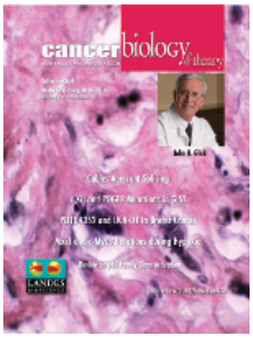

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Research Paper

Prostaglandin E_2 Drives Cyclooxygenase-2 Expression via Cyclic AMP Response Element Activation in Human Pancreatic Cancer Cells

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KEY WORDS

COX-2, CREB, PGE_2, PKA, cAMP, EP_2, pancreatic cancer

ABBREVIATIONS

COX-2	cyclooxygenase-2
PGE2	prostaglandin E2
PKA	protein kinase A
cAMP	adenosine 3', 5'-cyclic monophosphate
CREB	cAMP response element binding protein
NF-κB	nuclear factor κB
C/EBP	CCAAT/enhancer-binding protein
p-CREB	phosphorylated CREB
AA	arachidonic acid
IBMX	3 isobutyl-1-methylxanthene
EP	E prostanoid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
EMSA	electrophoretic mobility shift assay
ChIP	chromatin immunoprecipitation
PCR	polymerase chain reaction
FACS	fluorescence-activated cell sorting
siRNA	small interfering RNA

ACKNOWLEDGEMENTS

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ABSTRACT

Cyclooxygenase-2 (COX-2) is constitutively expressed in most human primary carcinomas and with its synthesized product, prostaglandin E_2 (PGE_2), appears to play important roles in tumor invasion, angiogenesis, resistance to apoptosis and suppression of host immunity. However, the molecular mechanisms that control COX-2 expression are unclear. The purpose of this study was to clarify the mechanism of basal and PGE_2-mediated COX-2 expression in the highly metastatic L3.6pl human pancreatic cancer cell line. Using RNA interference to disrupt the expression of CREB and the NF-κB p65 subunit, we found that both are involved in maintaining basal COX-2 expression in L3.6pl cells. We also demonstrated that PGE_2 increased the cyclic AMP concentration, thereby activating protein kinase A (PKA), which in turn phosphorylated the cyclic AMP response element binding protein (CREB), leading to interaction with the cyclic AMP response element in the promoter region of the COX-2 gene. Immunocytochemical analysis confirmed that PGE_2 stimulated the translocation of PKA to the nucleus and increased the immuno-reactivity of phosphorylated CREB. Pretreatment with the PKA selective inhibitor H 89 and the E-prostanoid receptor 2 inhibitor AH 6809 reduced COX-2 upregulation by PGE_2. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay results further suggested a role for CREB in COX-2 transcriptional control. Understanding the pathways that control COX-2 expression may lead to a better understanding of its dysregulation in pancreatic carcinomas and facilitate the development of novel therapeutic approaches.

INTRODUCTION

Cancer of the exocrine pancreas is one of the greatest therapeutic challenges for the clinical oncologist. The inability to detect pancreatic cancer at an early stage, the aggressiveness of the disease and the lack of effective systemic therapies are responsible for nearly identical incidence and mortality rates.[1,2] Research efforts have increasingly focused on the elucidation of the molecular processes and signaling pathways that become dysregulated in cancer cells and are responsible for the aggressive behavior of pancreatic cancer and its resistance to chemotherapy.

Prostaglandin endoperoxide synthase, commonly referred to as cyclooxygenase (COX), catalyzes the double oxygenation and reduction of arachidonic acid (AA), after its release from membrane glycerophospholipids by phospholipase A_2 (PLA_2), to the intermediate form prostaglandin H_2 which is further metabolized to form prostaglandins.[3] Currently, there are three known COX isoforms COX-1, COX-2 and COX-3 (a splice variant of COX-1). COX-1 is a ubiquitously and constitutively expressed isoform that is postulated to have "housekeeping" functions. In contrast, COX-2 is encoded by an early-response gene and can be rapidly induced by growth factors, cytokines, inflammatory mediators and tumour promoters.[4-6]

In recent years, overexpression of COX-2 has been reported in a variety of human malignancies, including lung, colorectal, prostate and breast cancer.[7-11] Increased expression of COX-2 has also been observed in pancreatic cancer, irrespective of histological type and grade, but the mechanisms that control the constitutive and induced expression of COX-2 in human pancreatic carcinoma cells are not completely understood.[12-17] The transcriptional activation of COX-2 is mediated by the binding of transcription factors to regulatory elements in the COX-2 promoters, such as nuclear factor κB (NF-κB), CCAAT/enhancer-binding protein (C/EBP), cyclic AMP response element-binding protein (CREB) and nuclear factor-activated T-cell/AP-1, which are involved in COX-2 induction in response to a variety of stimuli in different species and cell types.[18-23] For example, the C/EBP family of transcription factors plays an important role in COX-2 induction by lipopolysaccharide and phorbol ester in human vascular endothelial cells,[18] by tumor necrosis factor-α in

murine MC3T3-E1 osteoblastic cells,[19] and in mouse skin carcinoma cells.[20] Transcription factor NF-κB has been reported to mediate COX-2 induction by lipopolysaccharide in differentiated U937 monocytic cells[21] and by tumor necrosis factor-α in the MC3T3-E1 cell line.[19] CRE has been implicated in COX-2 induction by bradykinin in human pulmonary artery smooth muscle cells[22] and in phorbol 12-myristate 13-acetate-mediated differentiation of the human monocytoid U937 cells,[23] but none have looked at the involvement of the CRE in PGE_2-mediated COX-2 induction.

