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The EP4 receptor antagonist, L-161,982, blocks prostaglandin E₂-induced Signal Transduction and Cell Proliferation in HCA-7 colon cancer cells

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Abstract

Accumulating evidence indicates that elevated levels of prostaglandin E₂ (PGE₂) can increase intestinal epithelial cell proliferation, and thus play a role in colorectal tumorigenesis. PGE₂ exerts its effects through four G-protein coupled PGE receptor (EP) subtypes, named the EP1, EP2, EP3, and EP4. Increased phosphorylation of extracellular regulated kinases (ERK1/2) is required for PGE₂ to stimulate cell proliferation of human colon cancer cells. However, the EP receptor(s) that are involved in this process remain unknown. We provide evidence that L-161,982, a selective EP4 receptor antagonist, completely blocks PGE₂-induced ERK phosphorylation and cell proliferation of HCA-7 cells. In order to identify downstream target genes of ERK1/2 signaling, we found that PGE₂ induces expression of early growth response gene-1 (EGR-1) downstream of ERK1/2 and regulates its expression at the level of transcription. PGE₂ treatment induces phosphorylation of cyclic AMP response element binding protein (CREB) at Ser133 residue and CRE-mediated luciferase activity in HCA-7 cells. Studies with dominant negative CREB mutant (ACREB) provide clear evidence for the involvement of CREB in PGE₂ driven egr-1 transcription in HCA-7 cells. In conclusion, this study reveals that egr-1 is a target gene of PGE₂ in HCA-7 cells and is regulated via the newly identified EP4/ERK/CREB pathway. Finally our results support the notion that antagonizing EP4 receptors may provide a novel therapeutic approach to the treatment of colon cancer.

Keywords

PGE₂; colon cancer; ERK1/2; L-161,982; egr-1

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Introduction

Cyclooxygenases (COX-1 and -2) are mainly responsible for the production of prostaglandins such as PGE₂, PGD₂, PGI₂, PGF₂α and thromboxanes from arachidonic acid. Increased expression of COX-2, but not COX-1, was reported in human colorectal cancer tissues as well as in azoxymethane (AOM)-induced colonic tumors in rats [1,2]. Further, a genetic study with COX-2 knockouts in Apc^{Δ716} mice directly implicated a role for COX-2 in colorectal carcinogenesis [3]. Elevated levels of PGE₂ as a result of COX-2 overexpression were observed in human colorectal tumors as well as in carcinogen-treated rats [4-6]. PGE₂ treatment was shown to enhance incidence of colonic tumors in AOM-treated rats and attenuates non-steroidal anti-inflammatory drug (NSAID)-induced tumor regression in Apc^{Min/+} mice by increasing intestinal epithelial cell proliferation and reduction of apoptosis [7-9]. In addition, PGE₂ can increase cell survival, invasion, and migration of human colon cancer cells [10-12]. Collectively, these *in vivo* studies strongly implicated a role for elevated levels of PGE₂ in colorectal carcinogenesis.

PGE₂ signals are transduced via four G-protein coupled cell surface receptors, termed as EP1, EP2, EP3, and EP4 receptors. The EP1 receptors are coupled to G_{α_i} protein and are known to increase cytosolic Ca²⁺ levels in response to PGE₂ [13]. Both EP2 and EP4 receptors are coupled to G_{α_s} and can increase formation of intracellular cyclic AMP (cAMP) by activating adenylyl cyclase, whereas G_{α_i}-coupled EP3 receptors inhibit cAMP formation [13]. Thus, the effects of PGE₂ on cell proliferation appear to be mediated by its overall second messenger response, which in turn depends on receptor-ligand affinity and ligand concentration [14].

Signaling mediated through the EP4 receptor is associated with colon carcinogenesis. PGE₂ stimulates the proliferation and motility of LS174T adenocarcinoma cells through the EP4 dependent activation of phosphatidylinositol 3-kinase/AKT signaling [10]. Furthermore, premalignant aberrant crypt foci formation in EP4 deficient mice following azoxymethane treatment is suppressed compared to the EP4 wildtype mice [15]. This study also showed a reduction in colon adenomatous polyp formation in mice wild-type for the EP4 receptor but treated with the EP4 receptor antagonist ONO-AE2-227 [15]. Treatment with another EP4 antagonist, ONO-AE3-208, decreased liver metastases after intrasplenic injection of MC26 colon cancer cells [16]. Finally, increased expression of the EP-4 receptor has been associated with colon cancer progression as well as the stimulation of cell growth [17].

