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EP₃ Prostanoid Receptor Isoforms Utilize Distinct Mechanisms to Regulate ERK 1/2 Activation

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Summary

Prostaglandin-E₂ (PGE₂) is a hormone derived from the metabolism of arachidonic acid whose functions include regulation of platelet aggregation, fever and smooth muscle contraction/relaxation. PGE₂ mediates its physiological and pathophysiological effects through its binding to four G-protein coupled receptor subtypes, named EP₁, EP₂, EP₃ and EP₄. The EP₃ prostanoid receptor is unique in that it has multiple isoforms generated by alternative mRNA splicing. These splice variants display differences in tissue expression, constitutive activity and regulation of signaling molecules. To date there are few reports identifying differential activities of EP₃ receptor isoforms and their effects on gene regulation. We generated HEK cell lines expressing either the human EP_{3-Ia}, EP_{3-II} or EP_{3-III} isoforms. Using immunoblot analysis we found that nM concentrations of PGE₂ strongly stimulated the phosphorylation of ERK 1/2 by the EP_{3-II} and EP_{3-III} isoforms; whereas, ERK 1/2 phosphorylation by the EP_{3-Ia} isoform was minimal and only occurred at μM concentrations of PGE₂. Furthermore, the mechanisms of the PGE₂ mediated phosphorylation of ERK 1/2 by the EP_{3-II} and EP_{3-III} isoforms were different. Thus, PGE₂ stimulation of ERK 1/2 phosphorylation by the EP_{3-III} isoform involves activation of a Gα_i/PI3K/PKC/Src and EGFR-dependent pathway; while for the EP_{3-II} isoform it involves activation of a Gα_i/Src and EGFR-dependent pathway. These differences result in unique differences in the regulation of reporter plasmid activity for the downstream effectors ELK1 and AP-1 by the EP_{3-II} and EP_{3-III} prostanoid receptor isoforms.

Keywords

Prostaglandin E₂; E Prostanoid receptor; ERK 1/2; protein kinase C; phosphoinositide-3 kinase; epidermal growth factor receptor

Introduction

Prostaglandin E₂ (PGE₂) is an autocrine and paracrine hormone derived from the metabolism of arachidonic acid via the activities of cyclooxygenase. PGE₂ mediates its physiological and patho-physiological effects through its four G-protein coupled receptor subtypes EP₁₋₄. Four distinct genes encode the EP receptor subtypes and each receptor couples uniquely to G-proteins. In humans, the EP₁ receptor couples to G_q, EP₂ to G_s, EP₃ to Gα_i, Gα_s, Gα_q and Gα_{12/13} and EP₄ couples to Gα_s and Gα_i [1–4]. The EP₃ receptor is unique among the EP receptor subtypes, in that there are multiple isoforms generated through alternative mRNA

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splicing in the carboxyl tail of the EP₃ receptor gene. Thus far 10 mRNA splice variants of the human EP₃ receptor have been identified [4–8]. Previous studies have shown that the differences in the carboxyl tail impart differences in constitutive activity, G-protein coupling and agonist induced internalization [5,9,10]. Evidence of unique signal transduction pathways and regulation of gene expression among individual receptor isoforms has also been demonstrated in both published and unpublished studies [5,11,12].

Physiologically, the EP₃ receptor is important in a number of functions including the febrile response, gastro-duodenal bicarbonate secretion, vasoconstriction of the pulmonary arteries, growth inhibition in keratinocytes and inhibition of aromatase activity in breast fibroblasts [13–18]. Its expression has mostly been observed in clusters of multiple isoforms rather than singly. In human uterus, mRNAs for EP_{3-V} and EP_{3-VI} receptor isoforms have been detected, whereas, in primary keratinocytes EP_{3A1}, EP_{3C} and EP_{3D} splice variants are expressed [19, 20]. The EP_{3A1}, EP_{3C} and EP_{3D} splice variants correspond to EP_{3-Ia}, EP_{3-II} and EP_{3-IV} respectively, according to the Kotani nomenclature [21].