Using L3.6pl pancreatic cancer cell line, we explored the role of CREB in the constitutive and PGE_2-induced COX-2 expression. We found that not only was CREB involved in controlling the basal COX-2 expression, it also plays a critical role in PGE_2-induced COX-2 expression mainly through a PGE_2-cAMP-dependent mechanism.

MATERIALS AND METHODS

Reagents and antibodies. PGE_2, SC-19220, AH-6809, cAMP EIA kit and PKA assay kit were purchased from Cayman Chemicals (Ann Arbor, MI). PS-341 was provided by Millenium Pharmaceuticals (Cambridge, MA). H-89 was obtained from Calbiochem (La Jolla, CA) and forskolin and 3 isobutyl-1methylxanthene (IBMX) were purchased from Sigma (St. Louis, MO). siGenome SMARTpool reagent for human CREB1 and the p65 subunit of NF-κB were purchased from Dharmacon (Lafayette, CO) and oligofectamine was bought from Invitrogen (Carlsbad, CA). Anti-COX-2 monoclonal antibody was from Cayman Chemicals, anti-CREB polyclonal antibody and anti-phosphorylated CREB (p-CREB) (ser133p) monoclonal antibody were from Upstate Biotechnology (Lake Placid, NY) and anti-PKA catalytic subunit monoclonal antibody was from BD Pharmigen (San Diego, CA).

Cell line. The L3.6pl human pancreatic cancer cell line was established from Colo-357 cells as previously described.[24] Cells were maintained as adherent monolayers in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine and a vitamin solution in an atmosphere containing 5% CO_2 at 37°C under sterile conditions.

Small interfering RNA (siRNA) transfection. To evaluate the role of CREB and NF-κB in basal COX-2 expression, siRNA transfection was performed. Cells were grown in six-well plates in 10% MEM until they reached 40% confluence. siRNA was transfected using oligofectamine (Invitrogen) in serum-free media without antibody. After 48 h of incubation, the cells were harvested and western blot analysis was performed.

Western blot analysis. Cells were grown to 75–80% confluence in 10% MEM, serum starved for 24 h, treated and then lysed as described previously.[25] Lysates were mixed with Laemmli's reducing sample buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred onto nitrocellulose membranes. Blots were blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween 20 for 2 h at 4°C. The blots were then probed overnight with relevant antibodies, washed and probed with species-specific secondary antibodies coupled to horseradish peroxidase (Amersham Biosciences, UK). Immunoreactive material was detected by enhanced chemiluminescence. Quantification of band intensity was performed using UN-SCAN-IT gel software version 5.1 (Silk Scientific, Orem, UT).

cAMP assay. To study the effects of exogenous PGE_2 on intracellular cAMP concentrations, experiments were performed in MEM containing 1% serum with cells grown on 100 mm tissue culture dishes. For dose-response assays, cells were incubated for 3 min with 1 mM IBMX at 37°C and then exposed to different concentrations of PGE_2 (0–10 μM) for 10 min. For time-course studies, cells were treated with 10 μM PGE_2 for up to 120 min without exposure to IBMX. In all experiments, cells were lysed by the addition of 0.1 N HCl and incubated for 30 min at room temperature. The lysed cells were then scraped into microcentrifuge tubes and centrifuged at 1,000 x g for 10 min. The supernatants were collected and subjected to enzyme immunoassay for cAMP according to Cayman Chemical's recommendations.

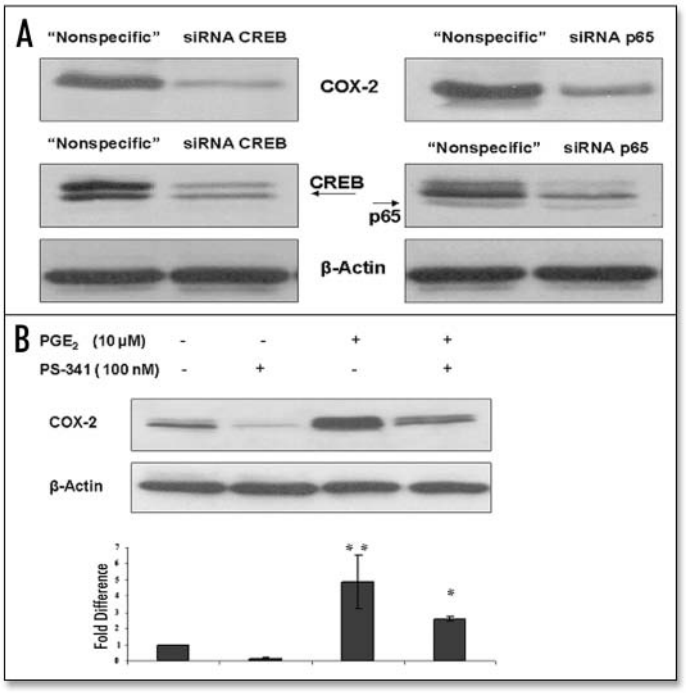

Figure 1. Role of NF-κB and CREB in the basal COX-2 expression and effect of NF-κB inhibition on PGE_2-induced COX-2 expression. (A)The role of CREB and NF-κB in the basal COX-2 expression was evaluated using siRNA against CREB and the NF-κB p65 subunit. Both are involved in maintaining basal COX-2 expression in L3.6pl cells, since a reduced COX-2 basal expression was observed. (B) Top panel L3.6pl cells were treated with 100 nM PS-341 for 12 h in the absence or presence of 10 μM PGE_2. Cells were lysed, and western blot analysis was performed using monoclonal anti COX-2 antibody. The decrease in COX-2 expression by PS-341 was reversed by 4 h of treatment with PGE_2. Equal amounts of proteins were loaded in all lanes, as confirmed by ≤ actin blotting. Bottom panel Corrected intensities relative to that of untreated cells (set at 1). Data are means ± S.E.M of three separate experiments. $p < 0.05$ and $p < 0.01$ vs. untreated cells.