Although there is evidence for the importance of the EP4 receptor in colon carcinogenesis and observations suggesting that inhibition of the EP4 receptor may be efficacious for colon cancer therapy, there have been no reports identifying the signaling events downstream of ERK stimulated by PGE₂-EP4 receptor activation and whether pharmacologic inhibition of the EP4 receptor blocks these mitogenic signaling events. In this present study, we elucidate the PGE₂/EP4 signal transduction pathway. We demonstrate that PGE₂ stimulation of the EP4 receptor leads to ERK/CREB activation. Furthermore, we show that PGE₂-activated signaling transduction pathway leads to CREB-dependent induction of EGR-1 in HCA-7 colon cancer cells. Finally, we also demonstrate that antagonism of the EP4 receptor with L-161,982 attenuates cell growth and is associated with the inhibition of ERK activation by PGE₂.

Materials and Methods

Cell culture and reagents

HCA-7 and MCF-7 cells were maintained in Dulbecco's modification of eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin at 37°C with 5% CO₂. 17-phenyltrilor prostaglandin E₂, AH 6809, and prostaglandin E1 alcohol (PGE1-OH) were purchased from Cayman chemicals (Ann Arbor, MI). PGE₂ is purchased

from Sigma (St. Louis, MO). U0126 and H-89 were purchased from Cell signaling technology (Beverly, MA) and Calbiochem (La Jolla, CA), respectively. A selective EP4 receptor antagonist L-161,982 [18] was from Merck-Frosst laboratories (Merck-Frosst, Canada). Antibodies against phospho-ERK (Tyr 304), and Egr-1 (sc-110) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against phospho-CREB (Ser 133) was from Upstate Biotechnology (Lake Placid, NY).

Plasmids

Human egr-1 promoter constructs pEgr-1A (-600/+ 12), pEgr-1B (-460/+ 12), and pEgr-1C (-164/+ 12) [19] were kindly provided by Dr. Stephen Safe (Texas A&M University). ACREB, a dominant negative CREB mutant [20], was a gift from Dr. Charles Vinson, (Laboratory of Biochemistry, NCI, NIH, Maryland). All the plasmid constructs were checked by digesting with appropriate restriction enzymes before use.

Drug treatments

HCA-7 cells were plated in 100 mm culture dish at 1.7×10^6 cells/plate and grown for 24 h in DMEM media. Later the cells were subjected to serum starvation for 24 h before stimulating the cells with PGE₂ or any other drugs used for the indicated time period. DMSO was maintained in the culture at <0.1% concentration.

Immunoblotting

Cells were lysed with appropriate volume of lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris, 1% Na deoxycholate, 1% Nonidet P-40) supplemented with protease inhibitor cocktail (Sigma), 1 mM sodium orthovanadate and 1 mM PMSF. Protein concentration was determined using BCATM protein assay kit (Pierce, Rockford, IL). Fifty micrograms of total cell lysate was separated on 10% SDS-PAGE gels and transferred to the PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with 5% non-fat dry milk followed by overnight incubation with primary antibody in 1% PBS containing tween-20 (0.1%) and 1% BSA. Later the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary IgG antibody and developed using chemiluminiscent system (Amersham Biosciences, Piscataway, NJ). The membrane was stripped and reprobed with mouse anti α -tubulin (Oncogene, San Diego, CA) as a loading control.

Cell proliferation assay

Cell proliferation in response to different treatments was measured using sulforhodamine B (SRB) assay as previously described [21]. Briefly, $\sim 8 \times 10^4$ HCA-7 cells were plated per well in a 6-well plate and allowed to grow for 24 h. Later the cells were serum starved for 24 h and pre-incubated with L-161,982 (10 μ M) for 2 h before stimulating the cells with PGE₂ for 72 h. Later the viable cells were fixed with cold 50% trichloroacetic acid (final concentration 10%) for 1 h at 4°C. Cells were washed with deionized water and stained by incubating with 0.4% SRB dye for 10 min at room temperature. Then the cells were washed with 1% acetic acid and the bound SRB dye was solubilized with 1M unbuffered Tris, mixed well, and the optical density (O.D.) was measured using a plate reader (Biomek @ 540 nm).