Studies of the activities of EP₃ receptors have uncovered a number of discrepancies especially related to its effects on proliferation and differentiation. An inhibitory role of EP₃ signaling on proliferation has been described using the EP₃ receptor agonist GR 63799X. GR 63799X inhibits the proliferation of keratinocytes and HCA-7 colon cancer cells and induces 3T6 fibroblast growth arrest [13,19,22,23]. In contrast, EP₃ receptor activation has also been reported to induce the proliferation of hepatocytes, endometrial stromal cells and A549-adenocarcinoma cells [20,24,25]. The divergent data regarding the influences of EP₃ receptor activation on proliferation indicate that EP₃ receptors may have different effects based on the tissue type in which they are expressed. However, few studies on EP₃ receptor activity have identified the specific receptor isoforms expressed, therefore, the divergent effects attributed to EP₃ receptor activation may actually be due to differences in isoform expression.

Mitogen activated protein kinase (MAPK) pathway activation is often associated with survival signals and increased cellular proliferation. The p44/p42 MAPKs, also known as ERK 1/2, are serine/threonine kinases activated by extracellular stimuli, typically growth factors, and involve the sequential activation of Ras, Raf and MEK. ERK 1/2 activities include regulation of cell proliferation, differentiation, survival and apoptosis. Upstream kinases modulating the activation status of ERK 1/2 include protein kinase C (PKC), phosphoinositide-3 kinase (PI3K), protein kinase A (PKA) and transactivation by receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) [26–28].

Previously, we described the activation of the ERK 1/2 pathway by the EP_{3-Ia} receptor expressed in COS-7 cells [11]. Here we characterize the differential mechanisms of ERK 1/2 pathway activation by three human EP₃ receptor isoforms stably expressed in human embryonic kidney cells (HEK). We also show that these differences in ERK 1/2 signaling mechanisms result in differences in the activation of both ELK1 and AP-1 transcription. ELK1 is an Ets-related transcription factor activated by MAPK that binds to the serum response element of a variety of genes that regulate growth, including *c-fos*. AP-1 is a transcription factor complex made up of heterodimers of members Jun (*c-jun*, JunB and JunD) and Fos (*c-fos*, *fosB*, Fra-1 and Fra-2) family of proteins. Like ELK1, AP-1 is regulated by MAPK phosphorylation and binds to serum response element (SRE) to mediate the transcription of proteins important for cell growth and survival [3,29–31]. Our present studies suggest a mechanism by which EP₃ receptor mediated signaling may be fine-tuned by regulation of isoform expression. Differences in the expression patterns of EP₃ receptor isoforms may help clarify the disparate data in the literature.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), DMEM without phenol red, OptiMEM, G418 and gentamicin sulfate were from Mediatech (Herndon, VA). Fetal bovine serum (FBS), hygromycin B, pCEP4 vector and HEK-EBNA cells were from Invitrogen (Carlsbad, CA). QIAQUICK gel purification kit was from Qiagen (Venlo, The Netherlands). T4 DNA ligase was from New England Biolabs (Ipswich, Massachusetts). Fugene 6 transfection reagent was from Roche Diagnostics (Indianapolis, IA). Horseradish peroxidase conjugated anti-rabbit IgG antibody, Dual Luciferase Assay and Promega CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kits were from Promega (Madison, WI). Horseradish peroxidase-conjugated anti-mouse IgG and nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). Antibodies against ERK 1/2 and phospho-ERK 1/2 were from Cell Signaling Technology (Waltham, MA). AP-1 and ELK1 *Trans*-Reporting System were from Stratagene (La Jolla, CA). PGE₂ and sulprostone were from Cayman Chemical Company (Ann Arbor, MI). Enhanced chemiluminescence substrate was from Pierce (Rockford, IL). PD98059, phorbol-12-myristate-13-acetate (PMA), forskolin, AG1478, bisindolylmaleimide I, pertussis toxin and PP2 were from Calbiochem (San Diego, CA). [³H]PGE₂ and [³H]cAMP were from Amersham/GE Healthcare (Buckinghamshire, England). Protein kinase A, 3-isobutyl-1-methylxanthine (IBMX) and cAMP were from Sigma (St. Louis, MO).

