Journal of Cellular Biochemistry 97:1317–1327 (2006)

Ghrelin Stimulates Interleukin-8 Gene Expression Through Protein Kinase C-Mediated NF-κB Pathway in Human Colonic Epithelial Cells

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Abstract Ghrelin, a newly identified gastric peptide, is known for its potent activity in growth hormone (GH) release and appetite. Although ghrelin is involved in several other responses such as stress and intestinal motility, its potential role in intestinal inflammation is not clear. Here, we show that expression of ghrelin and its receptor mRNA is significantly increased during acute experimental colitis in mice injected intracolonically with trinitrobenzene sulfate (TNBS). We found by PCR that ghrelin receptor mRNA is expressed in non-transformed human colonic epithelial NCM460 cells. Exposure of NCM460 cells stably transfected with ghrelin receptor mRNA to ghrelin, increased IκBα phosphorylation and its subsequent degradation. In addition, ghrelin stimulated NF-κB-binding activity and NF-κB p65 subunit phosphorylation, and induced IL-8 promoter activity and IL-8 protein secretion. Furthermore, our data show that ghrelin-induced IκBα and p65 phosphorylation was markedly reduced by pharmacological inhibitors of intracellular calcium mobilization (BAPTA/AM) and protein kinase C (GF 109203X). Pretreatment with BAPTA/AM or GF109203X also significantly attenuated ghrelin-induced IL-8 production. Together, our results strongly suggest that ghrelin may be a proinflammatory peptide in the colon. Ghrelin may participate in the pathophysiology of colonic inflammation by inducing PKC-dependent NF-κB activation and IL-8 production at the colonocyte level. J. Cell. Biochem. 97: 1317–1327, 2006.

Key words: ghrelin; protein kinase C; interleukin-8; inflammation

Ghrelin, a unique acylated 28 amino acid peptide, was identified recently from rat stomach extracts as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR) [Kojima et al., 1999]. In addition to its potent GH-releasing activity, ghrelin is a major regulator of food intake, energy production, and body weight. It stimulates food intake and induces obesity independent of its GH-releasing activity [Tschop et al., 2000; Nakazato et al., 2001]. In humans, the plasma levels of ghrelin increase before each meal and decrease afterwards [Cummings et al., 2002]. Furthermore, central administration of a ghrelin antibody inhibits appetite [Nakazato et al., 2001].

Ghrelin is also implicated in the stress response since it induces potent anxiogenic

Abbreviations used: GHSR, growth hormone (GH) secretagogue receptor; NF-κB, nuclear factor-κB; PKC, Protein kinase C; BAPTA/AM, 1,2-bis(*o*-aminophenooxy) ethane-N,N,N′,N′-tetraacetic acid, acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; IL-8, interleukin-8.

Grant sponsor: Clinical Nutrition Research Center at Harvard (to D.Z.); Grant sponsor: National Institute of Health (to D.Z.); Grant number: 1K01DK064920-01A1; Grant sponsor: National Institute of Health (to C.P.); Grant number: 5R01DK060729.

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Received 24 July 2005; Accepted 2 November 2005
DOI 10.1002/jcb.20744

activities in the elevated plus maze test and tail pinch stress. Furthermore, starvation stress induces ghrelin gene expression in the stomach [Asakawa et al., 2001]. The effects of ghrelin in stress appear to be mediated, at least in part, via corticotropin-releasing hormone (CRH) as administration of a CRH receptor antagonist significantly inhibits ghrelin-induced anxiogenic effects, and peripherally administered ghrelin significantly increases CRH mRNA in the hypothalamus [Asakawa et al., 2001].

In the intestine, ghrelin stimulates gastric acid secretion and motility [Masuda et al., 2000; Date et al., 2001], and accelerates gastric emptying and small intestinal transit of a liquid meal [Trudel et al., 2002]. Treatment with ghrelin also protects ethanol-induced gastric ulcers [Sibilia et al., 2003], and partially corrects hemodynamic and metabolic alterations in septic shock of rats [Chang et al., 2003]. However up to now, there is very little information about the potential role of ghrelin in intestinal inflammation, although it was reported that receptor density for ghrelin is elevated in the coronary artery of patients with coronary disease [Katugampola et al., 2002] and circulating ghrelin levels are higher in chronic liver disease patients [Tacke et al., 2003]. Here, we sought to determine whether expression of ghrelin and its receptor is altered in acute intestinal inflammation during experimental colitis in vivo and examined its effects on the NF-κB pathway and expression of the proinflammatory cytokine IL-8 in human colonic epithelial cells in vitro.