PKA kinase activity assay. Cells were washed twice with ice-cold PBS, resuspended in cold sample preparation buffer (50mM Tris-HCL, 50mM β-mercaptoethanol, 10mM EGTA, 5mM EDTA, 1mM PMSF, 10mM Benzamidine, pH 7.5), and sonicated with a sonic dismembrator (Fisher Scintific, Pittsburgh, PA) at 30% maximum power for five 10-s pulses on ice. Lysates were centrifuged at 10,000 x g for 60 min, after which the supernatant was collected and subjected to the PKA assay according to Cayman Chemical's recommendations.

Immunocytochemical analysis and confocal imaging. Translocation of the PKA catalytic subunit from the cytosol to the nucleus was determined by confocal microscopy. The subsequent activation (i.e., phosphorylation) of CREB in the nucleus was also evaluated. Untreated or stimulated adherent cells on slides were fixed with 4% formaldehyde for 15 min, washed with phosphate-buffered saline (PBS) and then permeabilized with 0.2% Triton X-100 for 10 min. After permeabilization, the cells were blocked with a solution of 1% normal goat serum and 5% normal human serum in PBS for 20 min and incubated overnight with a mouse monoclonal antibody against the PKA catalytic subunit or against p-CREB. Goat anti-mouse immunoglobulin G (IgG) antibody (Alexa 488; Molecular Probes, Eugene, OR) was used to visualize protein localization. The nucleus was counterstained with To-Pro-3 (Molecular Probes). Fluorescent bleaching was minimized by mounting slides with Prolong antifade agent (Molecular Probes). Confocal fluorescence microscopy was performed using a Zeiss LSM510 microscope (Oberkochem, Germany).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). After drug treatment, cells were washed twice with ice-cold PBS

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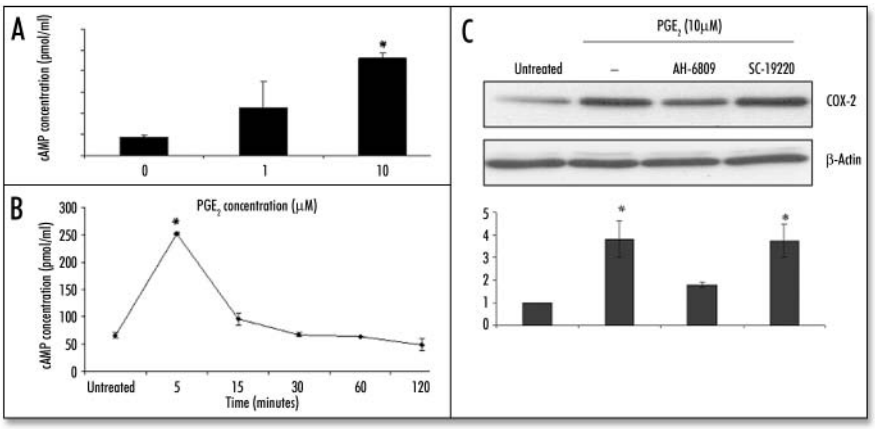

Figure 2. PGE_2 elevates intracellular cAMP concentrations through the EP_2 receptor activation in L3.6pl cells. (A) L3.6pl cells were incubated for three min with 1 mM IBMX at 37°C and then exposed to 0–10 μM PGE_2 for 10 min. A concentration dependent increase in cAMP was observed. (B) Cells were treated with 10 μM PGE_2 without IBMX for up to 120 min. cAMP production increased significantly within 5 min after PGE_2 application but decreased thereafter. Data are means ± S.E.M. of three separate experiments. $p < 0.05$ vs. untreated cells. (C) Top panel L3.6pl cells were pretreated for 30 min with the EP_1/EP_2 receptor inhibitor AH-6809 (25 μM) or with the EP_1 receptor antagonist SC-19220 (25 μM) before the addition of 10 μM PGE_2. Results from western blot analysis show that AH-6809 but not SC-19220 blocks PGE_2-induced COX-2 expression. Equal amounts of proteins were loaded in all lanes, as confirmed by actin blotting. Bottom panel Corrected intensities relative to untreated cells (set at 1). Data are means ± S.E.M. of three separate experiments. $p < 0.05$ vs. untreated cells.

and scraped. The cells obtained by centrifugation for five min at 1,500 x g were incubated for 30 min on ice in ice-cold buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$ and protease inhibitors). The nuclei obtained by centrifugation for 2 min at 8,000 x g were extracted by 30 min incubation in ice-cold buffer C (20 mM HEPES, 25% glycerol, 450 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and protease inhibitors). The extracts were then centrifuged at 14,000 x g for 10 min, and the supernatant was used for EMSA. The double-stranded oligonucleotide containing the consensus sequence for CRE (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and was then purified in Microspin G-25 columns (Amersham Biosciences) and used as probes for EMSA. The protein-DNA complexes were resolved by PAGE (5% gel) in EMSA buffer (500 mM Tris, 200 mM glycine and 1 mM EDTA) and visualized by autoradiography. In competition studies, a 100-fold excess of unlabeled oligonucleotide was included in the reaction mixture along with the radiolabeled probe.

