Journal of Cellular Biochemistry 102:1245–1255 (2007)

Protein kinase Cδ-mediated CREB Activation Regulates Ghrelin-induced Cyclooxygenase-2 Expression and Prostaglandin E2 Production in Human Colonic Epithelial Cells

Dezheng Zhao*

Gastroenterology Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Abstract Ghrelin, a newly identified gastric peptide, is known for its potent activity in growth hormone release and appetite. Our recent study showed that ghrelin could stimulate protein kinase C-mediated activation of nuclear factor-κB (NF-κB) and interleukin-8 secretion in human colonic epithelial cells transfected with a functional ghrelin receptor. In the present study, the effect of ghrelin stimulation on cyclooxygenese-2 expression and prostaglandin E2 production was examined. The data indicate that ghrelin significantly increased the levels of cyclooxygenase-2 (COX-2) protein as well as its promoter activity, which leaded to profound increase in prostaglandin E2 secretion. In order to examine the involvement of NF-κB and cAMP responsive element-binding protein (CREB) in this response, the NF-κB inhibitory protein IκBα or a dominant negative mutant of CREB was co-transfected into cells and the data show that transfection of either IκBα or DN-CREB significantly attenuated ghrelin-induced COX-2 expression. Moreover ghrelin stimulated phosphorylation of CREB, which was mediated primarily via protein kinase Cδ activation. Furthermore, inhibition of PKCδ function significantly attenuated ghrelin-induced COX-2 expression. In addition, ghrelin stimulates phosphorylation of PKCδ. Together, these results indicate that in addition to NF-κB, protein kinase Cδ-mediated CREB activation plays an important role in the cellular responses of ghrelin. J. Cell. Biochem. 102: 1245–1255, 2007.

Key words: ghrelin; cyclooxygenase-2; CREB; protein kinase C

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 growth hormone-releasing activity, ghrelin stimulates food intake and induces obesity independent of its GH-releasing activity [Tschop et al., 2000; Nakazato et al., 2001]. The functional ghrelin receptor was also shown to be expressed in a number of cancer cell lines to mediate several cellular responses. Ghrelin significantly increased motility of human astrocytloma cells [Dixit et al., 2006]. In human hepatoma cell lines HepG2 cells [Murata et al., 2002] and prostate cancer cells [Yeh et al., 2005], it increased mitogen-activated protein kinase activity and cell proliferation. In addition, ghrelin increased proliferation, motility, invasiveness, and Akt phosphorylation in pancreatic adenocarcinoma cell lines [Duxbury et al., 2003]. 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]. Interestingly the serum ghrelin levels in patients with inflammatory bowel diseases were three to four times higher than those in healthy people [Karmiris et al., 2006]. The endogenous role of

Abbreviations used: GHSR, growth hormone (GH) secretagogue receptor; CREB, cAMP-responsive element binding protein; NF-κB, nuclear factor-κB; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; COX-2, cyclooxygenase-2.

*Correspondence to: Dezheng Zhao, PhD, Division of Gastroenterology, Dana 501, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215.
E-mail: dzhao@bidmc.harvard.edu

Received 31 January 2007; Accepted 6 March 2007
DOI 10.1002/jcb.21355

ghrelin in intestinal inflammation has been demonstrated in ghrelin-deficient mice in which dextran sulfate sodium-induced colitis was profoundly inhibited [de Smet et al., 2006] although exogenously administered ghrelin appeared to attenuate DSS colitis [Gonzalez-Rey et al., 2006]. We recently showed that ghrelin and its receptor mRNA were significantly upregulated during the acute model of trinitrobenzene sulfonic acid (TNBS) colitis and that in GHSR-transfected human colonic epithelial cells, ghrelin stimulated protein kinase C-dependent NF-κB-mediated interleukin-8 production [Zhao et al., 2006]. Here we sought to determine whether ghrelin also stimulates expression of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) production and the signaling mechanisms mediating this effect.

COX-2 is a rate-limiting enzyme for biosynthesis of prostaglandins such as PGE2 [Ristimaki, 2004] and an important mediator of tumorigenesis [Telliez et al., 2006]. Unlike constitutively expressed COX-1, its expression can be rapidly induced by cytokines, growth factors and inflammatory cytokines [Ristimaki, 2004]. Analysis of the COX-2 promoter sequence has identified several putative transcriptional regulatory sequences including two NF-κB sites, two AP-2 sites, three SP1 sites, one C/EBP motif, and one CRE site [Appleby et al., 1994] as well as two STAT-binding GAS sites [Yamaoka et al., 1998; Koon et al., 2006]. Although NF-κB appears to be a major transcriptional factor mediating COX-2 transcription induced by many factors such as IL-1β [Crofford et al., 1997], TNFα [Chen et al., 2000], and LPS [D'Acquisto et al., 1997], other transcription factors, in particular, CREB was also found to be important for Cox-2 transcription in response to many factors like IL-1β [Kirtikara et al., 2000], UV irradiation [Tang et al., 2001], clostridium difficile toxin A [Kim et al., 2005] and bradykinin [Nie et al., 2003].

In this study, we first show that ghrelin stimulated COX-2 gene expression and PGE2 synthesis in human colonic epithelial cells transfected with a functional ghrelin receptor GHSR1a. Ghrelin also significantly increased expression of COX-2 protein in a human astrocytoma cell line which endogenously express much higher levels of the functional ghrelin receptor than primary normal human astrocytes [Dixit et al., 2006]. The induction of COX-2 expression was largely mediated by both NF-κB and CREB as shown by co-transfection experiments with the inhibitory protein IκBα and a dominant negative mutant of CREB. Moreover, ghrelin significantly stimulated CREB/ATF1 phosphorylation which was mediated by protein kinase Cδ activation. Furthermore, inhibition of PKCδ blocked ghrelin-induced COX-2 expression. The results demonstrate a new pathway in which ghrelin stimulates COX-2 expression via protein kinase Cδ-mediated CREB signaling.

MATERIALS AND METHODS

Materials

Non-transformed human colonocytes expressing human ghrelin receptor (NCM460-GHSR cells) have been described previously and cultured in M3D media containing 10% fetal bovine serum (INCELL Co., San Antonia, TX) [Zhao et al., 2006]. Human astrocytoma cell line U-87 was from ATCC and cultured according to the manufacturer's instruction. Ghrelin was purchased from Pheonix Pharmaceuticals, Inc. (Belmont, CA). The antibody against COX-2 was from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against phospho-Ser133-CREB and phospho-Thr 505-PKCδ were from Cell Signaling (Beverly, MA). The actin antibody was from Sigma. Dominant negative mutant of CREB was from BD Bioscience Clontech (Mountain View, CA) and also subcloned into our retroviral vector pCMBP [Zhao et al., 2001] to prepare retroviruses for infection experiments. The pharmacological inhibitors NS398, FR122047, GF109203X and rottlerin were purchased from EMD Bioscience-Calbiochem (San Diego, CA).