MATERIALS AND METHODS

TNBS Colitis

Eight to twelve-week-old CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) matched for sex and weight were housed under controlled conditions on a 12–12 h light-dark cycle. Mice were acclimated for at least 3 days before any experimental manipulation and fasted 12–18 h before the experiments to avoid formation of stool, but had free access to tap water. The procedure for induction of colitis in mice was performed as previously reported by us [Castagliuolo et al., 2002]. Briefly, mice were anesthetized with a mixture of ketamine (0.9 ml–100 mg/ml)) and xylazine (0.1 ml–100 mg/ml) in 9 ml of saline at a dose of 0.15 ml/20 gm body weight. A 50 μl enema of 2, 4, 6 trinitrobenzene sulfonic acid (TNBS) (Fluka, Buchs, Switzerland) in 40% ethanol or 40% ethanol alone were then injected into the rectum (3.5 cm from the anal verge) via a 1-ml syringe (Becton Dickinson, Franklin Lakes, NJ) fitted with a polyethylene cannula (Intramedic PE-20 tubing; Becton Dickinson). Body weight of individual animals at the indicated times (Fig. 1) was recorded. Mice were sacrificed at different time intervals (Fig. 1) by cervical dislocation and the colon was then removed and processed for macroscopic score analysis as described [Castagliuolo et al., 2002]. Pieces of the distal colon were weighed, measured, and snap-frozen in liquid nitrogen for RNA isolation. Animal studies were approved by the institutional animal care and use committee of the Beth Israel Deaconess Medical Center.

RNA Extraction and Real-Time PCR

Total RNA was prepared based on a standard procedure described previously [Chomczynski and Sacchi, 1987]. Equal amount of total RNA were used to detect the expression of ghrelin and GHS-R mRNA by standard RT-PCR [Date

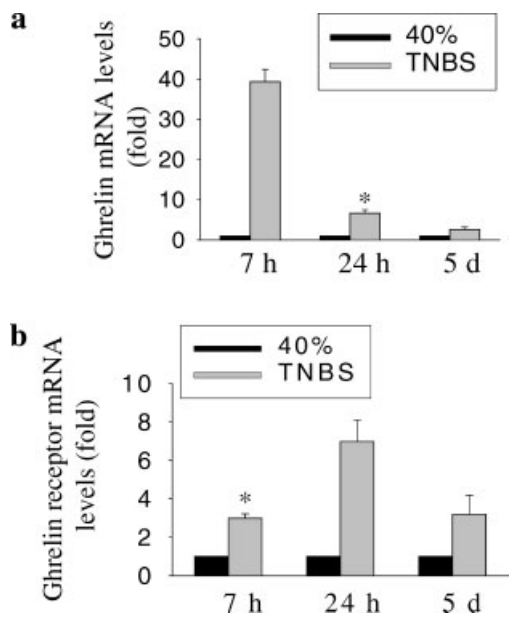

Fig. 1. Upregulation of ghrelin and its receptor during acute TNBS colitis. Mice were fasted overnight, anesthetized, and intracolonically instilled with 100 mg/kg TNBS or its vehicle (40% ethanol). All animals were then fed on regular chow diet. At the indicated times, animals were sacrificed, colons were removed, and RNA were isolated. Equal amounts of total RNA were used to measure the levels of ghrelin and ghrelin receptor mRNA by the real-time PCR technique. The relative levels of these mRNA were normalized to the equal levels of GAPDH mRNA. *denotes $P < 0.05$. Results are representative of three separate experiments, each with triplicate determinations.

et al., 2000] and to quantify the levels of ghrelin and GHS-R mRNA by real-time PCR. The procedure for real-time PCR was based on the manufacturer's instructions using single step real-time PCR reagent (Applied Biosystem Co.). The primers for mouse GHSR-1a were: 5′-CGTCCGCCTCTGGCAGTA-3′ (forward), 5′-TGGAAGAGTTTGCAGAG CAGG-3′ (reverse) and 5′-/TET/CGGCCCTGGAACTTCGGCG/36-TAMTph/-3 (probe). The primers for mouse ghrelin were: 5′-AGCCCAGCAGAGAAAGGAATC-3′ (forward), 5′-AGCCAGCCTTCCAG AGCTC-3′ (reverse) and 5′-/5TET/AAGAAGCCACCAGCTA AACTGCAGCCA/36-TAMTph/-3′ (probe).

NCM460-GHSR Cell Line

The coding region of GHSR-1a was isolated from human brain mRNA (Strategene, CA) by RT-PCR using the primers 5′-GCCTCTCACCTCCCTCTCTTTC-3′(forward) and 5′-CTCGCAATGTGCTAGGTCATG-3′ (reverse). The PCR fragment was subcloned into a TA cloning vector (Invitrogen) and its identity was confirmed by DNA sequencing. The coding region was then isolated from the TA vector and subcloned into a retroviral vector (kindly provided by Dr. Richard A. Mulligan, Children's Hospital Boston). Preparation of retroviruses expressing GHSR and infection of human colonic epithelial cells NCM460 were done as previously described by us [Zhao et al., 2001].

IL-8 Measurements

IL-8 protein levels in colonic epithelial cell conditioned media were determined by a double-ligand enzyme-linked immunosorbent assay (ELISA) using goat anti-human IL-8 (R & D Systems, Inc., MN) as described previously [Zhao et al., 2001]. Results were expressed as mean ± SE (ng/ml). At least three independent experiments were performed for each experimental condition, each with triplicate determinations.