Construction of Expression Vector

The EP₃ receptor isoforms were amplified from the pBC plasmid constructs previously generated by polymerase chain reaction (PCR) [7]. The following primers were used: a common EP₃ sense primer includes nucleotides 1–23 (bold) and contains a HindIII site (italics): 5'-ATTATTAAGCTTATGAAGGAGACCCGGGGCTACGG-3'. All EP₃ anti-sense primers contain an Xho-1 site (italics). EP_{3-Ia} anti-sense primer represented nucleotides 1154–1173 (bold) 5'-CCGCCGCTCGAGTTATCTTTCCAAATGGTCGC-3'. EP_{3-II} anti-sense primer represented nucleotides 1143–1167 (bold) 5'-CCGCCGCTCGAGTCATGCTTCTGTCTGTATTATTTC-3'. EP_{3-III} anti-sense primer represented nucleotides 1077–1098 (bold). 5'-CCGCCGCTCGAGTTAATTTCCCCAAAATTCTCTCC-3'. PCR products were electrophoresed on 1.5% agarose gel, excised and purified using QIAQUICK gel purification kit. Following purification, the individual EP₃ receptor PCR products were sequenced at the University of Arizona Genomic Analysis and Technology Core facility. After sequence confirmation, the individual EP₃ receptor fragments and the pCEP4 expression vector were digested using the restriction enzymes XhoI and Hind III. EP₃ receptor fragments were then ligated into the pCEP4 vector using T4 DNA ligase. The plasmids generated were named as follows: pCEP4/EP_{3-Ia}, pCEP4/EP_{3-II} and pCEP4/EP_{3-III}.

Cell Culture and Generation of Stable EP₃ Transfectants

HEK-EBNA cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 250 µg/ml G418 and 100 µg/ml gentamicin sulfate and incubated at 37°C and 5% CO₂. Transfected cells HEK-EBNA cells were grown in the same media as above, but with 200 µg/ml Hygromycin B. One million HEK cells were seeded in 10cm plates and the following day transfected with 10 µg pCEP4/EP_{3-Ia}, pCEP4/EP_{3-II} and pCEP4/EP_{3-III} plasmid DNA using Fugene 6 transfection reagent. One day after transfection, cells were selected for pCEP4/EP₃ receptor expression using 200 µg/ml hygromycin B. Approximately one week after hygromycin selection, 3–5 clones for each receptor isoform were expanded and examined for functional analysis. Whole cell radioligand binding, cAMP assay and inositol phosphate assays were used to select a clone for each EP₃ receptor isoform.

Radioligand Binding

Competition binding experiments were conducted as previously described by Regan *et al.* using HEK-EBNA cells stably expressing either the pCEP4 plasmid alone, pCEP4/EP_{3-Ia}, pCEP4/EP_{3-II} or pCEP4/EP_{3-III} [7]. Assays were conducted by incubating whole cells for one hour at room temperature in a final concentration of 2.5 nM [³H]PGE₂.