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% nonfat, 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 a COX-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-CREB-S133, phosphor-PKCδ 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).

PGE2 Measurements

PGE2 levels in colonic epithelial cell conditioned media were determined by a competitive immuno-assay kit from R&D Systems (Minnesota). Results were expressed as mean ± SE (pg/ml). At least three independent experiments were performed for each experimental condition, each with triplicate determinations.

COX-2 Promoter-Luciferase Assay

A reporter construct containing 2003-bp (nucleotides −2068 to −66 bp upstream of the translation start site) of the promoter region of the human COX-2 gene has been previously described [Koon et al., 2006]. To determine the COX-2 promoter activity in response to ghrelin, cells were seeded in 12-well plates (0.2×10^6 cells/well) overnight and transiently transfected using Effectene Transfection Reagent (Qiagen) with COX-2 promoter luciferase constructs plus a control luciferase construct pRL-TK (Promega) or along with dominant negative CREB plasmid (BD Biosciences Clontech, Palo Alto, CA). Transfected cells were serum starved for 24 h followed by exposure to ghrelin for 8 h. 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 COX-2 promoter-driven Firefly luciferase activity to control Renilla luciferase activity. Data from all experiments were presented as relative luciferase activity (mean ± SE).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA experiments were conducted based on our previously described procedure [Zhao et al., 2001] using the oligonucleotide probe derived from the human COX-2 promoter containing the CRE was used: CRE-sense, 5-AAC AGT CAT TTC GTC ACA TGG GCT TG-3′ (sense) and 5′-CA AGC CCA TGT GAC GAA ATG ACT GTT-3′ (antisense)′. Probes were annealed and 5′-overhangs were labeled by incorporation of [^{32}P]dATP (PerkinElmer Life Sciences) with T4 polynucleotide kinase. Equal amounts of nuclear protein extracts were incubated with the labeled probe and DNA/protein complexes were separated on 6% polyacrylamide gel, dried under vacuum, and visualized with a PhosphorImager (Amersham Biosciences).

In Vitro PKCδ Kinase Assay

PKCδ kinase activity in cell extracts was measured as previously described [Blois et al., 2004]. Briefly, cells were washed once in 1× phosphate-buffered saline, and solubilized in a lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 plus proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Lysates were incubated for 1 h at 4°C with anti-PKCδ antibody and then with a 40-μl slurry of protein A/G-agarose beads. The beads were washed five times with lysis buffer. The anti-PKCδ immune complexes were used to phosphorylate 1 μg of histone H1 substrate in 30 μl of kinase buffer supplemented with 5 μCi of [γ-^{32}P]ATP for 15 min at 30°C. The kinase reactions were terminated by the addition of SDS-PAGE sample buffer, boiled (5 min), and separated by SDS-PAGE. The phosphorylated histone H1 was detected by autoradiography. To control equal protein loading, equal amount of cell lysates was also subjected to Western blotting using an actin antibody.

Knowckdown of PKCδ

NCM460-GHSR cells were transfected with PKCδ siRNA vector or a control vector (kindly provided by Dr. Alex Toker in our institution, Ref. 29) by lipofectamine 2000 reagent (Invitrogen) for 48 h and then selected with neomycin for 14 days. Individual clones were isolated and expression of endogenous PKCδ, PKCα and actin was measured by Western blotting. Western blotting using PKCα antibody and actin antibody was used to control the specificity of the PKCδ siRNA specificity and equal loading.

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

Induction of COX-2 Expression and PGE2 Synthesis by Ghrelin

COX-2 is an important mediator responsible for colorectal carcinoma formation primarily by

catalyzing prostaglandin synthesis [Reddy et al., 2005]. As it was shown previously that ghrelin could stimulate production of IL-8, a potent chemokine and angiogenic factor in human colonic epithelial cells NCM460 cells transfected with a functional ghrelin receptor (NCM460-GHSR cells) [Zhao et al., 2006], we sought to examine whether ghrelin could also induce COX-2 expression and PGE2 synthesis in the human colonic epithelial cells. The results show that ghrelin induced the expression of COX-2 protein in both time- and dose-dependent manner (Fig. 1A and B), but had no significant effect on the expression of COX-1 (data not shown). This response was evident at 2 h and peaked between 4 and 8 h. Ghrelin significantly stimulated COX-2 expression at the concentration of 10^{-8} M with a maximal induction of COX-2 expression with a dose ranging from 10^{-8} to 10^{-7} M. Since the physiologic levels of ghrelin in the blood after fasting are approximately 1.5 nM [Tschop et al., 2000], its local concentration in affected tissues could be higher than that in the circulation. Therefore, 10^{-8} M ghrelin was chosen for the rest of experiments. To further examine whether ghrelin also increased transcription of COX-2 gene, cells were transiently transfected with a construct containing the 2 kb human COX-2 promoter and then treated with ghrelin (10^{-8} M) for 8 h. Ghrelin significantly increased COX-2 promoter activity (Fig. 1C, left panel). Moreover the effect of ghrelin on COX-2 mRNA levels was also determined by the quantitative-PCR technique using COX-2 specific primers and the results indicate that ghrelin significantly increased COX-2 mRNA levels (data not shown). In order to determine whether ghrelin could increase COX-2 protein expression in human astrocytoma cells which are known to endogenously express much higher levels of the functional ghrelin receptor than primary normal human astrocytes [Dixit et al., 2006], the human astrocytoma cell line U87 cells were treated with ghrelin (10^{-8} M) for the indicated times and COX-2 protein was examined by Western blotting. The data show that ghrelin significantly increased COX-2 protein expression in these cells (Fig. 1D).