IL-8 Promoter-Luciferase Assay

A reporter construct containing 1,521-bp (nucleotides −1,481 to +40) of the promoter region of the human *IL-8* gene has been previously described [Zhao et al., 2001]. To determine the IL-8 promoter activity in response to ghrelin, NCM-GHSR cells were seeded in 12-well plates (0.2×10^6 cells/well) overnight and transiently transfected using Effectene Transfection Reagent (Qiagen) with IL-8 promoter luciferase constructs or a control luciferase construct pRL-TK (Promega) or other DNA constructs as indicated. Transfected cells were serum-starved for 24 h followed by exposure to ghrelin for various times. Firefly and *Renilla* luciferase activities in cell extracts were measured using a Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was calculated by normalizing IL-8 promoter-driven Firefly luciferase activity to control Renilla luciferase activity. Data from all experiments were presented as relative luciferase activity (mean ± SE).

Western Blotting

Equal amounts of cell extracts were separated by SDS–PAGE (10%), and proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% non-fat, dried milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) and then incubated with antibodies specific to dual phospho-IκBα or phospho-p65 (Cell Signaling, Beverly, MA) or β-actin (Sigma) at 4°C overnight. Blots were then washed and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h. Peroxidase activities on the blots were detected by Super Signal Chemiluminescent Substrate (Pierce).

Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear extracts were prepared for DNA-binding assays as described previously [Zhao et al., 2001]. Cells were washed in PBS, collected into TNE buffer (40 mM Tris (pH 7.4), 1 mM EDTA, 0.15 M NaCl), and centrifuged (5,000*g* for 10 s). The cell pellets were incubated with buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF] for 10 min before addition of 10% NP40 for an additional 2 min. Nuclei were centrifuged (5,000*g* for 10 s), incubated with buffer B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF] for 45 min, and centrifuged at 13,000*g* for 10 min. Nuclear extracts were incubated with poly(dI-dC), band shift buffer [50 mM $MgCl_2$, 340 mM KCl, and 8 μl of delta buffer (0.1 mM EDTA, 40 mM KCl, 25 mM N-2-hydroxyethylpiperazeine-N′-2-ethananesulfonic acid (HEPES; pH 7.6), 8% Ficoll 400, 1 mM dithiothreitol] at 4°C for 15 min. ^{32}P-labeled double-stranded oligonucleotide probe (100,000 cpm) was added to the reaction mixture and incubated for 30 min on ice.

Binding of specific nuclear protein to the probe was determined by fractionating the nuclear proteins through a non-denaturing 6% polyacrylamide gel at 200 volts for 2 h at room temperature in TBE buffer [80 mM Tris-borate, 2 mM EDTA (pH 8.0)]. The gel was dried at 80°C for 2 h under vacuum before exposure to X-ray autoradiography film. The NF-κB consensus oligonucleotide (Promega) was end-labeled by T4 DNA kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P] ATP (DuPont NEN, Boston, MA).

Statistical analyses. Results were expressed as means ± SEM. Data were analyzed using the SIGMA- STAT™ professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance with protected *t*-test (ANOVA) were used for intergroup comparisons.

RESULTS

Upregulation of Ghrelin and its Receptor Expression in TNBS-Induced Colitis

Recently it was shown by RT-PCR, immunohistochemistry, and in situ hybridization that ghrelin and its receptor are expressed in normal human and animal intestine cells [Date et al., 2000; Hosoda et al., 2000; Sakata et al., 2002; Wang et al., 2002]. To examine whether their expression is altered during intestinal inflammation, we utilized a commonly used model of intestinal inflammation elicited by intracolonic administration of TNBS. We rectally infused CD-1 mice with TNBS (100 mg/kg) or vehicle control (40% ethanol) (~3.0 cm from the anal verge), as previously described [Castagliuolo et al., 2002]. After recording body weight of individual animals, the colon was removed and processed for macroscopic score analysis. TNBS-treated animals showed typical weight loss (10–20% less), diarrhea, and severe ulceration 3–5 days after treatment, although there was no significant damage to the colons after 24-h treatment (not shown). In contrast, 40% ethanol-injected mice gained similar weight to the normal mice and showed no detectable macroscopic damage (not shown). To examine the expression of ghrelin and its receptor, total RNA was isolated from colons and their mRNA levels were determined by a real-time PCR as described in the Methods. The levels of GAPDH mRNA in each RNA sample were also measured. The relative levels of ghrelin and GHSR mRNA were then normalized to the levels of GAPDH mRNA. The results show that the levels of ghrelin mRNA rapidly increased 7 h after TNBS infusion as compared to controls and declined afterwards (Fig. 1a). The levels of GHSR increased 7 h after TNBS treatment, reached the maximal levels 24 h later, and declined as compared to 40% ethanol control (Fig. 1b). This rapid upregulation of ghrelin and its receptor implies that ghrelin and its receptor interaction may participate in TNBS-induced colonic inflammation.

