviewed by (30) and (31)). In most cell types, NF-κB is sequestered in the cytoplasm in an inactive complex with IκBα or IκBβ. Diverse signals induce the phosphorylation and degradation of IκB resulting in the nuclear translocation of NF-κB and thereby enhancing DNA sequence-specific gene transcription (32,33).

We demonstrated that leptin induces an increase in NF-κBp65 immunoreactive protein in the nucleus and the appearance of an additional IκB-α immunoreactive protein in cytosol. This nuclear translocation was followed by an increase of NF-κB DNA binding and transcriptional activities in an Ob-Rb-dependent manner in HT29 cells. These data suggest that leptin activation of NF-κB signaling pathways may enhance HT29 cell proliferation through an increase expression of cyclin D1. This well fits with the identification of NF-κB binding sites on cyclin D1 promoter (34). They are also in line with recent analysis of
transformed cells (35-37) showing that NF-κB plays a critical role in cell cycle regulation through modulation of cyclin D1 expression. Taken together, we conclude that leptin induces HT29 cell growth through activation of NF-κB which in turns increases expression of cyclin D1. Whether leptin directly affect the expression of cyclin D1 in our conditions remained to be further elucidated.

In this study, we also demonstrated that leptin prevents HT29 cells from sodium butyrate-induced apoptosis. This finding can be put forward our previous report showing that leptin enhances butyrate uptake in human intestinal Caco-2 cells through the proton-linked monocarboxylate transporter MCT-1 (16). The SCFA, sodium butyrate is a metabolic product of microbial fermentation of carbohydrates and dietary fibres in the large bowel. Butyrate has considerable physiologic relevance to the integrity and function of the colonic epithelium and may be an important factor in the pathogenesis of diseases of the colonic epithelium (38,39).

Butyrate is well known to inhibit growth and stimulate the differentiation of normal and carcinoma colonic cells both in vivo and in vitro leading to apoptosis (40, 41). Consistent with previous studies, we showed that sodium butyrate inhibits cell proliferation and induces apoptosis in colon cancer HT29 cells. Interestingly, we evidence an anti-apoptotic effect of leptin which was still associated with its ability to stimulate HT-29 cell proliferation. The precise mechanisms by which leptin promotes cell survival upon butyrate challenge remain unclear. However, the reduced phosphorylation of ERK-1/2 proteins, the unaltered activation of NF-κB and the ability of butyrate-treated cells to still proliferate in response to leptin, strongly suggest that NF-κB may be the critical mediator of this effect. We conclude that
leptin promotes intestinal epithelial cell survival upon butyrate challenge by counteracting the apoptotic programs initiated by this short chain fatty acid probably through NF-κB pathways. However, we cannot formerly exclude the involvement of ERK pathways in this anti-apoptotic effect. On the other hand, leptin has been shown to promote apoptosis in human bone marrow stromal cells through activation of ERK pathways (42) and in adipose tissue (43). We have no clear explanation for these cell types discrepancies but it might be dependent on the cell signaling being targeted.

In summary, we have shown that leptin acts as a potent mitogen and anti-apoptotic cytokine on colon cancer cells. We have identified NF-κB as an effector of leptin receptor in intestinal epithelial cells. The mitogenic and anti-apoptotic effects might involved both NF-κB and ERK-1/2 pathways. Further studies are required to unravel the precise mechanisms of leptin anti-apoptotic effects.
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ABBREVIATIONS LIST

HT29, human colon cancer cells

Ob-r, leptin receptor

NFκ-B, transcription factor

DMEM, Dulbecco's MEM with glutamax, with sodium pyruvate, with 4500MG/L glucose, with pyridoxine

PI, propidium iodide

ERK, extracellular signal-regulated kinase

PBS, phosphate buffered saline solution

NaB, sodium butyrate

EDTA, ethylenediaminetetraacetic acid
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FIGURE LEGENDS

Figure 1. Leptin promotes HT29 cell proliferation via Ob-R. Panels A and B: confocal views of double fluorescent labeling of HT29 cells with a specific goat polyclonal antibodies to human Ob-R (green staining), actin labeled with phalloidin-TRITC (red staining). Panel C: dose-response effect of leptin on HT29 proliferation. HT29 cells (10^5) were seeded in 12 wells/plate in triplicate samples. The cells were serum starved for 24 hr and medium supplemented with leptin (0 to 10 nM) was added for 24 hr. The cell viability was determined by the method of trypan blue exclusion. Values are expressed as number of viable cells and represent mean ± SD of four independent experiments performed in triplicates. Panel D: HT29 cell viability in the presence of 1 nM leptin or 5 mM sodium butyrate (NaB) or combined compounds (Leptin + NaB). After 24 hr serum starvation, compound was added to serum-free medium. The growth of cells was determined at 48 hrs. Viable cells were counted in the presence of trypan blue. Values are expressed as the average of growth normalized to the number input cells and represent the mean ± SD of four independent determinations in triplicate. Each bar corresponded to the mean ± 1SEM of four different experiments performed in duplicates with * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Control; ### P < 0.001 vs. NaB alone