Next, we examined whether ghrelin induces prostaglandin E_2 secretion in the colonocytes and determined whether the prostaglandin E_2 production is mediated by COX-2 using the selective COX-2 inhibitor NS-398. The data showed that ghrelin (10^{-8} M) increased PGE2 secretion by approximately threefold at 8 h

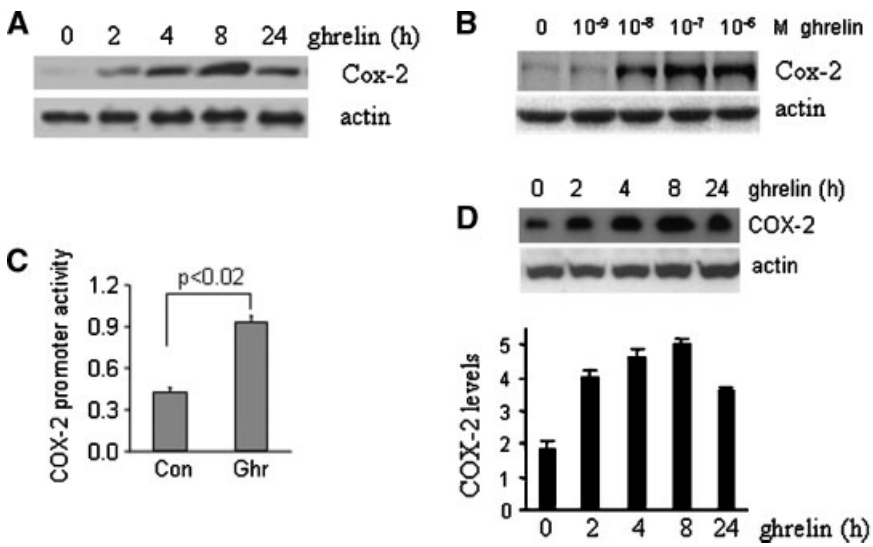

Fig. 1. Ghrelin stimulates COX-2 expression. **A:** NCM460-GHSR cells were treated with ghrelin (10^{-7} M) for the indicated times. **B:** NCM460-GHSR cells were treated with different doses of ghrelin for 8 h. Equal amount of cell protein was subjected to Western blot analysis using a monoclonal antibody against COX-2 as well as an actin antibody to show equal protein loading. **C**: NCM460-GHSR cells were transiently transfected with a COX-2 protein reporter construct along with an internal control plasmid and stimulated with ghrelin (10^{-8} M) for 8 h. The relative COX-2 promoter activity was measured. **D**: Human astrocytoma cells U-87 cells were treated with ghrelin (10^{-8} M) for the indicated times and analysis of the effect of ghrelin on expression of COX-2 and actin was carried out by immunoblotting as above. The **bottom panel** shows the quantitative analysis of ghrelin on COX-2 protein expression in U-87 cells. The results are representative of three independent experiments.

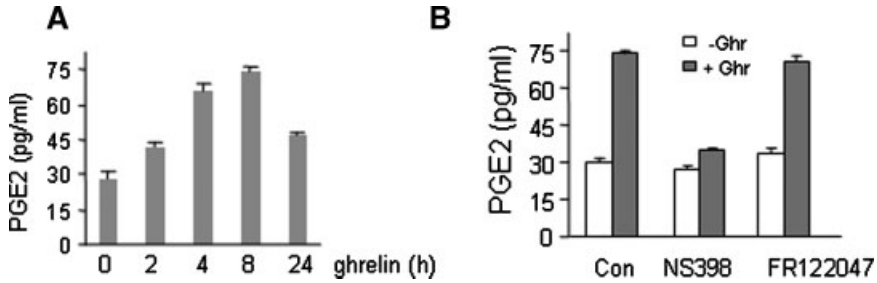

Fig. 2. Ghrelin-induced PGE2 secretion is mediated by COX-2 activity. **A**: NCM460-GHSR cells were stimulated with ghrelin (10^{-8} M) for the indicated times. **B**: NCM460-GHSR cells were pretreated with specific COX-2 inhibitors NS398 (50 μM) or the selective COX-1 inhibitor FR122047 (20 μM) for 30 min and then stimulated with ghrelin (10^{-8} M) for 8 h. The conditioned media were collected and PGE2 levels were measured as mean + SD (pg/ml). The results are representative of three independent experiments.

following the treatment (Fig. 2A), which was blocked by the selective COX-2 inhibitor NS-398 but not affected by the selective COX-1 inhibitor FR122047 (Fig. 2B). These results indicate that ghrelin-stimulated secretion of PGE2 in colonocytes required COX-2 induction.

Involvement of NF-κB in Ghrelin-induced Cox-2 Transcription and PGE2 Production

Since the transcriptional factor NF-κB is a major mediator of COX-2 gene expression in response to many extracellular factors, the importance of this NF-κB in ghrelin-induced COX-2 promoter activity was investigated. Cells were transiently transfected with the COX-2 promoter along with the inhibitory protein IκBα-expressing construct or a control plasmid and serum starved before ghrelin stimulation for 8 h. The data show that transfection of IκBα significantly reduced ghrelin-induced COX-2 promoter activity ($P < 0.01$) (Fig. 3A). To examine whether inhibition of NF-κB also affected PGE2 production, cells were infected with IκBα-expressing retroviruses or the control LacZ-expressing viruses and serum starved before exposure to ghrelin for 8 h. PGE2 secretion was measured as above. The data show that blockade of NF-κB activation also inhibited ghrelin-mediated PGE2 production (Fig. 3B).

Ghrelin-induced COX-2 Expression and PGE2 Production Involves CREB Activation

Since CREB is also involved in COX-2 expression in response to some extracellular stimuli as mentioned above, we examined whether ghrelin activates this transcription factor by looking at its serine 133 phosphorylation that is critical for CREB activation. Cells were treated with ghrelin as above for the indicated times and CREB phosphorylation was determined by western blotting using a

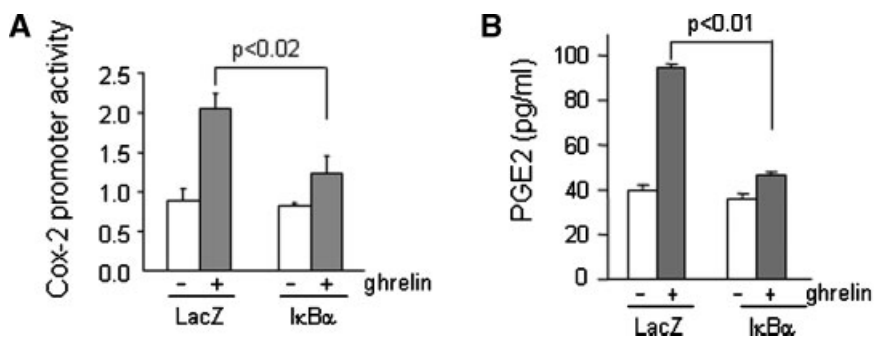

Fig. 3. Ghrelin-induced COX-2 expression and PGE secretion involves NF-κB activity. **A**: NCM460-GHSR cells were transiently transfected with a COX-2 protein reporter construct together with control LacZ or IκBα-expressing plasmid along with an internal control plasmid and stimulated with ghrelin (10^{-8} M) for 8 h. The relative Cox-2 promoter activity was measured. **B**: Cells were infected with control LacZ or IκBα-expressing retroviruses and serum starved before treated with ghrelin for 8 h. PGE2 levels in the conditioned media were measured. The results are representative of three independent experiments.