Activation of GHS-R Stimulates Expression of Interleukin-8

Increased expression of ghrelin receptor mRNA in the colons of animals treated with TNBS suggests that ghrelin receptor activation may play an important role in intestinal inflammation. To find out whether ghrelin has any proinflammatory effects, we examined the effect of activation of the ghrelin receptor on expression of IL-8, a potent chemotactic factor for neutrophils that is involved in the pathogenesis of intestinal inflammation [Grimm et al., 1996]. We first cloned the GHSR cDNA from human brain as described in the Methods and confirmed its identity by sequencing analysis. The cDNA was then ligated into a retroviral expression vector and the resultant plasmid was called pMMP-GHSR. Next, we examined by RT-PCR whether ghrelin receptor is endogenously expressed in human colonic epithelial cell lines including the non-transformed human colonic epithelial cell line NCM460 and colonic carcinoma cells HT-29 and Caco-2 cells. Our results indicate that ghrelin receptor mRNA is expressed in the human colonic epithelial cells (data not shown). To test whether increased ghrelin receptor levels are required for IL-8 expression in response to ghrelin, we overexpressed the ghrelin receptor in NCM460 cells using a retroviral expression vector since ghrelin receptor was dramatically upregulated in TNBS colitis (Fig. 1b). NCM460 cells were transiently transfected with pMMP-GHSR or a control plasmid pMMP-LacZ along with an IL-8 promoter-luciferase construct plus an internal control construct. The transfected cells were stimulated with ghrelin, or GHRP-6 (a functional GHSR agonist) or IL-1β (as a positive control) for 6 h, and IL-8 promoter activity was measured as described in the Methods. The results indicate that both ghrelin and GHRP-6

significantly stimulate IL-8 promoter activity, at levels comparable to that induced by IL-1β a potent IL-8 stimulus (Fig. 2a). We also examined whether ghrelin stimulates IL-8 protein production. To do this, the retroviruses expressing human GHSR were prepared using our previously described procedure [Zhao et al., 2001]. NCM460 cells were infected with the GHSR-expressing viruses (the cells named NCM460-GHSR cells). The levels of GHSR in NCM460-GHSR as well as in the parental NCM460 cells were measured by a ligand-binding assay using [^{3}H] labeled ghrelin according to the previously described procedure [Zhao et al., 2001]. The data show that the average numbers of GHSR were approximately 120,000 receptors per cell (NCM460-GHSR) compared to 1,300 receptors per cell (NCM4460). The increased levels of expression in the stable cell line may be close to its induced levels at the pathophysiological conditions. Stably transfected NCM-GHSR cells were serum starved and then treated with ghrelin (10^{-7} M), GHRP-6 (0.2 μM), or IL-1β (10 ng/ml) for 6 h. IL-8 secretion in the conditioned media was also measured as described in the Methods. The data show that ghrelin stimulation significantly induces IL-8 secretion in the NCM460-GHSR cells (Fig. 2b).

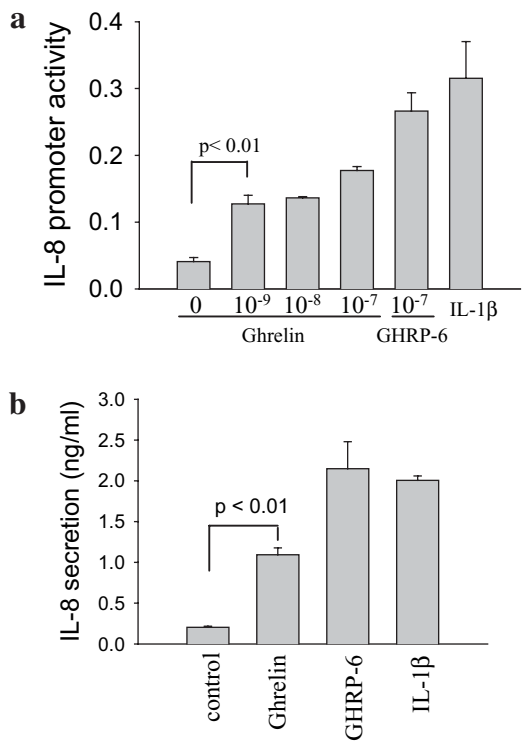

Fig. 2. Stimulation of ghrelin receptor induces IL-8 gene expression. NCM460 cells infected with GHSR-1a-expressing viruses (NCM460-GHSR) were generated as described in the Methods. **a**: NCM460-GHSR cells were transfected with the IL-8 promoter-luciferase construct together with an internal control plasmid and serum starved before stimulation with ghrelin (10^{-9}–10^{-7} μM), GHSR-1a agonist GHRP-6 (0.2 μM) or IL-1β (10 ng/ml) for 6 h. IL-8 promoter activity in cell extracts was measured. **b**: NCM460-GHSR cells were treated with ghrelin (0.1 μM), GHRP-6 (0.2 μM), or IL-1β (10 ng/ml) for 6 h. The conditioned media were used to measure IL-8 secretion by ELISA. Results are representative of three separate experiments, each with quadruplicate determinations.