Total RNA isolation and quantitative realtime RT-PCR (QRT-PCR)

Total RNA from HCA-7 cells was isolated using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. To avoid genomic DNA contamination, samples were incubated with RNase-free DNaseI (Qiagen) during RNA preparation. Two micrograms of total RNA was reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA). PCR primers used to determine EP receptor expression were: EP1: F-5'-GTCGTGCATCTGCTGGAG-3'; R-5'-CGCTGCAGGGAGGTAGAG-3'; EP2: F-5'-

TGGCTATCATGACCATCACC-3'; R-5'-GTTTCATTTCATATATGCAAAAATCGT-3'; EP4: F-5'-CTCCCTGGTGGTGCTCAT-3'; R-5'-GGCTGATATAACTGGTTGACGA-3'. The egr-1 mRNA levels in vehicle (DMSO) or PGE₂ treated samples were measured with iTaq™ SYBR® Green supermix with ROX (BioRad) using Gene Amp 5700 sequence detector (Applied Biosystems). Quantitative real time PCR was carried out in a volume of 25 µl consisting of 25 ng of cDNA and 200 nM each of forward and reverse egr-1 primers. PCR primers used to amplify egr-1 were: F-5'-AGCAGCACCTTCAACCCTCA-3'; R-5'-CAGCACCTTCTCGTTGTTTCAGA-3'. The egr-1 primers were originally designed by Ning et al [22]. Differences in egr-1 mRNA levels between the vehicle and PGE₂-treated samples were determined using the threshold cycle (C_T) method [23]. Each sample was run in quadruplicate and the values were normalized to the corresponding GAPDH values.

Transient transfection of egr-1 promoter constructs and luciferase assay

For transient transfection of egr-1 promoter-luciferase reporter constructs, HCA-7 cells were plated at 85,000 cells/well in 24-well plates and allowed to grow for 24 h. Later the medium was replaced with serum-free media and the cells were co-transfected with 0.5 µg/well of egr-1 promoter construct and 10 ng of renilla plasmid using LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA). After 20 h of transfection, cells were stimulated with either DMSO or PGE₂ (2.1 µM) for 6 h. Then the growth medium was removed, washed the cells with 1x PBS and lysed the cells with the passive lysis buffer (Promega, Madison, WI). Firefly and renilla luciferase activities were measured with Dual luciferase reporter assay system (Promega) using Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN) according to the protocol suggested by the manufacturer. Firefly luciferase values obtained with each egr-1 promoter construct were normalized to the corresponding renilla luciferase values. Transfection efficiency of HCA-7 cells was 67% as determined using EGFP constructs. Mean ± SD values shown for each treatment were derived from 4 wells in a single experiment.

Statistical analysis

All experiments were repeated at least three independent times. Level of significance between different treatments was measured using student's t-test. The probability values less than 0.05 were considered significant.

Results

EP4 receptor mediates PGE₂-induced phosphorylation of ERKs in HCA-7 cells

The effects of PGE₂ are mediated by four G protein-coupled receptors called the EP 1-4 [13]. We first characterized the expression of the prostaglandin receptors. We examined the expression of the EP receptors in HCA-7 cells and MCF-7 cells using real time PCR. The MCF-7 cells were used as a positive control since these cells have been shown to express the EP1, EP2, and EP4 receptors [24]. We found that the EP1, EP2, and EP4 mRNAs were expressed in HCA-7 cells (Fig.1). Among, the various EP receptors, expression of the EP4 receptor mRNA was abundant in both cell lines. Whereas the EP3 receptor was down-regulated in HCA-7 cells (data not shown). This finding was consistent with previous studies by Shoji and co-workers [25] who showed that the EP3 receptor subtype is down-regulated in colon cancer most likely by hypermethylation of the promoter region.

Next, we employed pharmacological approach to identify the EP receptor(s) that may be involved in PGE₂-induced phosphorylation of ERKs. Treatment of HCA-7 cells with PGE1-OH, a selective EP4 agonist [26], induced ERK phosphorylation. However, treatment with 17-phenyltrior PGE₂ (2.1 µM), an (EP1/EP3 agonist) [26] did not induce ERK phosphorylation (Fig. 2A). Furthermore, there is a concentration dependent increase in ERK phosphorylation following treatment with PGE1-OH (0.7-2.1 µM) (Fig. 2B). There was no difference in total

ERK levels. Next, pretreatment of HCA-7 cells with AH 6809 (EP1/EP2 antagonist) (10 μ M) [27] did not have any effect on PGE₂-induced ERK phosphorylation (Fig. 2C). Collectively, these data suggest the involvement of EP4 receptor in PGE₂-induced ERK activation.

L-161,982 blocks PGE₂-stimulated cell proliferation of HCA-7 cells

We evaluated whether pharmacological inhibition of EP4 receptor with L-161,982 inhibits cell proliferation of HCA-7 cells stimulated by PGE₂. Serum starved HCA-7 cells were pre-incubated with L-161,982 (10 μ M) and then stimulated with PGE₂ for 72 h. There was significant increase in proliferation of HCA-7 cells when cells were treated with PGE₂ alone compared to the vehicle treatment, while pretreatment with L-161,982 blocked PGE₂-induced cell proliferation (Fig 3). Treatment with L-161,982 alone also showed significant decrease in cell proliferation compared to the vehicle treated HCA-7 cells. Similar findings were observed in LS174T colon cancer cells (data not shown). These results demonstrate that PGE₂-induced cell proliferation of HCA-7 cells can be suppressed by L-161,982.