Adenosine 3'-5'-cyclic monophosphate (cAMP) Assay

HEK 293 cells stably expressing pCEP4 plasmid alone, pCEP4/EP_{3-Ia}, pCEP4/EP_{3-II} and pCEP4/EP_{3-III} were seeded into 6 well plates at a density of one million cells per well in normal culture media and incubated overnight at 37°C, 5% CO₂. In experiments using pertussis toxin, cells were dosed with 100 ng/ml PTX 8 hrs after seeding cells and 16 hrs before experiments were conducted. The following day cells were rinsed twice with phosphate buffered saline (PBS) then incubated with 2 mls Opti-MEM reduced serum media containing 0.1 mg/ml of the phosphodiesterase inhibitor, IBMX, for 15 mins at 37°C, 5% CO₂. After IBMX treatment cells were incubated with DMSO or sulprostone for 1 hour followed by treatment with 3 µM forskolin at 37°C, 5% CO₂. Following drug treatment, media was aspirated and 720 µl cold Tris/EDTA buffer (TE) (50 mM Tris/HCl, 4 mM EDTA, pH 7.5) was added to each well. Samples were scraped and cell lysates were transferred into labeled screw cap microcentrifuge tubes and boiled for 8 mins. Samples were then placed on ice for 5 mins then tubes were centrifuged 12,000 rpm for 2 mins. A standard curve was prepared to determine the concentration of cAMP in each sample. The standard curve reactions were prepared as follows in microcentrifuge tubes; 50 µl of 0.125-64 picomoles of cAMP diluted in TE or TE alone, 50 µl of 0.9 pmol [³H]cAMP diluted in TE, 100 µl of 0.06 mg/ml protein kinase A diluted in 1% BSA or 1% BSA alone. Experimental sample reactions were prepared similarly except 50 µl of cell lysates was added instead of untritiated cAMP. Reactions were incubated for two hrs at 4°C. Following reaction incubation, 100 µl of 2.5 mg/ml activated charcoal in 2% BSA was added to each microcentrifuge tube. Samples were vortexed, then centrifuged at room temperature for 1 minute, and 200 µl of supernatant was transferred into labeled scintillation vials containing 9 ml of Safety-Solve scintillation cocktail. Vials of samples were vortexed then radioactivity was detected using a Beckman LS100 scintillation counter.

Immunoblotting

Forty-eight hrs prior to experiments $\sim 0.5 \times 10^6$ HEK cells expressing EP_{3-Ia}, EP_{3-II} and EP_{3-III} were seeded into 10 cm plates and incubated at 37°C and 5% CO₂. On the day of the experiment, cells were treated with either vehicle (DMSO) or PGE₂ or phorbol-12-myristate-13-acetate for 10 mins. Following treatment cells were lysed on ice with 300 µl RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mg/ml leupeptin and 10 µg/ml aprotinin. Samples were sonicated on ice then centrifuged at 15,000 g for 15 mins. Fifty µg of sample lysates were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for one hour in 5% nonfat milk in tris buffered saline and 0.1% Tween 20 (TBS-T) at room temperature. Membranes were then incubated at 4°C overnight with anti-phospho-ERK 1/2 primary antibody diluted in 5% non-fat milk in TBS-T. Following incubation in primary antibody, membranes were washed three times with TBS-T, then incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase in 5% nonfat milk TBS-T for one hour at room temperature. After washing membrane three times, immunoreactivity was detected using SuperSignal enhanced chemiluminescence. Membranes were stripped using 2% SDS, 62.5 mM Tris (pH 7.6) and 100 mM β-mercaptoethanol for 30 mins at 55°C, then re-probed with an anti-ERK 1/2 antibody to determine total protein loading.

ELK1 and AP-1 Luciferase Assay

HEK-EBNA cells stably expressing EP_{3-Ia}, EP_{3-II} and EP_{3-III} isoforms were seeded at $\sim 0.35 \times 10^6$ cells in triplicate in 6-well plates. Twenty-four hrs later cells were transfected with 50 ng ELK1 hybrid fusion protein containing the DNA binding domain of the yeast transcription factor Gal4 plasmid, 1 μ g Gal4-luciferase plasmid and 12.5 ng of renilla (pRL-TK) plasmid. For AP-1 experiments, 1 μ g AP-1 luciferase reporter plasmid and 10 ng of pRL-TK plasmid were used. Cells were pre-treated with either vehicle or MAPK inhibitors for 30 mins followed by treatment with 50 nM PGE₂ for 16–24 hrs. Cells were then lysed with 200 μ l Passive Lysis Buffer® (Promega, Madison WI), centrifuged at 15,000g and supernatant transferred to fresh tubes. Samples were analyzed on a Turner Design TD-20 Dual-Luciferase luminometer. In AP-1 and ELK1 experiments, renilla luciferase activities for each individual transfection were used to normalize firefly luciferase activity.