Requirement of the IκB-NF-κB Pathway in Ghrelin-Induced IL-8 Gene Expression

To examine whether ghrelin-induced IL-8 gene expression involves the NF-κB pathway, NCM460-GHSR cells were transiently transfected with an IL-8-luciferase construct along with an internal control plasmid. The quiescent cells were pretreated with 10 μg/ml CAPE (a pharmacological inhibitor of the NF-κB pathway) for 30 min, treated with ghrelin (10^{-7} M) for 6 h, and then IL-8 promoter activity was measured. We found that pretreatment with CAPE markedly inhibited ghrelin-induced IL-8 promoter activity (Fig. 3a). To confirm the involvement of the NF-κB pathway in the response, we used a molecular approach by employing an endogenous inhibitor of NF-κB; IκBα NCM460-GHSR cells were transiently transfected with an IL-8-luciferase construct along with an internal control plasmid, as well as a super-repressor IκBαM- or LacZ (control)-expressing plasmid. Transfected cells were serum starved, treated with ghrelin (10^{-7} M) for 6 h, and IL-8 promoter activity in cell extracts was measured. The results indicate that expression of IκBαM significantly inhibited ghrelin-induced IL-8 promoter activity (Fig. 3b). Next, we examined whether ghrelin stimulates the signaling events in the NF-κB pathway including phosphorylation and degradation of IκBα and NF-κB DNA-binding activity as well as p65 phosphorylation. Cells were treated with ghrelin for various times, and equal amounts of cell proteins were subjected to Western blot analysis using a monoclonal antibody against dual phospho-Ser32/ Ser36-IκBα or antibodies against total IκBα or phospho-65. The results indicate that ghrelin stimulates phosphorylation and degradation of IκBα in a time-dependent manner (Fig. 4a) as well as p65 phosphorylation (Fig. 4b). To examine whether

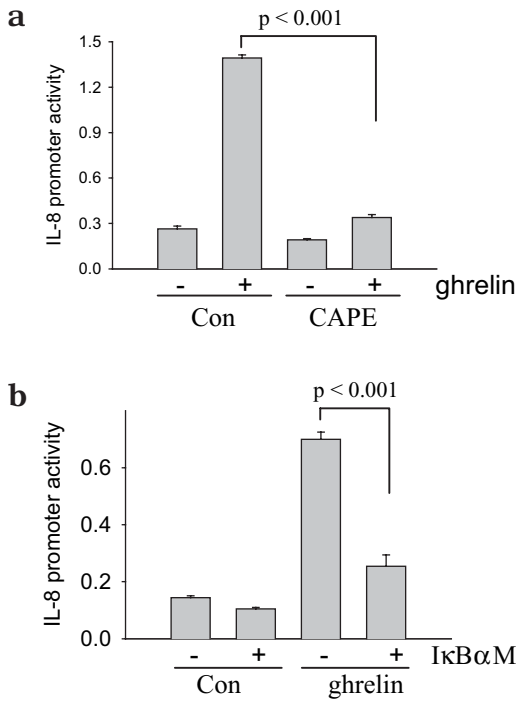

Fig. 3. Ghrelin-induced IL-8 gene expression is dependent on NF-κB activation. **a**: Quiescent NCM460-GHSR cells transfected with the IL-8 promoter construct were pretreated with CAPE (10 μg/ml) or DMSO for 30 min and then treated with ghrelin (10^{-7} M) for 6 h. **b**: Cells were transfected with the IL-8 promoter construct together with IκBαM- or LacZ-expressing plasmids and then treated with ghrelin (10^{-7} M) for 6 h. Results are representative of three separate experiments, each with triplicate determinations.

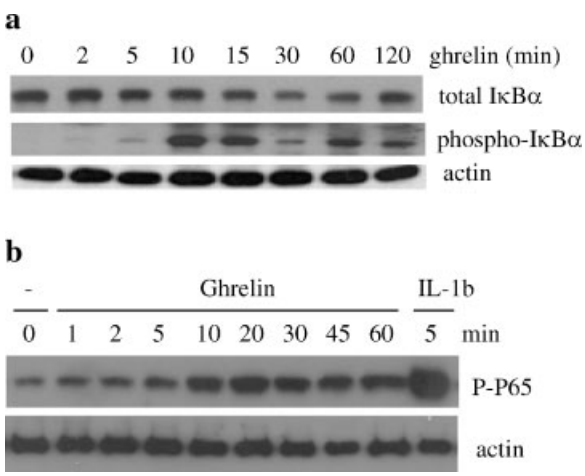

Fig. 4. Ghrelin induces IκBα phosphorylation and degradation. NCM460-GHSR cells were treated with ghrelin (0.1 μM) for the indicated times. Equal amounts of cell proteins were subjected to Western blot analysis using antibodies against either total IκBα or phosphorylated IκBα. Results are representative of three separate experiments.

ghrelin stimulates the DNA-binding activity of NF-κB, equal amount of nuclear extracts were used in electrophoretic gel mobility shift assays as previously described [Zhao et al., 2001]. We found that ghrelin induces a time-dependent increase in DNA-binding activity of NF-κB (Fig. 5).