L-161,982 blocks PGE₂-stimulated ERK phosphorylation

Next, we investigated if selective inhibition of the EP4 receptor would result in suppression of ERK activation assessed by ERK phosphorylation status. We first determined concentration of PGE₂ necessary to elicit a robust ERK response in HCA-7 cells. Dose-response studies with PGE₂ (0.7-2.1 μ M) showed strong phosphorylation of ERK1/2 at a dose of 2.1 μ M, and therefore, 2.1 μ M PGE₂ dose was used in all experiments (Supplemental Figure S1). Next, we studied the kinetics of ERK phosphorylation in response to PGE₂ treatment in HCA-7 cells. In time-course studies following stimulation of serum starved HCA-7 cells with PGE₂ (2.1 μ M), increased phosphorylation of ERK1/2 was seen as early as 15 min, and reached peak levels at 30 and 60 min followed by the decline at 120 min (Fig. 4A). There was no change in total ERK levels. Therefore, a dose of 2.1 μ M PGE₂ for 30-60 min were used in all subsequent experiments. Similar concentrations of PGE₂ have been used by others to study the effects of PGE₂ [28].

We then used the selective EP4 antagonist, L-161,982 for these studies. HCA-7 cells were pretreated with L-161,982 (10 μ M) for 60 min and then stimulated with PGE₂ for another 60 min. As shown in Fig. 4B, PGE₂ treatment increased the phosphorylation levels of ERKs 1 and 2 by 3.5 and 5- fold, respectively at 60 min. However, there was a complete blockade of ERK phosphorylation in HCA-7 cells pretreated with L-161,982. In addition, L-161,982 at 2.5 and 5.0 μ M also attenuated PGE₂-induced ERK phosphorylation (data not shown). Based on these data, we conclude that EP4 receptor mediates PGE₂-induced ERK phosphorylation in HCA-7 cells. The concentrations of L-161,982 used for our studies were based on similar studies using this agent investigating the role of the EP4 receptor in PGE₂-induced cell survival of glomerular epithelial cells and hypertrophy of cardiac myocytes [29,30]. Further, pretreatment of HCA-7 cells with a small molecule epidermal growth factor receptor (EGFR) inhibitor, ZD 1839 (10 μ M) completely blocked PGE₁-OH induced ERK phosphorylation (Supplemental Figure S2). These results suggest the requirement of cross-talk between EP4 and EGFR to induce ERK phosphorylation in HCA-7 cells.

CREB phosphorylation by ERK is attenuated by L-161,982

Activation of ERKs is known to phosphorylate CREB at ser133 residue [31]. Phosphorylation of CREB at its critical Ser133 residue was shown to increase its DNA binding activity [32]. Therefore, we analyzed phosphorylation status of CREB in a time-course experiment in PGE₂-stimulated HCA-7 cells. Upon PGE₂ treatment, increased CREB phosphorylation at Ser 133 was observed at 30 and 60 min and return to baseline levels at 120 min (Fig. 5A). Next we asked the question that whether PGE₂-stimulated CREB phosphorylation was mediated through EP4 receptor. We observed complete blockade of CREB phosphorylation in HCA-7

cells pretreated with L-161,982 (10 μ M) followed by PGE₂ treatment for 30 min (Fig. 5B). Phosphorylation of CREB at ser133 residue increases its DNA binding activity to cAMP response element (CRE) and initiates transcription of its target genes. Therefore, we next studied whether PGE₂ was able to transactivate CRE-mediated transcription. HCA-7 cells were transfected with the luciferase reporter vector containing four tandem CRE- repeats. Following PGE₂ stimulation, CRE-luciferase activity was increased by 3-fold (Fig. 5C).