phosphor-Ser 133-specific antibody. The data show that ghrelin significantly induced CREB phosphorylation (Fig. 4A). Next we also examined whether ghrelin-induced CREB phosphorylation resulted in the increase in nuclear CREB DNA binding activity. To do this, equal amounts of nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled oligonucleotide probe corresponding to the CREB site of COX-2 promoter as described in the Methods. The results show that ghrelin significantly stimulated CREB binding to the CREB site of COX-2 promoter which was blocked by excess of the cold CREB probe, indicating the specificity of the binding (Fig. 4B). To further study whether CREB activation regulates ghrelin-induced COX-2 transcription, we used a commonly used dominant negative mutant of CREB (DN-CREB). Cells were transiently transfected with COX-2 promoter together with a control vector or DN-CREB-expressing plasmid plus an internal control plasmid and serum starved. Cells were then treated with ghrelin for 8 h. Cell lysates were harvested and luciferase activity was measured. As shown in Figure 5A, compared to control cells, co-transfection of dominant negative CREB significantly reduced ghrelin-induced COX-2 promoter activity, indicating that CREB activation is involved in ghrelin signaling to COX-2 transcription. To further determine whether the effect of DN-CREN on COX-2 gene transcription would cause corresponding reduction of COX-2 protein and PGE2 secretion, cells were infected with DN-CREB-expressing viruses or its control LacZ-expressing viruses and serum-starved before stimulation with ghrelin for 8 h. The results show that expression of DN-CREB also inhibited ghrelin-induced COX-2 protein expression (Fig. 5B) and PGE2 secretion (Fig. 5C). To further examine whether the NF-κB pathway and the CREB pathways coordinately regulate ghrelin-induced COX-2 gene transcription, cells were also transfected with both IκBα- and DN-CREB-expressing constructs together along with the COX-2-promoter construct and stimulated with ghrelin as above. The results show that inhibition of both NF-κB and CREB pathway completely diminished ghrelin-induced COX-2 promoter activity (Fig. 5D), indicating that these two pathways work independently at least in the ghrelin signaling.

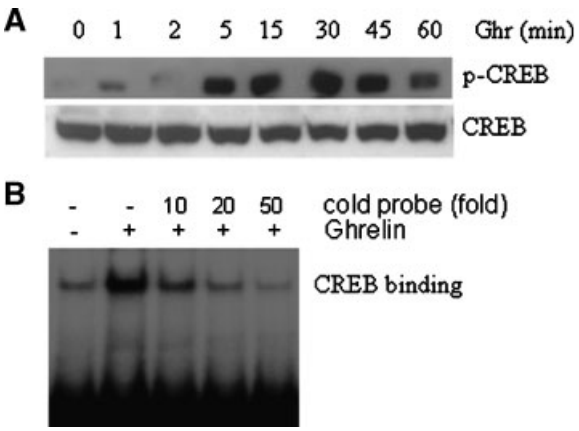

Fig. 4. Ghrelin stimulates CREB phosphorylation and DNA binding activity. **A**: NCM460-GHSR cells were treated with ghrelin as above for the indicated times. Equal amount of cell protein was subjected to Western blot analysis using rabbit polyclonal antibodies against phospho-CREB or CREB. **B**: Cells were treated with ghrelin (10^{-8} M) for 30 min and equal amounts of nuclear extracts were subjected to electrophoretic mobility shift assay using a ^{32}P-labelded oligonucleotide probe corresponding to the CREB sequence of the COX-2 promoter. Excess amounts of cold probe were also included in the binding mixture to determine the binding specificity. The results are representative of three independent experiments.

Ghrelin-mediated CREB Phosphorylation Requires Protein Kinase Cδ Activity

It was previously shown that protein kinase C is involved in ghrelin-induced NF-κB activity and IL-8 production [Zhao et al., 2006]. Thus we determined whether PKC activity is involved in ghrelin signaling to the CREB pathway. Since CREB activation in response to several extracellular stimuli involves MAP kinase pathways such as ERK or p38 MAP kinase [Iordanov et al., 1997; Swart et al., 2000; Kim et al., 2005], NCM460-GHSR cells were first pretreated with the ERK inhibitor PD98059 (20 μM) or the p38 inhibitor SB203580 (10 μM) for 30 min and then treated with ghrelin for 15 min and the CREB phosphorylation was examined. The data show that PD98059 or SB203580 only slightly affected the CREB phosphorylation (each approximately 20% inhibition, $P < 0.05$, data not shown). Our data also indicate that ghrelin significantly stimulated activation of ERK1/2 and p38 (data not shown). To examine whether ghrelin-induced CREB activation primarily involves PKCs, cells were pretreated with PMA overnight to deplete PKCs or with a general PKC inhibitor GF109203X or the PKCδ inhibitor rottlerin for 30 min and then treated with ghrelin for 15 min. The data indicate that