Statistics

All data are presented as the mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA with Bonferroni post hoc test using GraphPad Prism software.

Results

Radioligand binding experiments with [³H]PGE₂ were undertaken to characterize the parameters of PGE₂ binding to the human EP₃ receptor isoforms stably expressed in HEK cells. Cells were prepared and incubated with [³H]PGE₂ and either vehicle (DMSO) or final concentrations of 1 nM, 10 nM, 100 nM, 1 μ M, or 10 μ M nonradioactive PGE₂. Figure 1A shows the results for binding to cells expressing the EP_{3-Ia}, EP_{3-II} and EP_{3-III} receptor isoforms and for cells stably transfected with the empty vector. The IC₅₀ values for EP_{3-Ia}, EP_{3-II} and EP_{3-III} receptor isoforms were 23 nM, 69 nM and 29 nM, respectively. The maximal binding (B_{max}) of [³H]PGE₂ to cells expressing EP_{3-Ia}, EP_{3-II} and EP_{3-III} were 857 fmol/mg protein, 1169 fmol/mg protein and 1087 fmol/mg protein, respectively. There was no specific binding of [³H]PGE₂ to cells stably transfected with the vector alone.

The EP₃ receptor is often characterized as G α_i coupled and inhibits the activation of the enzyme, adenylyl cyclase, and reduces intracellular cAMP levels. To assess G α_i coupling potential following activation of the EP₃ receptor isoforms, cAMP assays were completed as described in Methods. Following pre-treatment with the EP₃ agonist, sulprostone, activation of all three isoforms inhibits forskolin stimulated cAMP production. Figure 1B shows that in all EP₃ receptor isoform cell lines forskolin treatment increases cAMP levels above those observed with vehicle treatment alone. Pre-treatment with sulprostone inhibits forskolin stimulated cAMP production in all three cells lines, but the inhibition is greatest in the EP_{3-Ia} and EP_{3-III} expressing cells. Inhibition of G α_i with pertussis toxin pre-treatment reverses the effects of sulprostone on forskolin stimulated cAMP production. In EP_{3-II} expressing cells, pertussis toxin pre-treatment increased cAMP levels above the levels observed in forskolin treated cells. Sulprostone treatment, alone, had no effect on cAMP production.

Immunoblot assays were utilized to assess possible differences in the phosphorylation of ERK 1/2 among the cells expressing the EP₃ receptor isoforms. Phorbol-12-myristate 13 acetate (PMA) induces PKC activation and was used as a positive control to ensure all cell lines were capable of inducing ERK 1/2 phosphorylation. As shown in Figure 2, treatment of cells expressing the EP_{3-II} isoform with PGE₂ resulted in robust phosphorylation of ERK 1/2 at all concentrations tested. For cells expressing the EP_{3-III} isoform, treatment with PGE₂ clearly stimulated phosphorylation of ERK 1/2, but at lower levels than those observed for the EP_{3-II} isoform. For cells expressing the EP_{3-Ia} isoform, there was no significant PGE₂ induced phosphorylation of ERK 1/2, especially as compared with cells expressing the EP_{3-II} or

EP_{3-III} isoforms. Additionally, it should be noted that while the autoradiographs for the EP_{3-II} and EP_{3-III} isoforms were exposed for similar lengths of time, the autoradiographs for the EP_{3-Ia} isoform had to be exposed 5–10 times longer in order to see anything on the film. Thus, at physiological concentrations of PGE₂, in the range of 1–100 nM, the EP_{3-II} and EP_{3-III} receptor isoforms clearly signal through the activation of ERK 1/2; whereas, the EP_{3-Ia} isoform does not. Further experiments, therefore, utilized 50 nM PGE₂ to determine the mechanism of this activation and focussed solely on the EP_{3-II} and EP_{3-III} isoforms.