Transcriptional regulation of EGR-1 induction by PGE₂ in HCA-7 cells

EP4-mediated PGE₂ signaling has been implicated in the induction of EGR-1. EGR-1 is a zinc finger transcription factor that binds to the GC-rich sequences in the regulatory region of its target genes and regulates their expression in response to the external stimulus. The transcription factors involved in the regulation of EGR-1 by PGE₂/EP4 receptor have not been identified. Furthermore, there are CRE elements within the human EGR-1 promoter [19]. Therefore, we speculated that EP4 mediated PGE₂ induction of EGR-1 may occur through CREB-dependent transcription. First, we investigated whether PGE₂ is able to induce expression of EGR-1 in HCA-7 cells. To address this issue, we conducted a time-course study (0-120 min) to examine induction of EGR-1 protein by PGE₂ (2.1 μ M) in HCA-7 cells. Endogenous EGR-1 protein levels were markedly increased at 30 and 60 min after PGE₂ treatment, and by 120 min, EGR-1 protein levels were comparable to those seen at 0 min (Fig. 6A). We next investigated whether the observed induction of EGR-1 by PGE₂ in HCA-7 cells involves phosphorylation of ERK. For these experiments, HCA-7 cells were pre-incubated with either U0126 (MEK inhibitor) (10 μ M) or H-89 (PKA inhibitor) (10 μ M) for 60 min, and then stimulated with PGE₂. As shown in Fig. 6B, pretreatment with U0126, but not H-89, inhibited EGR-1 induction by PGE₂, suggesting direct involvement of MEK pathway in PGE₂-induced EGR-1 expression in HCA-7 cells. We then measured *egr-1* mRNA levels using *egr-1* specific primers in a time course study by QRT-PCR. Following PGE₂ treatment, a rapid and significant increase in *egr-1* mRNA levels were seen at 15 and 30 min. *egr-1* mRNA levels reached near control levels by 60 min after PGE₂ treatment (Fig. 6C). The kinetics of *egr-1* mRNA expression in response to PGE₂ in HCA-7 cells correlate well with the observed EGR-1 protein levels, indicating that increased mRNA levels would account for the induction of EGR-1.

To determine if EGR-1 expression is induced at the transcriptional level, HCA-7 cells were transiently transfected with human *egr-1* promoter constructs coupled to the luciferase reporter gene followed by stimulation with PGE₂ (2.1 μ M) for 6 h. The -600/+12 pEgr-1A construct contains several critical regulatory elements, such as Sp-1 binding site, NF-kB binding site, serum response elements (SRE), and one cAMP response element (CRE) site (Fig. 7A) [33, 34]. PGE₂ treatment of HCA-7 cells transiently transfected with pEgr-1A construct resulted in significant increase in luciferase activity compared to the vehicle-treated cells (Fig. 7B), indicating that PGE₂ regulates EGR-1 induction at the level of transcription. Stimulation of HCA-7 cells transfected with the promoterless basic vector by PGE₂ did not have any effect.

The involvement of the CRE- site within the EGR-1 promoter region

To further delineate the cis-acting elements that are required for PGE₂ to induce *egr-1* transcription, HCA-7 cells were transiently transfected with deletion constructs of *egr-1* promoter (pEgr-1B, -460/+12 and pEgr-1C, -164/+12) and then stimulated with PGE₂ for 6 h. As shown in Fig. 7B, in cells transfected with pEgr-1B (-460/+12), basal luciferase activity was 62% of that seen with pEgr-1A, indicating that deletion of Sp1 binding sites had effect on basal luciferase activity of *egr-1*. However, the luciferase activity of pEgr-1B was increased significantly upon stimulation with PGE₂. Similar to the pEgr-1A and 1B constructs, PGE₂ treatment resulted in significant increase in luciferase activity in HCA-7 cells transiently transfected with pEgr-1C. The basal levels of pEgr-1C were 50% and 80% to that of pEgr-1A

and pEgr-1B, respectively. These results clearly indicate that PGE₂ responsive cis-acting elements are located in the -164/+12 bp region of the egr-1 promoter and further suggests the possible involvement of trans-acting factors that binds either to the CRE-site, or to the proximal SRE- site or cooperativity between the transcription factors that binds to CRE- and SRE-sites in PGE₂-induced egr-1 transcription.

A dominant negative inhibition of CREB completely inhibits PGE₂ stimulation of egr-1 transcription

Involvement of CRE site (-138 to -131 bp) in the egr-1 promoter in PGE₂-stimulated egr-1 transcription has been previously reported [35]. The observations of induction of CREB phosphorylation at ser133 residue and transactivation of CRE-mediated transcription in by PGE₂ in HCA-7 cells further supports the possible involvement of the CRE- site in PGE₂-induced egr-1 transcription in HCA-7 cells. To determine whether CREB contributes to the PGE₂ increased egr-1 transcription in HCA-7 cells, we cotransfected HCA-7 cells with pEgr-1C construct and with either empty vector CMV 500 or ACREB. ACREB heterodimerizes with endogenous CREB and selectively inhibits its binding to DNA [20]. After 24 h of transfection, cells were stimulated with PGE₂ for 6 h. A significant increase in luciferase activity upon PGE₂ stimulation was observed in HCA-7 cells transfected with pEgr-1C construct alone and also in cells co-transfected with pEgr-1C and CMV 500 (Fig. 8). However, cotransfection of pEgr-1C with ACREB reduced the basal expression of pEgr-1C by 19% and completely blocked PGE₂- induced luciferase activity. These studies with ACREB indicate the involvement of CREB in egr-1 transcription induced by PGE₂.