Intracellular Calcium Mobilization and Protein Kinase C Activation Are Involved in Ghrelin-Induced NF-κB Activation and IL-8 Production

It is known that several extracellular stimuli can activate the NF-κB/IκB system through diverse signaling pathways, including intracellular calcium and protein kinase C activation. Since intracellular calcium and protein kinase C activation have been also implicated in ghrelin signaling [Kojima et al., 1999; Garcia et al., 2001], we sought to determine whether these signaling molecules are involved in ghrelin-induced NF-κB activation and IL-8 production. First, cells were pretreated with vehicle control, or different concentrations of BAPTA/AM (intracellular calcium blocker) or GF109203X (broad PKC inhibitor) for 30 min and then treated with ghrelin (10^{-7} M) for 15 min. Equal amounts of cell protein were used to determine the levels of phosphorylation of IκBα and p65. The data show that BAPTA/AM or GF109203X dramatically inhibited ghrelin-induced phosphorylation of IκBα (Fig. 6a) and p65 (Fig. 6b). Then, we also examined the effect of BAPTA/AM or GF109203X pretreatment on ghrelin-induced IL-8 production. Cells pretreated with BAPTA/AM or GF109203X were stimulated with ghrelin (10^{-7} M) for 6 h and IL-8 secretion in the conditioned media was measured. The results show that pretreatment with either BAPTA/AM or GF109203X markedly inhibited ghrelin-induced IL-8 production (Fig. 6c).

Ghrelin Potentiates Proinflammatory Cytokine-Induced IL-8 Expression

Two recent studies have showed that ghrelin pretreatment exerted an inhibitory effect on cytokine expression induced by IL-1β, TNFα, leptin, or LPS in human umbilical vein endothelial cells, human monocytes, and T cells [Dixit et al., 2004; Li et al., 2004]. To examine the effect of ghrelin pretreatment on proinflammatory cytokine-induced IL-8 expression in human colonic epithelial cells, NCM460-GHSR cells

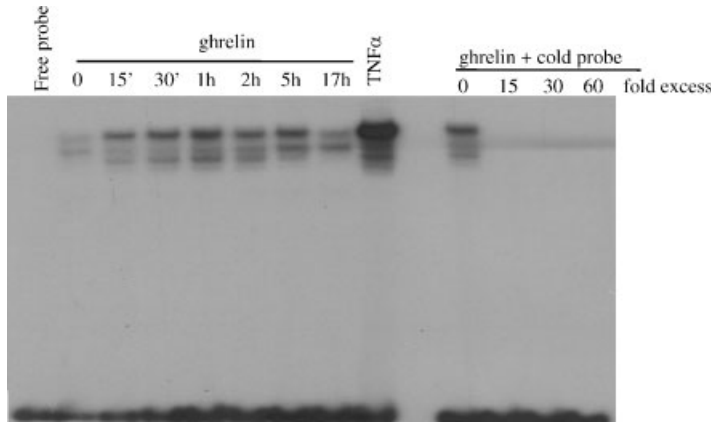

Fig. 5. Ghrelin stimulates NF-κB DNA-binding activity. NCM460-GHSR cells were treated with ghrelin (0.1 μM) for various times or TNFα (20 ng/ml) for 15 min. Equal amounts of nuclear proteins were used to determine the DNA binding activity of NF-κB by electrophoretic mobility shift assay. Results are representative of three separate experiments.

transfected with the IL-8 promoter were pretreated with ghrelin (10^{-7} M) for 2 h, then stimulated with TNFα for 6 h, and IL-8 promoter activity was determined. Consistent with our above observations, pretreatment with ghrelin alone induced IL-8 gene transcription and further increased TNF-induced IL-8 transcriptional activity (Fig. 7).