Chromatin immunoprecipitation (ChIP) assay and polymerase chain reaction (PCR). To detect the in vivo association of nuclear proteins with the human COX-2 promoter, ChIP assay was conducted using the protocol described by Upstate Biotechnology with some modifications. In brief, L3.6pl cells were serum starved for 24 h, and then protein-DNA complexes were fixed by 1% formaldehyde in PBS. The cells were washed twice with ice-cold PBS containing protease inhibitors and scraped into a conical tube, centrifuged for five min at 2,000 x g at 4°C, resuspended in 300 μl of lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1), and placed on ice for 10 min. The cell lysates were then sonicated at 30% maximum power for four 10-s pulses on ice. The debris was removed by centrifugation, and the supernatant was diluted with 1.1 ml of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 16.7 mM NaCl and proteinase inhibitors, pH 8.0). Chromatin solutions were incubated overnight at 4°C with anti-CREB antibody, and the immune complexes were mixed with 60 μl of salmon sperm DNA/protein A agarose slurry for 1 h. Normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used instead of specific antibody in the negative control. The protein A agarose-antibody-histone complex was pelleted by gentle centrifugation (2,000 x g at 4°C for 1 min), and the pellet was extensively washed and eluted by resuspension in freshly made elution buffer (1% SDS and 50 mM $NaHCO_3$). Twenty microliters of 5 M NaCl was added to the supernatant, and the mixture was incubated for 4 h at 65°C to reverse histone-DNA cross-linking. The DNA was extracted using a Qiagen kit (Qiagen, Santa Clarita, CA) and analyzed by PCR using specific COX-2 promoter primers: 5' primer– 709CTGTTGAAAGCAACTTAGCT–690 and 3' primer–32AGACTGAAAACCAAGCCCAT–51. The resulting product, which was 678 bp long, was separated by agarose gel electrophoresis.

Statistical analysis. All assays performed in this study were conducted at least three times, and the results were found to be reproducible. Numeric data are presented as means ± S.E.M. Comparisons between test and control groups were evaluated using Student's t test. Statistical significance was set at $p < 0.05$.

RESULTS

Role of NF-kB and CREB in basal COX-2 expression and effect of NF-kB inhibition on COX-2 expression induced by PGE_2. The transcriptional activity of COX-2 is regulated by several transcription factors, and the promoter elements responsible for COX-2 transcription differ depending on the cell type studied.[18-23] By using RNA interference to disrupt expression of CREB and the NF-kB p65 subunit, we showed that both are involved in maintaining basal COX-2 expression in L3.6pl cells (Fig. 1A). NF-kB is constitutively activated in most (>70%) human pancreatic cancer cell lines and primary tumor specimens.[26] The promoter region of the human COX-2 gene contains two NF-kB consensus sites, so to evaluate the contribution of NF-kB on COX-2 expression induced by PGE2 in pancreatic cancer cells, we treated L3.6pl cells with 100 nM bortezomib (PS-341), a dipeptidyl boronic acid that specifically inhibits the 26S proteosome and, hence, blocks IJB protein degradation and subsequent NF-kB activation.[27] In our experiment, 12 h of treatment with PS-341 reduced baseline COX-2 expression; however, this inhibitory effect was reversed by treatment with 10 μM PGE_2 (Fig. 1B). These findings suggest a redundancy in the signaling pathways and promoter elements regulating COX-2 expression in PGE_2 treated L3.6pl cells.

Blocking EP_2 receptor activation suppresses PGE_2-dependent COX-2 expression. PGE_2 elicits different signaling pathways depending on the receptor to which it is coupled.28 This work focused on EP_2 as the major PGE_2 receptor, which upon ligand binding, activates intracellular adenylyl cyclase with subsequent accumulation of cAMP and activation of protein kinase A (PKA).[29] To determine whether PGE_2 was increasing cAMP production and thereby activating the PKA pathway and CRE-mediated transcription in our experiment, we measured cAMP levels in L3.6pl cells after PGE_2 treatment. The concentrations were significantly higher after treatment with 10 μM PGE_2 than with no PGE_2 (Fig. 2A). In an experiment in which cells were treated with 10 μM PGE_2 for up to 120 min, the cAMP concentration peaked at five min and returned to baseline by 15 min (Fig. 2B). To determine whether EP_2-mediated the upregulation of COX-2 expression by PGE_2, we stimulated L3.6pl with 10 μM PGE_2 in the presence or absence of a combined EP_1/EP_2 receptor antagonist (AH-6809) or selective EP_1 receptor antagonist (SC-19220). While AH-6809 (25 μM) reduced the effect of PGE_2 on COX-2 expression level, the selective EP_1 receptor antagonist SC-19220 (25 μM) had no effect, suggesting that the EP_2 receptor was involved in PGE_2-mediated COX-2 expression (Fig. 2C).