Discussion

Cyclooxygenase-2 and prostaglandin E₂ (PGE₂) levels are increased in colorectal cancers and in a subset of adenomas. PGE₂ signaling through the EP4 receptor has previously been associated with colorectal cancer. For example, PGE₂ has been reported to stimulate the proliferation and motility of LS174T colorectal cancer cells via EP4 dependent stimulation of PI- 3-K/AKT signaling [10]. In addition Pozzi et al [36] demonstrated EP4 mediated PI-3-K/ ERK signaling pathway in mouse colon carcinoma cells. Furthermore, Chell et al [17] demonstrated increased EP4 receptor expression in human colon cancer progression and increased proliferation of EP4 expressing cells in response to PGE₂. However, there are no reports of the signaling events downstream of ERK stimulated by PGE₂-EP4 receptor activation or whether pharmacologic inhibition of the EP4 receptor blocks mitogenic signaling events.

In the present study we demonstrated concentration and time dependent activation of ERK in HCA-7 cells. These data are consistent with previous findings. Furthermore, we demonstrated that a selective EP4 receptor antagonist, L-161,982 suppresses PGE₂-induced cell proliferation and is associated with the inhibition of ERK phosphorylation. In this context, our results suggest that selective targeting of EP4 receptor with EP4 receptor antagonists such as L-161,982 may be beneficial to block PGE₂ effects on cell proliferation of human colon cancer cells. We plan to evaluate the efficacy of L-161,982 analogs *in vivo*.

PGE₂ signals are transduced via four G-protein coupled cell surface receptors, termed as EP1, EP2, EP3, and EP4. The EP1 receptors are coupled to G_{α_q} protein and are known to increase cytosolic Ca²⁺ levels in response to PGE₂. Both EP2 and EP4 receptors are coupled to G_{α_s} and can increase formation of intracellular cyclic AMP (cAMP) by activating adenylyl cyclase, whereas G_{α_i}-coupled EP3 receptors inhibit cAMP formation [13]. Significant reduction of AOM- induced colonic aberrant crypt foci (ACFs) were observed in mice with homozygous deletions in EP1, EP2 and EP4 receptors, but not EP3 [15,37,38]. In addition, selective antagonists for EP1 and EP4 receptors significantly inhibited AOM- induced ACFs in mice

and F344 rats as well as formation of intestinal polyps in Apc^{Min} mice [15,39]. Taken together, these results suggest that PGE₂-activated signaling pathways mediated by EP1, EP2, and EP4 receptors play a central role in animal models of colon cancer. However, exactly which receptor subtype plays a role in human colon carcinogenesis is only now becoming clear. Although recent studies suggest a role for the EP2 receptor in PGE₂-induced cellular response, a caveat with these studies is the use of cell culture systems in which the EP2 receptor is ectopically expressed [40]. Our studies, which are in agreement with several other reports [36], indicate an important role for the EP4 receptor for the following reasons: 1) PGE1-OH (relatively selective EP4 agonist), but not 17-phenyltrilor PGE₂ (EP1/EP3 agonist) induced ERK phosphorylation; 2) pretreatment of HCA-7 cells with AH6809 (EP1/EP2 antagonist) did not have any effect on PGE₂-induced ERK phosphorylation; 3) L-161,982 at concentrations that does not bind to either EP1 or EP2 receptors [18] abrogated PGE₂-induced ERK phosphorylation. Nevertheless, the relative contribution of the EP2 and EP4 receptors to human colon cancer will require further study.