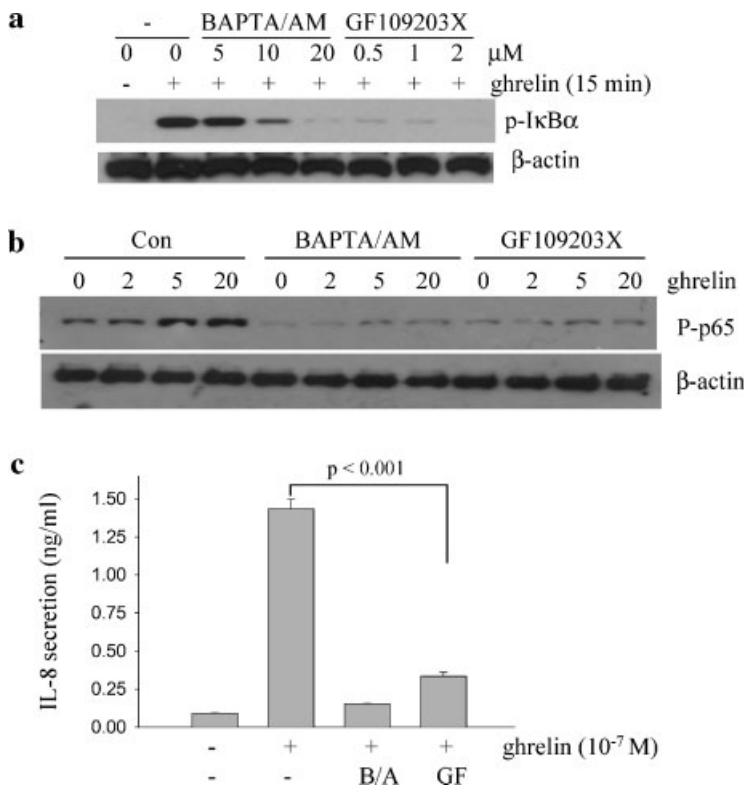

Fig. 6. Role of intracellular calcium mobilization and protein kianse C in ghrelin-induced NF-κB activation and IL-8 production. **a**: Cells were pretreated with vehicle control, or BAPTA/AM or GF109203X at the indicated concentrations for 30 min and then treated with ghrelin (10^{-7} M) for 15 min. Equal amounts of cell protein were used to determine the levels of phosphorylation of IκBα. **b**: Cells were pretreated with vehicle control, or 25 μM BAPTA/AM or 0.5 μM GF109203X for 30 min, and then treated with ghrelin (10^{-7} M) for various times. p65 phosphorylation was determined as above. **c**: Cells pretreated with 25 μM BAPTA/AM or 0.5 μM GF109203X for 30 min were stimulated with ghrelin (10^{-7} M) for 6 h. The conditioned media were used to measure IL-8 secretion. Results are representative of three separate experiments, each with triplicate determinations.

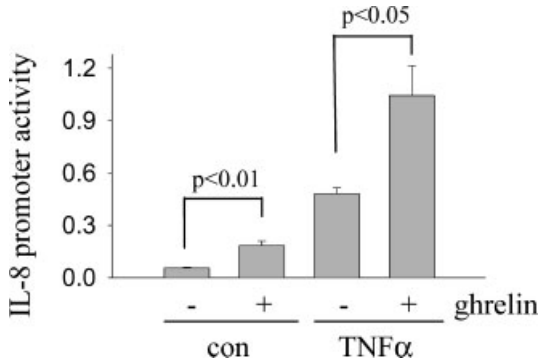

Fig. 7. Ghrelin potentiates TNFα-induced IL-8 gene expression. Quiescent NCM460-GHSR cells transfected with the IL-8 promoter construct along with an internal control plasmid were pretreated with ghrelin (0.2 μM) for 2 h and then treated TNFα (5 ng/ml) for 6 h. Equal amount of cell lysates were used to determine the relative IL-8 promoter activity as described above. Results are representative of three separate experiments, each with triplicate determinations.

DISCUSSION

In this study, we demonstrate that the levels of ghrelin and ghrelin-receptor mRNA are significantly elevated in the colon of mice during the acute phase of experimental colitis (Fig. 1). Although an increase in the mRNA levels of a gene usually correlates with a similar increase in its protein levels, it is important to confirm whether the level of ghrelin and its receptor protein are also increased during the acute phase of TNBS colitis. We will examine their in vivo expression and cellular localization by immunohistochemistry when the appropriate antibodies become available. Our RT-PCR experiment indicates that ghrelin receptor mRNA is present in non-transformed colonic epithelial NCM460 cells (not shown). Using NCM460 cells transfected with the functional GHSR, we further show the ability of the ghrelin receptor to elicit trigger a proinflammatory response, such as activation of the NF-κB/IκB pathway (Figs. 4, 5) and secretion of the potent chemoattractant IL-8 (Fig. 2) following ghrelin exposure. Our results suggest that ghrelin may play an important role in the pathophysiology of colonic inflammation. The direct involvement of ghrelin and its functional receptor interaction in intestinal inflammation will be investigated when ghrelin-deficient mice or the anti-ghrelin inhibitor (such as NOX-B11) becomes available.

We show here that ghrelin mRNA is expressed in mouse colon and highly upregulated 7 and 24 h after induction of TNBS colitis in mice, an animal model for Crohn's disease. Although previous studies have indicated the presence of ghrelin mRNA and protein in the colon of animals and humans [Date et al., 2000; Gnanapavan et al., 2002], the exact cell types synthesizing ghrelin in this organ have not been fully identified. Moreover, the mechanism(s) of regulation of ghrelin expression in the large or small bowel physiologically or during pathophysiologic conditions have not been studied. Gastric ghrelin mRNA, however, is increased after fasting or following leptin administration [Toshinai et al., 2001] and leptin levels are elevated in the early stages of experimental intestinal inflammation, including TNBS-induced colitis [Barbier et al., 1998] and *C. difficile* toxin A enteritis [Mykoniatis et al., 2003]. Thus, it is possible that increased leptin levels during acute TNBS colitis in our experiments to mediate the increased colonic ghrelin mRNA expression (Fig. 1). Alternatively, proinflammatory cytokines released during colonic inflammation could directly upregulate ghrelin expression during colitis by activating the transcription factor NF-κB or other transcription factors. This notion is strongly supported by a putative NF-κB site (−1,057 to −1,048) present on the promoter of the human ghrelin gene upstream of the translational start site [Kishimoto et al., 2003]. However, several other, not yet identified mechanisms may mediate this response.