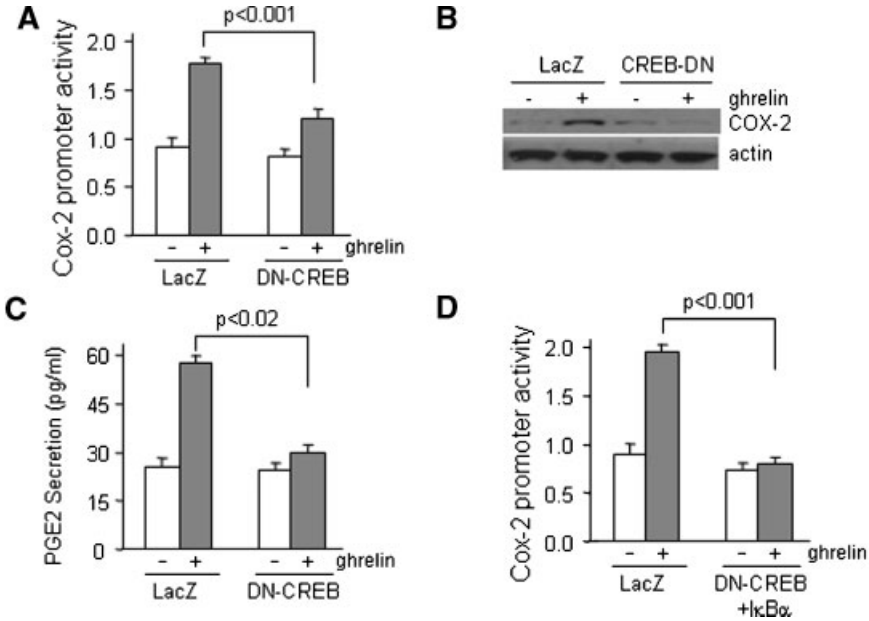

Fig. 5. Ghrelin-induced COX-2 expression and PGE2 secretion involves CREB activity. **A**: Cells were transiently transfected with the COX-2 promoter construct together with control LacZ or dominant negative CREB (K-CREB)-expressing plasmid along with an internal control plasmid and stimulated with ghrelin (10^{-8} M) for 8 h. The relative COX-2 promoter activity was measured. **B**, **C**: Cells were infected with control LacZ- or DN-CREB-expressing viruses and starved and then treated with ghrelin (10^{-8} M) for 8 h. Cell lysates and conditioned media were used to determine COX-2 protein expression (**B**) and PGE2 secretion (**C**), respectively as above. **D**: Cells were transiently transfected with the COX-2 promoter construct together with control LacZ or K-CREB and IκBα constructs along with an internal control plasmid and stimulated with ghrelin (10^{-8} M) for 8 h. The results are representative of three independent experiments.

blockade of PKCs dramatically inhibited ghrelin-induced CREB activation (Fig. 6A). The effect of rottlerin on ghrelin-induced CREB activation was concentration-dependent with an effective dose as low as to 5 μM (Fig. 6B), indicating that PKCδ is involved in the CREB pathway in response to ghrelin. To prove that PKCδ is involved in ghrelin-induced CREB activation, the state-of-art RNA interference approach was utilized. To do this, cells were transiently transfected with PKCδ-specific siRNA plasmid or control siRNA plasmid and selected with G418 to create stable cell lines expressing either PKCδ siRNA or its control siRNA. The expression of endogenous PKCδ but not PKCα was shown to be effectively knocked down by PKCδ siRNA (Fig. 6C, upper panel). Next we determined the effect of PKCδ siRNA on ghrelin-induced CREB activation. Cells expressing PKCδ siRNA or control siRNa were treated with ghrelin for 15 min. The data show that knockdown of PKCδ expression significantly inhibited ghrelin-mediated CREB phosphorylation (Fig. 6C, lower panel). To further determine whether ghrelin stimulates PKCδ activity, cells were treated with ghrelin for various times and PKCδ kinase activity was measured by an in vitro kinase assay using histone H1 as a substrate as described in the Methods. The data show that ghrelin significantly stimulated PKCδ enzymatic activity (Fig. 7A). PKCδ phosphorylation that is associated with its activity was also measured and the results show that ghrelin significantly stimulated PKCδ phosphorylation in a time-dependent manner (Fig. 7B).

PKCδ Mediates Ghrelin-induced COX-2 Expression and PGE2 Production

As PKCδ mediated ghrelin-induced CREB activity that was involved in COX-2 expression, the role of PKCδ activation in ghrelin-induced COX-2 expression was further examined. Cells were pretreated with rottlerin (10 μM) for 30 min and then treated with ghrelin for 8 h. The results showed that inhibition of PKCδ significantly reduced ghrelin-induced COX-2 expression (Fig. 8A). To further determine the role of PKCδ in ghrelin-induced COX-2 expression and PGE2 secretion, we also made use of the above described cell lines expressing PKCδ siRNA or control siRNA. The data show that compared to control siRNA-expressing cells, PKCδ siRNA transfected cells had significant low levels of

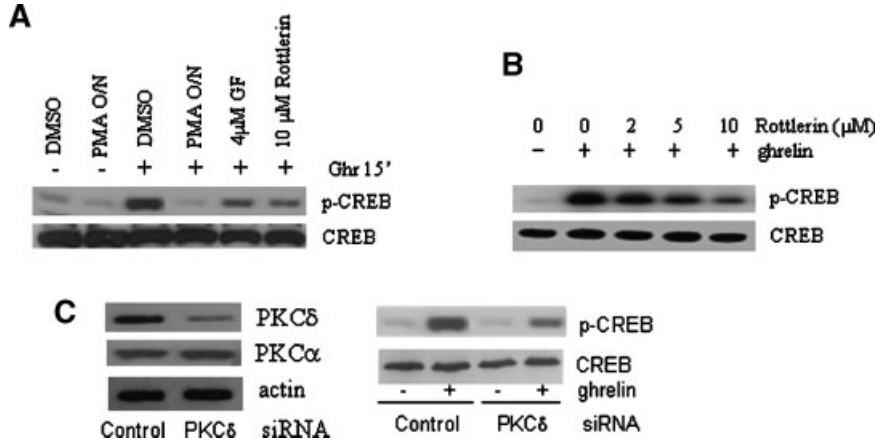

Fig. 6. Protein kinase Cδ mediates ghrelin-induced CREB phosphorylation. **A**: NCM460-GHSR cells were pretreated with PMA (1 μM) overnight to deplete PKCs or with the PKC inhibitor GF109203X (4 μM) or PKCδ inhibitor rottlerin (10 μM) for 30 min followed by exposure to ghrelin as above for 15 min. **B**: NCM460-GHSR cells were pretreated with different doses of rottlerin for 30 min and then treated with ghrelin (10^{-8} M) for 15 min. **C**: NCM460-GHSR cells expressing PKCδ siRNA or its control siRNA were prepared as in the Methods. The **left panel** shows expression of the endogenous PKCδ and PKCα as well as actin. The siRNA-expressing cells were then treated with ghrelin for 15 min (**right panel**). Equal amount of cell protein was subjected to western blotting using a phospho-CREB specific antibody or a CREB antibody to control equal protein loading. The results are representative of three independent experiments.