The downstream signaling events in the PGE₂/EP4 receptor/ERK pathway in colon cancer cells have not been elucidated. Although there is evidence for the importance of the EP4 receptor in colon carcinogenesis, there have been no reports delineating the signaling pathway activated by the EP4 receptor. Phosphorylation of CREB at its critical ser133 residue by ERK has been reported to increase its DNA binding activity [32]. In this report, we demonstrate that PGE₂ activation of ERK leads to the phosphorylation of CREB at Ser 133 (Fig. 9) in HCA-7 cells. Furthermore, we showed that L-161,982 could block CREB phosphorylation at Ser 133. It is well documented that PGE₂ via EP2 and EP4 receptors can increase intracellular cAMP levels, which acts as a second messenger molecule and leads to PKA activation and robust serine phosphorylation of CREB [13]. However, in our studies, we did not observe increased intracellular cAMP levels in HCA-7 cells in response to PGE₂ (data not shown). This may explain why only modest phosphorylation of CREB was seen in our studies. Nevertheless, we did see 3-fold increase in transactivation of CRE-luciferase vector in HCA-7 cells in response to PGE₂. The data from the present investigation suggests that CREB transcription may be important for the mitogenic signal transduction cascade elicited by EP4-mediated PGE₂ activation (Fig. 9). Cyclooxygenase-2 derived PGE₂ production also has a strong association with cell survival, migration, invasion, and angiogenesis [41]. The role of PGE₂/EP4 receptor/ERK/CREB in regulating these responses may warrant further investigation.

It has been reported that transfection of HEK 293 cells with the EP4 receptor leads to EGR-1 induction mediated by ERK [42]. In the present study, we confirm this observation in HCA-7 cells that endogenously express the EP4 receptor. Also there is a CRE element in the human EGR-1 promoter [19]. In addition, we extend these findings and demonstrate the involvement of the transcription factor CREB in PGE₂ induction of EGR-1 mRNA and protein levels. These data are consistent with previous reports that demonstrate PGE₂-induced induction of EGR-1 in inflammation. However, our studies suggest the involvement of ERK/CREB pathway rather than p38/ATF-2 pathway [35]. Hence, PGE₂ signaling via the MAP kinases in immune cells and epithelial cells appears to be different. Early growth response factor-1 (EGR-1) is a member of the zinc finger family of transcription factor and plays a key role in cell growth and differentiation. It is recognized as an immediate early gene that regulates the expression of several downstream genes such as matrix metalloproteinase expression [43], as well as cytokines such as IL-1 β and IL-6 [44]. In addition, it has been reported that the expression of cyclin D1 is regulated by EGR-1 through a PI3K and ERK dependent pathway [45]. Furthermore, it has been demonstrated that PGE₂ synthase is up-regulated by the binding of EGR-1 to the promoter region of the murine gene encoding PGE₂ synthase [46]. Therefore, signaling through the EP4 receptor has the potential to increase the expression of several genes involved tumorigenesis. In regards to PGE₂ synthase, it has been proposed that the PGE₂/EP4/

ERK/EGR1 pathway could provide a “positive feedback loop” that accelerates carcinoma growth in an autonomous manner [42], since the product of PGE₂ synthase is PGE₂ itself.

In summary, the data presented in these studies strengthen an important role for the EP4 receptor in colorectal carcinogenesis. Since PGE₂ is frequently elevated in colorectal cancer, signaling mediated by the EP4 receptor could represent an important step in the clonal evolution of colonic epithelial cells during the adenoma-carcinoma sequence and contribute to malignancy. Finally, in light of the recent side effects associated with the use of COX-2 inhibitor, our studies support the notion that inhibition of EP4 receptor signaling events may represent an alternative therapeutic target for the prevention and treatment of colorectal cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of EP receptors in HCA-7 cells

Total RNA isolated from HCA-7 cells was reverse transcribed, and the cDNA was used to detect expression levels of EP receptors using real time quantitative RT-PCR. The samples were run in quadruplicate and normalized to the corresponding GAPDH values. MCF-7 breast cancer cells were used as a positive control.

Figure 2. PGE1 alcohol induces ERK phosphorylation in HCA-7 cells

A) HCA-7 cells were serum starved for 24 h and then treated with PGE1OH (2.1 μM) or 17-phenyltrilor prostaglandin E₂ (2.1 μM) for 60 min. Fifty micrograms of total protein was electrophoresed on 10% SDS-PAGE gels, transferred to PVDF membrane and then probed with mouse anti-phospho ERK1/2 antibody (1:1000). The membrane was stripped, and reprobed with rabbit anti-p42/44 MAPK antibody (1:1000). **B)** Serum starved HCA-7 cells were stimulated with PGE1-OH at the indicated concentrations for 60 min and then analyzed for phospho-ERK levels. Alpha tubulin levels were served as a loading control. **C)** Serum starved HCA-7 cells were pre-treated with AH6809 (EP1/EP2 receptor antagonist) (10 μM) for 60 min and then stimulated with PGE₂ for 60 min. Phospho-ERK levels were determined by western analysis.