Our finding on the presence of ghrelin receptor mRNA in normal colonic tissue (Fig. 2) is not surprising since previous studies demonstrated the presence of ghrelin receptor mRNA and protein in human colon preparations [Gnanapavan et al., 2002]. Although the cell types containing ghrelin receptor mRNA in the human colon have not yet been identified, our results demonstrate that human colonic epithelial cells contain mRNA for this receptor (not shown). Although the functional significance of ghrelin receptor overexpression in the pathogenesis of colitis has not been directly addressed in our study, the fact that ghrelin receptor stimulation activates the NF-κB/IκB system and leads to a NF-κB-dependent increase of the potent cytokine IL-8 in colonocytes opens the possibility that ghrelin could be a proinflammatory peptide in the gut. Along these lines, both activation of NF-κB and increased expression of IL-8 have been associated with the

pathogenesis and treatment of Inflammatory Bowel Disease (IBD) [Grimm et al., 1996; Jobin and Sartor, 2000].

Our results suggesting a proinflammatory role for ghrelin in intestinal inflammation are different from its potential protective function suggested by several recent studies. For example, central administration of ghrelin was able to protect ethanol-induced gastric ulcers [Sibilia et al., 2003] and isoproterenol-induced injury in myocardium [Chang et al., 2004]. Ghrelin also attenuates the development of acute pancreatitis in rats [Dembinski et al., 2003]. It is likely that this protective function may be mediated at least partially through its potent central GH-releasing activity as administration of GH is known to reduce the levels of inflammation in experimental animals [Chen et al., 1997] and IBD patients [Slonim et al., 2000], and GH deficiency is associated with increased incidence of IBD in children [Ogura et al., 1995]. Moreover the evidence presented by Sibilia et al. [2003] indicates that the inhibitory effect of centrally injected ghrelin on ethanol-induced gastric ulcers is mediated via endogenous nitric oxide release and capsaicin-sensitive sensory nerves. Other evidence suggests that the protective effect of ghrelin in some types of inflammatory processes could also be mediated by its inhibitory act on proinflammatory cytokine expression. In this regard, ghrelin was shown to inhibit leptin and LPS-induced expression of proinflammatory cytokines such as IL-1β and IL-6 and TNFα in isolated human monocytes and T cells as well as LPS-cytokine expression in vivo [Dixit et al., 2004; Li et al., 2004]. Similarly pretreatment with ghrelin inhibits IL-8 and MCP-1 production in response to TNFα or H_2O_2 in HUVEC, which appeared to be mediated via attenuation of NF-κB nuclear translocation and transcriptional activity [Li et al., 2004].

However, it is somehow difficult to imagine how ghrelin exerts the inhibitory effect on the NF-κB activation and the downstream gene expression as it was also shown to activate several signaling events including the MAP kinase and PI-3 kinase pathway in several different cell types such as preadipocytes 3T3-L1 cells [Kim et al., 2004] as well as cardiomyocytes and endothelial cells [Baldanzi et al., 2002]. In addition, ghrelin stimulates intracellular calcium mobilization and PKC activation [Kojima et al., 1999; Garcia et al., 2001]. Activation of MAP kinase, PI-3 kinase, and PKC as well as intracellular calcium release is known to promote the NF-κB pathways in many systems [Ozes et al., 1999; Zhao et al., 2001, 2005; Lilienbaum and Israel, 2003]. In consistence with these well-established observations, our results that ghrelin stimulation activates PKC-mediated NF-κB pathway in human colonic epithelial cells, although it is different from the above described cell types [Dixit et al., 2004; Li et al., 2004]. Moreover, we also examined the effect of ghrelin pretreatment on IL-8 secretion in response to proinflammatory cytokine TNFα in HUVECs at early passages. Our data indicate that ghrelin pretreatment alone had no significant effect on IL-8 secretion, however if combined with TNFα, it increased rather than inhibited TNF-α-induced IL-8 secretion in this cell type (data not shown), which support our findings that ghrelin has a proinflammatory effect.

In summary, our results indicate that in addition to its major role in body homeostasis and food intake, ghrelin may also be involved in the pathogenesis of colonic inflammation. Our results indicating that both ghrelin and its G protein-coupled receptor are upregulated locally in the colon during gut inflammation coupled with the ability of ghrelin and its human receptor to activate the global mediator of inflammation NF-κB and to stimulate release of a common proinflammatory cytokine from target cells opens up the possibility that ghrelin may be a mediator of inflammation in other organs. Our findings suggest that ghrelin may be an important peptide linking obesity and inflammation [Das, 2001].

ACKNOWLEDGMENTS

H. Zeng is a recipient of NCI Career Development Award (1K01CA098581). The authors also acknowledge Dr. Richard A. Mulligan for providing the retroviral expression system.

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