PGE_2-stimulated induction of COX-2 Is mediated by PKA-dependent CREB phosphorylation. To test whether the COX-2 induction by PGE_2 was accompanied by PKA activation, translocation of its catalytic subunit to the nucleus and successive CREB phosphorylation in L3.6pl cells, PKA kinase activity assay, immunocytochemical and western blot analyses were performed. An increase on PKA activity was observed within 15 min of

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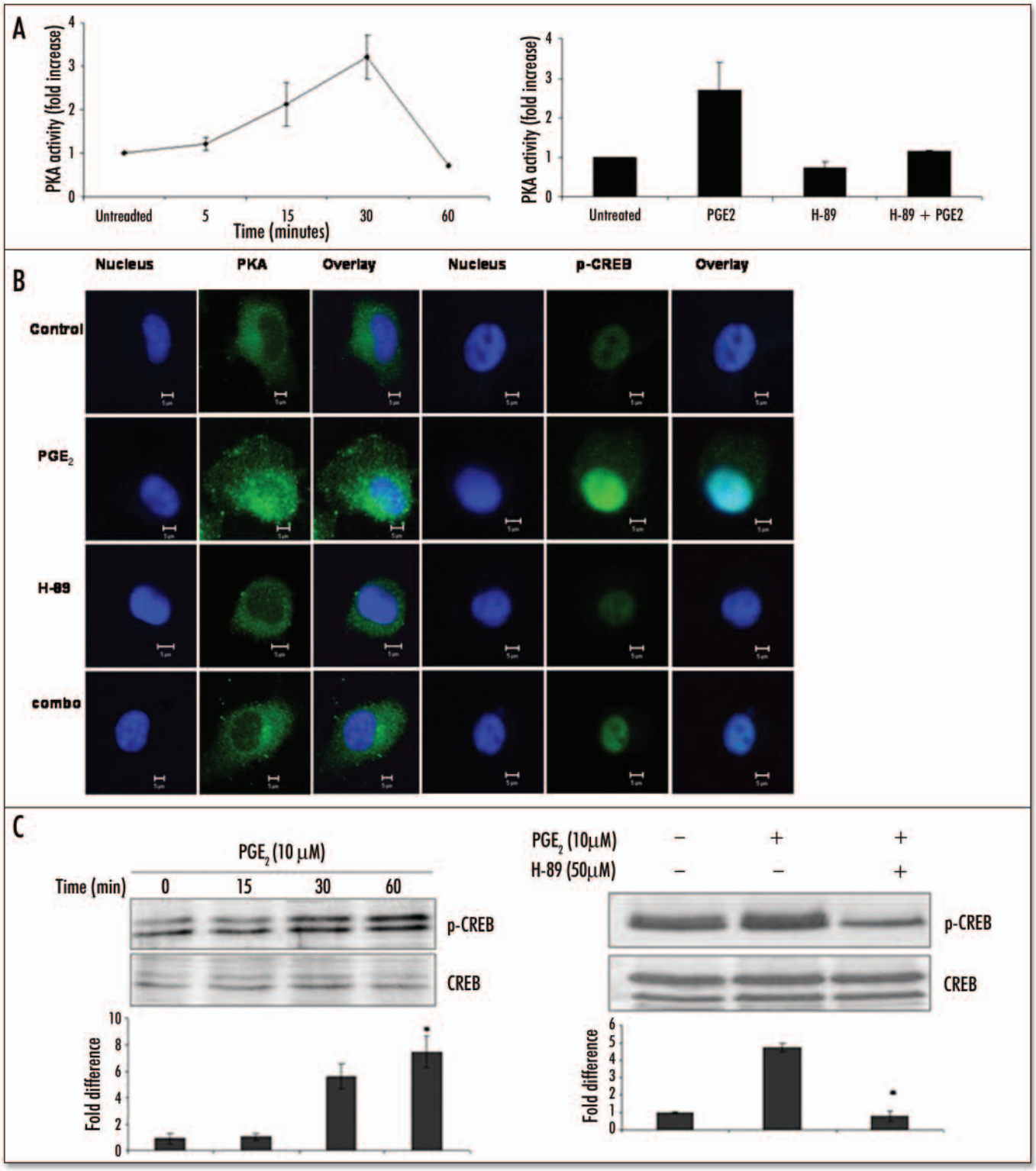

Figure 3. COX-2 expression by PGE_2 is associated with PKA activation, translocation of its catalytic subunit to the nucleus and successive CREB phosphorylation in L3.6pl cells. (A, left panel) An increase on PKA kinase activity was observed within 15 min of PGE_2 addition, reached a peak after 30 min, after which it decreased. (A, right panel) PGE_2 increases PKA activity whereas pretreatment with H-89 completely blocked PKA activation. (B) Confocal images of PKA catalytic subunit staining after 30 min stimulation with 10 μM PGE_2 revealed a redistribution of the catalytic subunit from a primarily perinuclear distribution in untreated L3.6pl cells to a nuclear localization in PGE_2 treated cells. Pretreatment with H-89 inhibited nuclear translocation. Thirty min of treatment with 10 μM PGE_2, strongly increased p-CREB immunoreactivity in the nuclei whereas treatment with 50 μM H-89 for 30 min alone or before the addition of PGE_2 reduced p-CREB levels. Scale bar, 5 μm. (C, left panel) Phosphorylation of CREB was induced by 30 min of treatment with 10 μM PGE_2 and was still seen after 60 min of treatment. The ratio between p-CREB and CREB protein (untreated cells set at 1) is also shown. Data are means ± S.E.M. of three separate experiments. $p < 0.05$ vs. untreated cells. (C, right panel) Pretreatment with 50 μM H-89 for 30 min before the addition of PGE_2 reduced CREB phosphorylation levels. The ratio between p-CREB and CREB protein (untreated cells set at 1) is shown. Data are means ± S.E.M. of three separate experiments. $p < 0.05$ vs. cells treated with PGE_2 but not H-89.