To examine the pathways responsible for PGE₂ mediated phosphorylation of ERK 1/2 by the EP_{3-II} and EP_{3-III} receptor isoforms, we utilized several well characterized pharmacological inhibitors of signaling pathways that could potentially be activated by the EP₃ receptor isoforms. Figure 3 shows the results of immunoblot assays for the PGE₂ mediated phosphorylation of ERK 1/2 following the pre-treatment of cells expressing the EP_{3-II} and EP_{3-III} isoforms with the various pathway inhibitors. The data include both a representative autoradiograph and the results of densitometry with the data normalized to PGE₂ stimulated cells that were not pre-treated with inhibitors. As can be seen, pre-treatment of cells with either the PKC inhibitor, BIM I, or the PI3K inhibitor, wortmannin, strongly decreased ERK 1/2 phosphorylation induced by the EP_{3-III} isoform, but not by the EP_{3-II} isoform. Inhibition of G α_i by pre-treatment of the cells with pertussis toxin (PTX) completely abrogated PGE₂ stimulated ERK 1/2 phosphorylation mediated by both the EP_{3-II} and EP_{3-III} isoforms. These data indicate that both isoforms couple to G α_i , but that the EP_{3-II} isoform does not utilize the PKC and PI3K pathways to induce ERK 1/2 phosphorylation following treatment with PGE₂ in this model system. Pre-treatment of the cells with genistein, a nonspecific inhibitor of tyrosine kinases had little effect on the PGE₂ stimulated phosphorylation of ERK 1/2; whereas, selective inhibition of the Src tyrosine kinase with PP2 completely blocked PGE₂ stimulated ERK 1/2 phosphorylation by both receptor isoforms. Selective inhibition of the EGFR tyrosine kinase with AG1478 partially blocked PGE₂ stimulated ERK 1/2 phosphorylation mediated by the EP_{3-II} and EP_{3-III} isoforms. These data show that the EP_{3-II} and EP_{3-III} receptor isoforms did not differ with respect to the potential involvement of receptor tyrosine kinases in PGE₂ stimulated phosphorylation of ERK 1/2, but there was an absolute requirement for the activation of Src by both isoforms. Pre-treatment with the PKA inhibitor, H-89, did not result in any significant inhibition of PGE₂ stimulated ERK 1/2 phosphorylation in cells expressing either the EP_{3-II} or EP_{3-III} isoforms (*data not shown*).

In Figure 4 luciferase reporter assays were used to investigate whether differences in the mechanism of the PGE₂ stimulation of ERK 1/2 phosphorylation by the EP_{3-II} and EP_{3-III} isoforms translated into differences in the activation of the downstream targets ELK1 and AP-1. For these experiments the same pharmacological inhibitors used in Figure 3 were again employed to determine which pathways contributed to activation of ELK1 and AP-1 reporter gene activity. As shown in Panel A of Figure 4, treatment of cells with 50 nM PGE₂ resulted in a significant stimulation of both ELK1 and AP-1 luciferase activity in cells expressing the EP_{3-II} and EP_{3-III} isoforms, but not in cells expressing the EP_{3-Ia} isoform. These results agree nicely with the PGE₂ stimulated phosphorylation of ERK 1/2 by the three isoforms as shown in Figure 2; thus, in the range of 10–100 nM, PGE₂ stimulated ERK 1/2 phosphorylation in cells expressing the EP_{3-II} and EP_{3-III} isoforms, but not in cells expressing the EP_{3-Ia} isoform.

As shown in Panel B of Figure 4, pre-treatment of cells with the inhibitors PD98059, AG1478, PP2 and pertussis toxin, significantly reduced PGE₂ stimulated ELK1 luciferase reporter activity in EP_{3-II} and EP_{3-III} isoform expressing cells. ELK1 luciferase activity in cells expressing the EP_{3-III} isoform was also significantly inhibited by pre-treatment with wortmannin and BIM I. For both EP_{3-II} and EP_{3-III} expressing cells, ELK1 luciferase reporter activity was MAPK dependent and mirrored the results obtained in Figure 3 for the PGE₂ mediated induction of ERK 1/2 phosphorylation by these isoforms.