COX-2 promoter activity (Fig. 8B), COX-2 protein (Fig. 8C) and PGE2 production (Fig. 8D) in ghrelin-treated conditions.

DISCUSSION

Involvement of gastrointestinal neuropeptides such as neurotensin in progression of both colorectal cancer and inflammatory bowel diseases has been realized during the past decade [Yoshinaga et al., 1992; Zhao et al., 2001; Rozengurt et al., 2002; Moody et al., 2003; Chao et al., 2005]. One of mechanisms by which these neuropeptides promote colon cancer growth may involve their stimulation of COX-2 and PGE2 production, important mediators of colorectal carcinoma growth [Brun et al., 2005] in addition to the direct effect on mitogen-activated protein kinase activation [Zhao et al., 2004]. Although the newly identified gastric peptide ghrelin has not been studied for its role in colorectal cancer growth, its growth-promoting function in other types of cancer cells such as hepatoma cell line HepG2 [Murata et al., 2002] and pancreatic adenocarcinoma cell lines [Duxbury et al., 2003] has been shown recently. Interestingly, Wolf showed most recently that serum levels of ghrelin were significantly increased in cancer cachexia in breast and colon cancer patients [Wolf et al., 2006]. In addition, our recent work indicated that ghrelin receptor is expressed in human colonic epithelial cells including human colonic carcinoma cells HT29 and Caco-2 cells although its levels are relatively low and that ghrelin and its receptor were upregulated in colonic mucosa during colitis [Zhao et al., 2006]. This study also showed that ghrelin receptor stimulation induced expression of IL-8, potent tumor angiogenic factor and neutrophil chemotactic factor from human colonic epithelial cells expressing ghrelin receptor [Zhao et al., 2006], suggesting a potential role in tumor growth and colitis. The role of ghrelin in colitis is supported by two recent studies, one of which showed that serum ghrelin levels were much higher in the

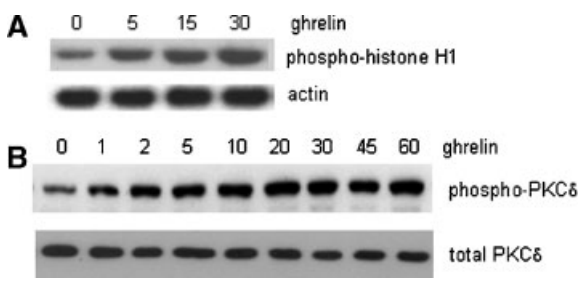

Fig. 7. Ghrelin activates PKCδ. **A**: NCM460-GHSR cells were treated with ghrelin for the indicated times. Equal amount of cell protein was subjected to PKCδ in vitro kinase assay as described in the Methods. **B**: NCM460-GHSR cells were treated with ghrelin for the indicated times. Equal amount of cell protein was subjected to Western blot analysis using an antibody against phospho-PKCδ as well as PKCδ antibody to control equal protein loading. The results are representative of three independent experiments.

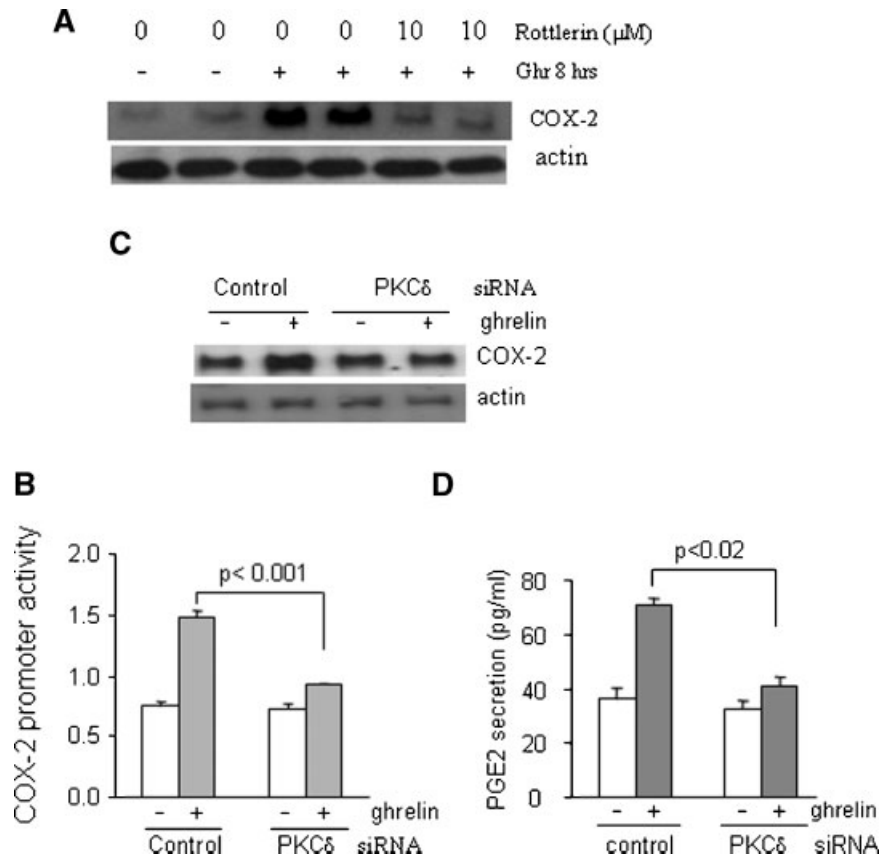

Fig. 8. PKCδ mediated ghrelin-induced COX-2 expression. NCM460-GHSR cells were pretreated with 10 μM rottlerin for 30 min and then treated with ghrelin (10^{-8} M) for 8 h. Expression of COX-2 and actin was examined as above (**A**). NCM460-GHSR cells expressing PKCδ siRNA or the control siRNA were transiently transfected with the COX-2 promoter construct as above and then treated with ghrelin for 8 h. The relative COX-2 promoter-driven luciferase activity was measured as above (**B**). NCM460-GHSR cells expressing PKCδ siRNA or the control siRNA were treated with ghrelin for 8 h. Equal amounts of cell lysates and conditioned media were used to determine COX-2 protein expression (**C**) and PGE2 secretion (**D**), respectively. The results are representative of three independent experiments.

patients with inflammatory bowel diseases than in healthy people [Karmiris et al., 2006]. More importantly, de Smet et al. have shown that dextran sulfate sodium (DSS) colitis was profoundly inhibited in ghrelin-deficient mice compared to the wild-type control [de Smet et al., 2006]. Our present study further indicates that ghrelin also stimulated expression of another important inflammation-related gene COX-2 and PGE2 production in human colonic epithelial cells by a novel PKCδ-dependent CREB pathway.