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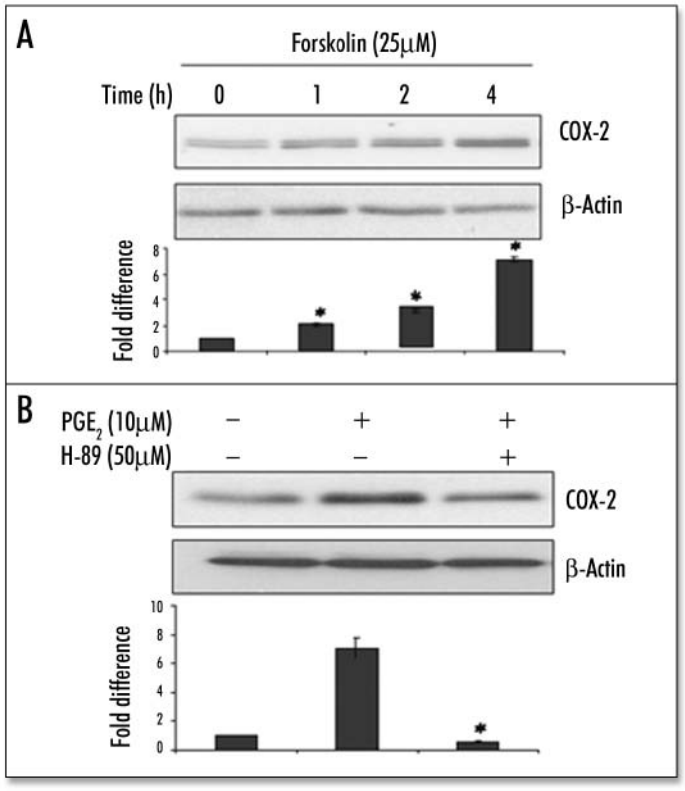

Figure 4. Expression of COX-2 by PGE_2 is a PKA-dependent event. (A) Top panel L3.6pl cells were treated with 25 μM forskolin for up to 4 h. The cells were lysed, and western blot analysis was performed using monoclonal anti COX-2 antibody. An increase on COX-2 expression in a time-dependent manner was observed. Bottom panel Corrected intensities relative to those in untreated cells (set at 1). Data are means ± S.E.M. of three separate experiments. $p < 0.05$ vs. untreated cells. (B) Top panel L3.6pl cells were pretreated for 30 min with the PKA inhibitor H-89 (50 μM) in the presence of 10 μM PGE_2. The increase in COX-2 expression by PGE_2 was suppressed by H-89. Bottom panel Corrected intensities relative to those in untreated cells (set at 1). Data are means ± S.E.M. of three separate experiments. $p < 0.05$ vs. cells treated with PGE_2 but not H-89.

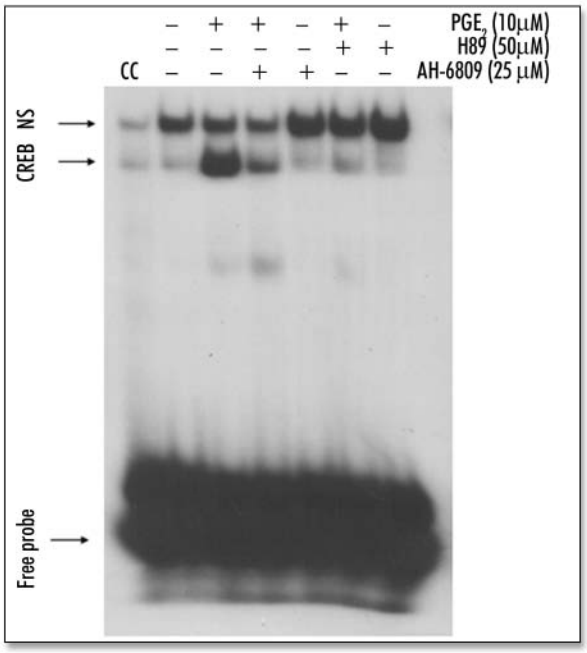

Figure 5. Effect of PGE_2 on induction of CREB DNA binding. L3.6pl cells were preincubated with 50 μM H-89 or 25 μM AH-6809 for 30 min before treatment with or without 10 μM PGE_2 for 1 h, and nuclear extracts were prepared for EMSA. PGE_2-induced formation of CREB complexes, but the activation of CREB binding by PGE_2 was decreased by both inhibitors. Competition assay with excessive unlabeled, cold COX-2 CRE probe (CC) completely abolished the complex formed with labeled probe, suggesting that the complex is specific to the COX-2 CRE. NS, nonspecific.

PGE_2 addition, and this activity reached a peak after 30 min, after which it decreased. Also whereas 10 μM PGE_2 increases PKA activity, treatment with 50 μM H-89 for 30 min or pretreatment with H-89 before the addition of PGE_2 completely blocked PKA activation (Fig. 3A). As shown in Fig. 3B, confocal microscopic analysis demonstrated a redistribution of the α-catalytic subunit from a primarily perinuclear distribution in untreated cells to a nuclear localization in cells treated with 10 μM PGE_2 for 30 min. Pretreatment with the specific PKA inhibitor H-89 inhibited the dissociation of the catalytic subunit from its regulatory subunit. Results from the immunocytochemical studies also revealed the effect of PGE_2 on CREB phosphorylation. Treatment with 10 μM PGE_2 for 30 min strongly increased p-CREB immunoreactivity in the nuclei, and in cells treated with H-89 for 30 min or pretreated with H-89 before the addition of PGE_2, p-CREB levels were substantially lower. The same results were observed by immunblot analysis (Fig 3C). Cells were treated with 10 μM PGE_2 for up to 60 min and enhanced CREB phosphorylation after 30 min of treatment was observed. Immunoblot analysis of the same membrane with an antibody that recognizes total CREB revealed that PGE_2 did not effect changes in the CREB level. Consistent with the previous data, pretreatment with H-89 before the addition of PGE_2 reduced CREB phosphorylation levels.