Again, as shown in Figure 4B, pre-treatment of cells with PD98059, BIM I, PP2 and pertussis toxin significantly decreased AP-1 luciferase reporter activity in EP_{3-II} and EP_{3-III} expressing cells. Although the PGE₂ stimulation of AP-1 luciferase activity clearly involved the activation of MAPK signaling, since it was inhibited by pre-treatment with PD98059, there were several unexpected findings. First, the PKC inhibitor, BIM I, failed to reduce PGE₂ mediated ERK 1/2 phosphorylation in EP_{3-II} expressing cells (Figure 3), but nevertheless significantly inhibited AP-1 luciferase reporter activity. Also, wortmannin pre-treatment failed to inhibit EP_{3-III} activation of AP-1 luciferase reporter activity, whereas, it reduced PGE₂ dependent ERK 1/2 phosphorylation by 50% (Figure 3). Finally, AG1478 pre-treatment partially inhibited ERK 1/2 phosphorylation in both EP_{3-II} and EP_{3-III} expressing cells, but had no effect on PGE₂ stimulated AP-1 luciferase activity in these cells.

Discussion

Several laboratories have undertaken the task of elucidating the functional differences between the EP₃ receptor isoforms. Because the EP₃ receptor isoforms are usually found expressed in multiple combinations, rather than singly, it has been nearly impossible to identify the signaling properties of the individual isoforms without the use of recombinant expression systems. Here we show that in HEK cells stably expressing either the human EP_{3-Ia}, EP_{3-II} or EP_{3-III} isoforms, there are distinct differences in the PGE₂ mediated activation of ERK 1/2 phosphorylation. In addition, these differences in the induction of ERK 1/2 phosphorylation appear to translate into differences in the activation of gene expression by these isoforms, as evidenced by their stimulation of AP-1 and ELK1 luciferase reporter gene activity. For example, in cells expressing the EP_{3-Ia} isoform, the induction of ERK 1/2 phosphorylation by PGE₂ was negligible as compared with cells expressing the EP_{3-II} and EP_{3-III} isoforms. Likewise, there was no significant PGE₂ mediated stimulation of AP-1 or ELK1 reporter gene activity in cells expressing the EP_{3-Ia} isoform; whereas, AP-1 and ELK1 reporter gene activity was markedly stimulated by PGE₂ in cells expressing the EP_{3-II} and EP_{3-III} isoforms. These signaling differences were not due to significant differences in receptor number as the B_{max} values for the three cell lines were very similar (857 fmol/mg protein, 1169 fmol/mg protein and 1087 fmol/mg protein for cells expressing the EP_{3-Ia}, EP_{3-II} and EP_{3-III}, respectively).

In addition to signaling differences between the EP_{3-Ia} isoform versus the EP_{3-II} and EP_{3-III} isoforms, the mechanisms underlying the PGE₂ mediated induction of ERK 1/2 phosphorylation by the EP_{3-II} and EP_{3-III} isoforms also differed. Thus, both the EP_{3-II} and EP_{3-III} isoforms utilize a pathway involving a pertussis toxin-sensitive activation of MAPK signaling, which is dependent on the activation of Src and EGFR. However, the induction of ERK 1/2 phosphorylation by the EP_{3-III} isoform is additionally dependent upon the activation of PKC and PI3K signaling, whereas, this is not the case for the EP_{3-II} isoform.