The molecular mechanism by which ghrelin stimulated COX-2/PGE2 was also investigated in the present study. Our results show that in addition to a previous described NF-κB pathway, ghrelin also activates a novel protein kinase Cδ and CREB, which further transactivates the promoter of the COX-2 gene leading to increased expression of COX-2 protein and PGE2 secretion. The gene expression of the COX-2 is known to be controlled primarily at the transcriptional level through several cis-acting elements including the κB site, the CRE site as well as the upstream STAT sites. For example, NF-κB mediates COX-2 induction by lipopolysaccharide and many other stimuli [Inoue and Tanabe, 1998]. The CRE element in the COX-2 promoter was shown to be important in the induction of COX-2 transcription mediated by protein kinase C [Pham et al., 2006], nitric oxide [Park et al., 2005], lipopolysaccharide [Caivano and Cohen, 2000], UVB irradiation [Tang et al., 2001] and *C. Difficile* toxin A [Kim et al., 2005]. Consistent with the importance of the NF-κB and CREB binding sites in COX-2 gene transcription, our results provide first evidence that these two transcriptional factors are also important for the COX-2 expression in response to ghrelin.

The molecular mechanism responsible for activation of CREB protein has been extensively studied. One of the critical mechanisms for this activation is mediated through its Serine 133 phosphorylation of CREB. A number of protein kinases have been demonstrated to mediate the Serine 133 phosphorylation. For example, MAP kinase family members p38 mediates the Serine 133 phosphorylation of CREB in response to a number of stimuli [Iordanov et al., 1997; Swart et al., 2000; Kim et al., 2005]. Protein kinase A also phosphorylates CREB [Gonzalez et al., 1989]. The present work shows that the MAP kinase members ERK1/2 and p38 are not the major protein kinases in ghrelin-mediated activation of CREB (data not shown). Instead, inhibition of PKCs largely attenuated ghrelin-induced CREB phosphorylation. Identity of the PKC isoform was also found to be a novel PKCδ. The results from the in vitro PKCδ kinase activity assay and the phospho-PKCδ immunoblotting further show that ghrelin stimulates PKCδ activation. In support of these findings, Blois et al. recently demonstrated that PKCδ-mediated CREB phosphorylation is important for B cell receptor (BCR) signaling mature B cells [Blois et al., 2004]. Phosphorylation of CREB by protein kinase C and the subsequent dimerization and transcriptional activation of CREB has been previously shown using purified CREB and protein kinase C in an in vitro setting [Yamamoto et al., 1988]. And the N-terminal fragment of CREB contains consensus recognition sequence for PKC [Gonzalez et al., 1989]. These results demonstrate that in addition to MAP kinase family, PKCs, especially PKCδ represents a novel signaling molecule in CREB activation in response to extracellular stimuli including ghrelin and B cell receptor.

In summary, our study shows for the first time that ghrelin stimulates COX-2-mediated PGE2 production and induces CREB phosphorylation and its DNA binding to the COX-2 promoter. Furthermore ghrelin-induced COX-2 gene expression involves both NF-κB and CREB pathways. Moreover, ghrelin increases protein kinase Cδ activity which leads to CREB activation and COX-2-mediated PGE2 production.

ACKNOWLEDGMENTS

We would like to thank Dr. Alex Toker for the PKCδ siRNA constructs. This work was supported by the grant from the National Institute of Health 1K01DK064920 (D. Zhao).

REFERENCES

Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. 1994. Structure of the human cyclo-oxygenase-2 gene. Biochem J 302(Pt 3):723–727.

Blois JT, Mataraza JM, Mecklenbrauker I, Tarakhovsky A, Chiles TC. 2004. B cell receptor-induced cAMP-response element-binding protein activation in B lymphocytes requires novel protein kinase Cdelta. J Biol Chem 279:30123–30132.

Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, Castagliuolo I. 2005. Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288:G621–G629.

Caivano M, Cohen P. 2000. Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. J Immunol 164:3018–3025.

Chao C, Tallman ML, Ives KL, Townsend CM Jr, Hellmich MR. 2005. Gastrointestinal hormone receptors in primary human colorectal carcinomas. J Surg Res 129:313–321.

Chen CC, Sun YT, Chen JJ, Chiu KT. 2000. TNF-alpha-induced cyclooxygenase-2 expression in human lung epithelial cells: Involvement of the phospholipase C-gamma 2, protein kinase C-alpha, tyrosine kinase, NF-kappa B-inducing kinase, and I-kappa B kinase 1/2 pathway. J Immunol 165:2719–2728.

Crofford LJ, Tan B, McCarthy CJ, Hla T. 1997. Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. Arthritis Rheum 40:226–236.

D'Acquisto F, Iuvone T, Rombola L, Sautebin L, Di Rosa M, Carnuccio R. 1997. Involvement of NF-kappaB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. FEBS Lett 418:175–178.

Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 280:904–907.

de Smet B, Thijs T, Moechars D, Podlers L, Coulie B, Peeters TL, Depoortere I. 2006. Role of endogenous ghrelin in acute and chronic DSS-induced colitis in mice. Gastroenterology 130:A229.

Dixit VD, Weeraratna AT, Yang H, Bertak D, Cooper-Jenkins A, Riggins GJ, Eberhart CG, Taub DD. 2006. Ghrelin and the growth hormone secretagogue receptor constitute a novel autocrine pathway in astrocytoma motility. J Biol Chem 281:16681–16690.

Duxbury MS, Waseem T, Ito H, Robinson MK, Zinner MJ, Ashley SW, Whang EE. 2003. Ghrelin promotes pancreatic adenocarcinoma cellular proliferation and invasiveness. Biochem Biophys Res Commun 309:464–468.

Gonzalez GA, Yamamoto KK, Fischer WH, Karr D, Menzel P, Biggs W III, Vale WW, Montminy MR. 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337:749–752.

Gonzalez-Rey E, Chorny A, Delgado M. 2006. Therapeutic action of ghrelin in a mouse model of colitis. Gastroenterology 130:1707–1720.

Inoue H, Tanabe T. 1998. Transcriptional role of the nuclear factor kappa B site in the induction by lipopoly-

saccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. Biochem Biophys Res Commun 244:143–148.