Activators of adenylate cyclase increase COX-2 protein expression, and the stimulatory effect of PGE_2 on COX-2 expression is blocked by PKA inhibitor H-89. We showed that PGE_2 increased COX-2 expression through PKA-dependent CREB phosphorylation. If cAMP-activated PKA is the downstream effector of PGE_2, then the activation of this signaling pathway should mimic PGE_2 to stimulate COX-2 expression. Consistent with this model, COX-2 expression was enhanced on treatment of L3.6pl cells with the adenylate cyclase activator forskolin (25 μM) as measured by immunoblotting. The effect of forskolin was time-dependent and peaked at 4 h (Fig. 4A). To further test whether increased PKA activity was responsible for the increase in COX-2 expression induced by PGE_2, we treated L3.6pl cells with 50 μM H-89 before exposing them to PGE_2. A significant inhibitory effect by H-89 on the up regulation of COX-2 by PGE_2 was detected (Fig. 4B). Together, these results demonstrate that PGE_2-induced activation of the downstream mediators of EP_2 receptor signaling, cAMP and PKA, is responsible for the increase in COX-2 expression.

In vitro binding of CREB to its cis-acting element in the COX-2 promoter. We then analyzed the in vitro binding of CREB to its regulatory element in the human COX-2 promoter by EMSA. Nuclear extracts were prepared from L3.6pl cells treated for 1 h with 10 μM PGE_2 alone or after 30 min of treatment with 50 μM H-89 or 25 μM AH-6809. The consensus oligonucleotides for the CRE binding sites were used as labeled probes. Compared with the control, PGE_2 induced the formation of a major complex with the consensus CRE probe, which was markedly decreased by preincubation with H-89 or AH-6809. Competition assay with excessive unlabeled, cold COX-2 CRE probe completely abolished the complex formed with labeled probes, suggesting that the complex is specific to the COX-2 CRE (Fig. 5).

In vivo association of the transcription factor CREB with the COX-2 promoter in PGE_2 stimulated L3.6pl cells. To further understand the roles of CREB and the COX-2 promoter regulatory element in PGE_2-induced COX-2 transcription, the in vivo association of this transcription factor with the COX-2 promoter was evaluated by the semiquantitative ChIP assay. To determine whether the transcription factor could specifically associate with the COX-2 promoter, PCR amplifications were conducted on immunoprecipitated DNA with 40 cycles of PCR using specific COX-2 promoter

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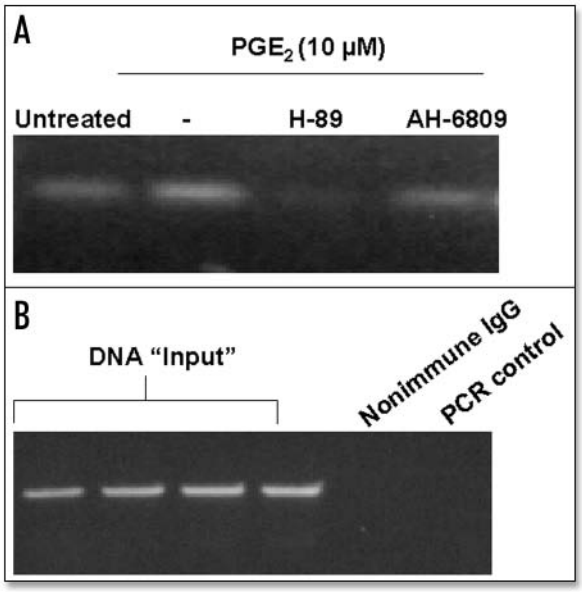

Figure 6. Effect of PGE_2 on the association of CREB with the human COX-2 promoter. In vivo association of CREB with the COX-2 promoter was evaluated by ChIP assay. (A) Confluent and serum starved L3.6pl cells in 150 mm dishes were pretreated with 50 μM H-89 and 25 μM AH-6809 for 30 min and then treated with 10 μM PGE_2 for 1 h. The in vivo protein DNA complexes were cross linked, and chromatin pellets were extracted and sonicated. CREB was immunoprecipitated with a specific antibody, and the associated COX-2 promoter DNA was amplified by PCR. After PGE_2 treatment, immunoprecipitates showed an enrichment of the COX-2 promoter DNA compared with the nonimmune IgG control, but the effect of PGE_2 was blocked by pretreatment with H-89 and AH-6809. (B) Input represents PCR products from chromatin pellets before immunoprecipitation. The results are representative of three independent experiments with similar results.