Figure 3. The effects of selective EP4 receptor antagonist on cell growth

A) HCA-7 cells were preincubated with L-161,982 (10 μ M) for 2 h. Later the cells were stimulated with PGE₂ (2.1 μ M) and then incubated for an additional 72 h. Cell proliferation was measured using the SRB assay. Differences between PGE₂ stimulated cells and cells treated with L-161,982 were significant with * $p < 0.05$.

Figure 4. The effects of selective EP4 receptor antagonist on ERK phosphorylation

A) HCA-7 cells were serum starved for 24 h and then stimulated with PGE₂ for the indicated times and subjected to western blot analysis for phospho ERK and total ERK. Alpha tubulin levels were served as a loading control. **B)** HCA-7 cells were pre-incubated with L-161,982 (10 μM) for 60 min. Later the cells were stimulated with PGE₂ (2.1 μM) for the indicated time points, harvested, and lysed in RIPA buffer. Proteins were then subjected to western blot analysis with primary antibodies to phosphorylated ERK (pERK) and total ERK.

A **B**

C

Figure 5. The effects of selective EP4 receptor antagonist on CREB phosphorylation

A) HCA-7 cells were treated with PGE₂ (2.1 μ M) for the indicated time points. CREB phosphorylation at Ser133 residue was detected by western blot analysis. **B)** HCA-7 cells were pretreated with L-161,982 (10 μ M) for 60 min followed by stimulation with PGE₂ (2.1 μ M) for 30 min. CREB phosphorylation was determined by western analysis using anti-phospho CREB primary antibody. Immunoblotting for α -tubulin levels served as a loading control. **C)** HCA-7 cells were plated and were cotransfected with CRE-reporter vector coupled to luciferase and renilla plasmid in serum-free media. After overnight transfection, cells were stimulated with either vehicle or PGE₂. Firefly luciferase values were normalized to the corresponding renilla luciferase values.

A **B** **C** **Figure 6. The effects of PGE₂ stimulation on the expression of EGR-1**

A) HCA-7 cells were stimulated with PGE₂ (2.1 μ M) for the indicated time points, 0-120 min. Next, the cells were harvested and proteins were subjected to western blot analysis with primary antibody to EGR-1. The blot was stripped and incubated with primary antibody to tubulin as

loading control. **B)** HCA-7 cells were pre-incubated with either U0126 (10 μ M) or H-89 (10 μ M) for 60 min followed by stimulation of cells with PGE₂ (2.1 μ M) for 30 and 60 min. EGR-1 protein levels were detected by western blot analysis. **C)** HCA-7 cells were treated with PGE₂ (2.1 μ M) and the cells were collected at the indicated time points. Total RNA was isolated, converted to cDNA and cDNA was used as a template to amplify egr-1 with egr-1 specific primers using QRT-PCR. For each treatment, the samples were run in quadruplicate and normalized to the corresponding GAPDH values. Differences between control (0 time point) and PGE₂ treated samples were significant at 15 min and 30 min *p<0.05.

Figure 7. The effects of PGE₂ on human EGR-1 promoter constructs

A) Schematic diagram of the *egr-1* promoter constructs. Binding sites for various transcription factors are indicated, **B)** HCA-7 cells were transiently co-transfected with an *egr-1* promoter construct and renilla plasmid in serum free media using LipofectAMINE 2000. After 20 h, cells were treated with PGE₂ (2.1 μ M) for 6 h. Firefly luciferase values were normalized to the corresponding renilla luciferase values. Differences between PGE₂ stimulated cells and vehicle control cells were significant with * $p < 0.05$.

Figure 8. The effects of a dominant negative CREB on EGR-1 induction by PGE₂

The effects of a dominant negative CREB construct on the induction of EGR-1 following PGE₂ stimulation. HCA-7 cells were cotransfected with pEgr-1C (-164/+12) (0.5 µg) and renilla plasmid (10 ng) and either the empty vector CMV 500 (25 ng) or the expression vector encoding the dominant-negative CREB (ACREB) (25 ng) in serum-free media. After 20 h of transfection, cells were stimulated with either vehicle or PGE₂ (2.1 µM) for 6 h. Firefly luciferase activities were normalized to the corresponding renilla luciferase values. Differences between vehicle control and PGE₂ treated cells were significant *p<0.05.

Figure 9. Proposed model for the PGE₂/EP4 receptor signal transduction

Activation of EP4 receptor by PGE₂ leads to phosphorylation of ERK via EGFR and its downstream MEK pathway. Upon phosphorylation, ERK1/2 translocates into the nucleus and phosphorylates CREB at Ser133 residue. Phosphorylation at ser133 residue enhances DNA binding activity of CREB, which in turn binds to the proximal CRE-site in the human egr-1 promoter and initiates egr-1 transcription.