Iordanov M, Bender K, Ade T, Schmid W, Sachsenmaier C, Engel K, Gaestel M, Rahmsdorf HJ, Herrlich P. 1997. CREB is activated by UVC through a p38/HOG-1-dependent protein kinase. EMBO J 16:1009–1022.

Karmiris K, Koutroubakis IE, Xidakis C, Polychronaki M, Voudouri T, Kouroumalis EA. 2006. Circulating levels of leptin, adiponectin, resistin, and ghrelin in inflammatory bowel disease. Inflamm Bowel Dis 12:100–105.

Kim H, Rhee SH, Kokkotou E, Na X, Savidge T, Moyer MP, Pothoulakis C, LaMont JT. 2005. Clostridium difficile toxin A regulates inducible cyclooxygenase-2 and prostaglandin E2 synthesis in colonocytes via reactive oxygen species and activation of p38 MAPK. J Biol Chem 280: 21237–21245.

Kirtikara K, Raghow R, Laulederkind SJ, Goorha S, Kanekura T, Ballou LR. 2000. Transcriptional regulation of cyclooxygenase-2 in the human microvascular endothelial cell line, HMEC-1: Control by the combinatorial actions of AP2, NF-IL-6 and CRE elements. Mol Cell Biochem 203:41–51.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660.

Koon HW, Zhao D, Zhan Y, Rhee SH, Moyer MP, Pothoulakis C. 2006. Substance P stimulates expression of cyclooxygenase-2 and prostaglandin E2 through JAK-STAT activation. J Immunol 176:5050–5059.

Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun 276:905–908.

Moody TW, Chan D, Fahrenkrug J, Jensen RT. 2003. Neuropeptides as autocrine growth factors in cancer cells. Curr Pharm Des 9:495–509.

Murata M, Okimura Y, Iida K, Matsumoto M, Sowa H, Kaji H, Kojima M, Kangawa K, Chihara K. 2002. Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. J Biol Chem 277:5667–5674.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. 2001. A role for ghrelin in the central regulation of feeding. Nature 409:194–198.

Nie M, Pang L, Inoue H, Knox AJ. 2003. Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: Involvement of different promoter elements, transcription factors, and histone h4 acetylation. Mol Cell Biol 23:9233–9244.

Park SW, Sung MW, Heo DS, Inoue H, Shim SH, Kim KH. 2005. Nitric oxide upregulates the cyclooxygenase-2 expression through the cAMP-response element in its promoter in several cancer cell lines. Oncogene 24:6689–6698.

Pham H, Shafer LM, Slice LW. 2006. CREB-dependent cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression is mediated by protein kinase C and calcium. J Cell Biochem 98:1653–1666.

Reddy BS, Patlolla JM, Simi B, Wang SH, Rao CV. 2005. Prevention of colon cancer by low doses of celecoxib, a cyclooxygenase inhibitor, administered in diet rich in omega-3 polyunsaturated fatty acids. Cancer Res 65:8022–8027.

Ristimaki A. 2004. Cyclooxygenase 2: From inflammation to carcinogenesis. Novartis Found Symp 256:215–212; discussion 221–222 259–269.

Rozengurt E, Guha S, Sinnett-Smith J. 2002. Gastrointestinal peptide signalling in health and disease. Eur J Surg Suppl:23–38.

Swart JM, Bergeron DM, Chiles TC. 2000. Identification of a membrane Ig-induced p38 mitogen-activated protein kinase module that regulates cAMP response element binding protein phosphorylation and transcriptional activation in CH31 B cell lymphomas. J Immunol 164:2311–2319.

Tang Q, Chen W, Gonzales MS, Finch J, Inoue H, Bowden GT. 2001. Role of cyclic AMP responsive element in the UVB induction of cyclooxygenase-2 transcription in human keratinocytes. Oncogene 20:5164–5172.

Telliez A, Furman C, Pommery N, Henichart JP. 2006. Mechanisms leading to COX-2 expression and COX-2 induced tumorigenesis: Topical therapeutic strategies targeting COX-2 expression and activity. Anticancer Agents Med Chem 6:187–208.

Trudel L, Tomasetto C, Rio MC, Bouin M, Plourde V, Eberling P, Poitras P. 2002. Ghrelin/motilin-related peptide is a potent prokinetic to reverse gastric postoperative ileus in rat. Am J Physiol Gastrointest Liver Physiol 282:G948–G952.

Tschop M, Smiley DL, Heiman ML. 2000. Ghrelin induces adiposity in rodents. Nature 407:908–913.

Wolf I, Sadetzki S, Kanety H, Kundel Y, Pariente C, Epstein N, Oberman B, Catane R, Kaufman B, Shimon I. 2006. Adiponectin, ghrelin, and leptin in cancer cachexia in breast and colon cancer patients. Cancer 106:966–973.

Yamamoto KK, Gonzalez GA, Biggs WH III, Montminy MR. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334:494–498.

Yamaoka K, Otsuka T, Niiro H, Arinobu Y, Niho Y, Hamasaki N, Izuhara K. 1998. Activation of STAT5 by lipopolysaccharide through granulocyte-macrophage colony-stimulating factor production in human monocytes. J Immunol 160:838–845.

Yeh AH, Jeffery PL, Duncan RP, Herington AC, Chopin LK. 2005. Ghrelin and a novel preproghrelin isoform are highly expressed in prostate cancer and ghrelin activates mitogen-activated protein kinase in prostate cancer. Clin Cancer Res 11:8295–8303.

Yoshinaga K, Evers BM, Izukura M, Parekh D, Uchida T, Townsend CM Jr, Thompson JC. 1992. Neurotensin stimulates growth of colon cancer. Surg Oncol 1:127–134.

Zhao D, Keates AC, Kuhnt-Moore S, Moyer MP, Kelly CP, Pothoulakis C. 2001. Signal transduction pathways mediating neurotensin-stimulated interleukin-8 expression in human colonocytes. J Biol Chem 276:44464–44471.

Zhao D, Zhan Y, Koon HW, Zeng H, Keates S, Moyer MP, Pothoulakis C. 2004. Metalloproteinase-dependent transforming growth factor-alpha release mediates neurotensin-stimulated MAP kinase activation in human colonic epithelial cells. J Biol Chem 279:43547–43554.

Zhao D, Zhan Y, Zeng H, Moyer MP, Mantzoros CS, Pothoulakis C. 2006. Ghrelin stimulates interleukin-8 gene expression through protein kinase C-mediated NF-κB pathway in human colonic epithelial cells. J Cell Biochem 97:1317–1327.