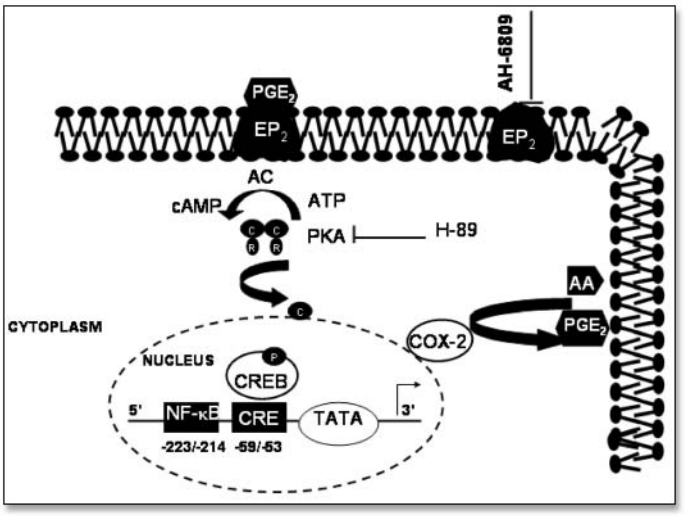

Figure 7. Model of PGE_2-induced COX-2 expression.

primers. We found that after PGE_2 treatment, CREB immunoprecipitates were enriched with COX-2 promoter DNA compared with the nonimmune IgG immunoprecipitate control (Fig. 6), indicating that the COX-2 promoter DNA precipitated by the CREB antibody is specifically associated with CREB. The effect of PGE_2 was blocked by the PKA inhibitor H-89, but the effect of pretreatment with AH-6809 was unnotable.

DISCUSSION

In this study, we demonstrate for the first time that the transcription factor CREB is involved in the basal and PGE_2-mediated COX-2 regulation in human pancreatic cancer cells. By using siRNA technology to disrupt CREB and NF-κB expression, we show a cooperative effect between these transcription factors in regulating basal COX-2 expression. NF-κB may play an important but not exclusive role in COX-2 expression, since targeting NF-κB was not sufficient to block the endogenous expression of COX-2. Also we investigated the role of CREB transcription factor in PGE_2-induced COX-2 expression in L3.6pl cell line and demonstrated that PGE_2 drives COX-2 expression through mechanisms involving activation of cAMP-linked PGE_2 receptor, PKA-dependent CREB phosphorylation, and activation of the COX-2 gene through CRE (Fig. 7). Constitutive CREB phosphorylation and further induction after PGE_2 stimulation were observed in other pancreatic cancer cell lines, including BxPc3, MiaPaCa-2 and Aspc1 (data not shown).

Although numerous upstream stimuli can induce COX-2 expression, PGE_2 itself has been found to promote the expression of COX-2 enzyme in human airway smooth muscle cells, human monocytes, murine keratinocytes and especially human prostate, breast and colon carcinoma cells.[30-33] PGE_2 elicits its autocrine and paracrine effects on target cells by interacting with G protein coupled receptors of at least four subtypes: EP_1 receptor couples to Gq and increases intracellular calcium concentrations and PKC activation, EP_2 and EP4 receptors couple to Gs to increase cAMP formation, and EP3 receptor couples to Gi to decrease cAMP formation.[34-36] Our work focused on the EP_2 receptor since recent observations have implicated this receptor in tumorigenesis. Rozic et al.[37] reported that EP_1/EP_2 receptors mediated the stimulatory effects of endogenous and exogenous PGE_2 on mammary tumor cell migration and invasiveness. In $APC\Delta^{716}$ mice, a murine model for familial adenomatous polyposis, enhanced angiogenic potential and microvascular density in intestinal polyps correlated strongly with COX-2, EP_2 receptor and angiogenic factor expression.[38,39] Ablation of the EP_2 receptor in these mice resulted in smaller intestinal polyps coincident with lower COX-2 and angiogenic factor expression. In human pancreatic cancer cells, PGE_2 produced by COX-2 increased vascular endothelial growth factor expression via the EP_2 receptor and subsequent accumulation of intracellular cAMP.[40] In agreement with these studies, our results support a role for the EP_2 receptor in mediating the effects of PGE_2 through PKA signaling in L3.6pl pancreatic cancer cells. Our results differ from those of researchers who reported that PGE_2 or other cAMP production-elevating agents inhibited COX-2 expression via stimulation of PKA. Karim et al.[41] found that PKA inhibited the phosphorylation of c-Jun transcription factor by c-Jun NH2-terminal kinase, which led to inhibition of c-Jun-mediated COX-2 transcription in porcine aortic smooth muscle cells; this effect was reversed by inhibition of the PKA activity. Reddy et al.[42] observed that PGE_2 suppressed C/EBPβ-mediated COX-2 transcription by activating PKA in murine mast cells. The discrepancies in these results may be explained by the use of different cell types and suggest that COX-2 regulation may be cell type and species specific and dependent on the amounts and activities of the transcriptional activators, repressors and cofactors and on the activities of signaling molecules regulating these factors.

In many cases, cAMP-PKA-induced transcription is mediated through the interaction of CREB with a consensus CRE found in

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the promoter of target genes. In this study, we provided several lines of evidence supporting the importance of CREB in PGE_2-mediated induction of COX-2 gene expression. Using immunocytochemical analysis, we demonstrated that treatment with PGE_2-induced translocation of the catalytic subunit of PKA to the nucleus of L3.6pl cells and consequently increased p-CREB immunoreactivity to above that seen in unstimulated cells. PGE_2 treatment also induced CREB phosphorylation and increased the binding of nuclear proteins to the COX-2 promoter CRE. Disrupting the PKA signaling cascade with H-89, a selective PKA inhibitor, and thereby blocking PKA translocation and CREB phosphorylation reduced COX-2 expression.

In summary, our data demonstrate that CREB plays a critical role in basal and PGE_2-induced COX-2 expression in L3.6pl pancreatic cancer cells mainly through a PGE_2-cAMP-related mechanism. Our study is focused on a single cell line observations, and exploring and understanding the role of CREB in the signaling mechanism involved in the regulation of COX-2 levels might lead to a better understanding of the dysregulations in pancreatic carcinomas and provide an alternate source for the development of novel therapeutic approaches.

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