Figure 2. Leptin increases cyclin D1 in HT29 cells. Panel A represents cytometric flow analysis. Shown a typical cell cycle profiles of control- (left panel) or leptin-treated HT29
cells (right panel). Cells were seeded at low density in 25 cm$^2$ flask and grown at 70-80% confluence reached. Cells were subjected to propidium iodide (PI) staining. Ten thousand events were counted. The data are expressed in % the cell population in different phase of cell cycle and represented the mean ± SD of duplicate samples from six different experiments [(control cells 92.4 ± 1.3% in G$_0$/G$_1$, 3.9 ± 0.5% in S and 2.8 ±0.6 % in G$_2$/M) (leptin-treated cells 70.6 ± 1.4 % in G$_0$/G$_1$, 8.2 ± 0.2% in S and 21.1 ±0.4% in G$_2$/M)]. Panel B: typical dots of two colours of immunofluorescence analysis of ethanol-fixed HT29 cells stained with anti-cyclin D1-FITC (x axis) and propidium iodide (y axis) after 24 hr of culture in the absence of serum (left panel B : control) or in the presence of 1nM leptin (right panel B). Representatives typical dot plots of five independent experiments. Cyclin D1 positive cells are present in the quadrant D2. The values expressed the mean ± SD of cyclin D1 positive cells (control cells: 61.9 ± 3.2% and leptin-treated cells: 98.2 ± 0.4%, P < 0.001).

**Figure 3. Leptin activates MAPkinase and NF-κB pathways in HT29 cells.** Serum-free cultured HT29 cells were exposed to leptin (1nM) or sodium butyrate (5mM) or both compounds. Panel A: Cellular phosphorylated ERK and total ERK proteins 15 min after exposure of the cells to compounds using an ELISA Kit. and represent the mean ± SD of 3 independent experiments performed in triplicates. Panel B: NF-κB DNA binding activity 30 min after exposure of the cells to compounds using an ELISA Kit. Each bar correspond to the mean ± 1SEM of four different experiments performed in duplicates with *** P < 0.001 vs. control; ## P < 0.01; ### P < 0.001 vs. NaB alone.
Figure 4. Leptin induces activation of the transcription factor NF-κB in HT29 cells. Panel A:
A representative immunoblot NF-κB from whole, cytoplasmic and nuclear proteins from leptin-treated (lanes 1-3) and control cells (lanes 4-6). 50 µg of whole (lanes 1 and 4), nuclear (lanes 2 and 5) and of cytoplasmic (lanes 3 and 6) proteins were double blotted with specific rabbit polyclonal antibodies to NF-κB p65 and to IκB-α. Star indicates an additional IκB-α immunoreactive protein in the cytoplasmic extracts of leptin-treated cells. Panel B: EMSA analysis of NF-κB/DNA binding activity. Nuclear extracts from leptin-treated cells (lanes 1 and 3) or control cells (lanes 2 and 4) were prepared and incubated with biotinylated probes corresponded to the consensus-binding site for NF-κB (wild type, lanes 3 and 4) or with a substitution G to C in the DNA binding motif (mutant type, lanes 1 and 2), and their respective DNA binding activity was determined. Panel C: Quantification of NF-κBp65 activation by Elisa Kit in control or leptin-treated cells and with (n) or without (¨) oligonucleotide competitor. Panel D: in vivo NF-κB transcriptional activity, HT29 cells transfected with (kappaB)₃ IFN-luciferase plasmid were starved for 24hr, then treated with (+FBS) or without (control) serum or with 1nM of leptin for 30 min. The results are expressed as “fold increase of luciferase activity” which was calculated relative to the basal level of (kappaB)₃ reporter activity set to 1 unit and corrected for empty vector effects.

Figure 5. Leptin prevents the sodium butyrate-induced apoptosis. Starved HT29 cells were cultured in free-serum medium (panels A-D) and were exposed for 48 hours to 1 nM leptin (panel B), 5 mM of sodium butyrate (panel C), or to both compounds (panel D). Cells were
stained with propidium iodide (PI) and examined by fluorescence microscopy with a rodhamine filter set. *White arrows* indicate the condensed nuclei of apoptotic cells. Corresponding flow cytometric analysis of HT29 cell PI staining is represented on the right panels (E-H).

**Figure 6: Leptin inhibits sodium butyrate-induced DNA fragmentation in HT29 cells.**

Serum-free cultured HT29 cells were exposed to leptin (1nM) or sodium butyrate (5mM) or both compounds. Typical DNA profiles from extracted DNA of NaB-treated cells (lane 2), leptin-treated cells (lane 3), combined leptin/NaB-treated cells (lane 4) analysed by agarose gel electrophoresis. Only NaB-treated cells exhibited a typical DNA ladder. **Lane 1**: 123bp DNA size marker.
A

B

C

D

Cell number x 10^6

0 0.1 1 10
Leptin nM

Cell number x 10^6

Control Leptin NaB Leptin NaB

* ** ***

*** ###

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Figure 2

A

Control

1 nM Leptin

B

Cyclin D1-FITC
Figure A: Graph showing the ratio of phospho-ERK/total-ERK in different conditions: Control, Leptin, NaB, and Leptin + NaB. The data points are represented as bars with error bars for each condition. The comparison between groups is indicated by P<0.05 and significance levels.

Figure B: Graph showing NF-kBp65 activation (OD450 nm) in different conditions: Control, Leptin, NaB, and Leptin + NaB. The data points are represented as bars with error bars for each condition. The comparison between groups is indicated by NS and significance levels.
Leptin counteracts sodium butyrate-induced apoptosis in human colon cancer HT-29 cells via NFkappa-B signaling
Patricia Rouet-Benzieneb, Thomas Aparicio, Sandra Guilmeau, Cecile Pouzet, Veronique Descatoire, Marion Buyse and Andre Bado

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