The involvement of Gα_i, PKC, Src and EGFR demonstrated here for the induction of ERK 1/2 phosphorylation by the EP_{3-III} isoform has previously been shown for the induction of ERK 1/2 phosphorylation by the thromboxane-A₂ (TxA₂) prostanoid receptor [32]. However, unlike the TxA₂ receptor, the phosphorylation of ERK 1/2 mediated by the EP_{3-III} receptor is also dependent on the activation of PI3K signaling. In contrast, the induction of ERK 1/2 phosphorylation by the EP_{3-II} isoform is more similar that observed for the lysophosphatidic acid receptor [33,34]. Thus, both receptors utilize a mechanism involving the activation of Gα_i, Src and EGFR that is independent of the activation of PKC and PI3K signaling.

We have found that the stimulation of ELK1 and AP-1 luciferase reporter gene activity by both the EP_{3-II} and EP_{3-III} receptor isoforms is dependent upon the activation of MAPK signaling. Thus, pre-treatment with the MEK inhibitor, PD98059, significantly decreased the stimulation of ELK1 and AP-1 luciferase activity by PGE₂. As expected, many of the same signaling

pathway inhibitors that decreased the PGE₂ mediated phosphorylation of ERK 1/2 also inhibited PGE₂ stimulated AP-1 and ELK1 luciferase activity in cells expressing the EP_{3-II} and EP_{3-III} isoforms. The EP_{3-II} receptor isoform was found to activate ERK 1/2 and subsequently ELK1 via a G α_i -Src/EGFR dependent pathway, but its activation of AP-1 was found to involve a G α_i -Src-PKC dependent pathway. On the other hand, the EP_{3-III} isoform was found to activate ERK 1/2 and ELK1 by a G α_i -Src/EGFR-PKC-PI3K dependent pathway and its activation of AP-1 was essentially by the same mechanism, except for the involvement of PI3K. The inability of the EP_{3-Ia} receptor isoform to stimulate AP-1 and ELK1 luciferase activity is not surprising given its failure to stimulate the phosphorylation of ERK 1/2 at low concentrations of PGE₂. Even at higher concentrations of PGE₂, stimulation of AP1 and ELK1 would be unlikely since it has been found that AP-1 components, such as FRA-1, require elevated and sustained levels of ERK 1/2 activity for successful activation of transcription [35,36].

In conclusion, the present studies have explored the mechanisms of the PGE₂ mediated phosphorylation of ERK 1/2 by the EP_{3-Ia}, EP_{3-II} and EP_{3-III} receptor isoforms. Our findings contribute to a better understanding of the signaling differences between the EP₃ receptor isoforms that underlie their involvement in the regulation physiological systems. Of particular significance is the potential of these receptor isoforms to differentially regulate gene transcriptional activity.

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Figure 1. Competition for the binding of $[^3\text{H}]\text{PGE}_2$ by nonradioactive PGE_2 (A) and pertussis toxin sensitive inhibition of forskolin stimulated cAMP formation by sulprostone (B) in HEK cells stably expressing either the EP_{3-1a} , EP_{3-II} or EP_{3-III} receptor isoforms

(A) HEK cells stably expressing either the EP_{3-1a} , EP_{3-II} or EP_{3-III} receptor isoforms were incubated with either vehicle or 1 nM-10 μM PGE_2 and assayed for specific binding of $[^3\text{H}]\text{PGE}_2$ as described in Materials and Methods. (B) EP_{3-1a} , EP_{3-II} and EP_{3-III} isoform expressing cells were pre-treated with pertussis toxin (PTX) then assayed for inhibition of forskolin stimulated cAMP production following treatment with 1 μM sulprostone as described in Materials and Methods. Data are from three independent experiments and represent the mean $\pm$ SEM.

Figure 2. Phosphorylation of ERK 1/2 in HEK cells stably expressing either the EP_{3-1a}, EP_{3-II} or EP_{3-III} receptor isoforms following treatment with increasing concentrations of PGE₂ Cells were incubated with either vehicle (Veh), 1 nM-1 μ M PGE₂ or 100 nM phorbol-12-myristate-13-acetate (PMA) for 10 mins, then lysed on ice. Samples of the cell lysates were immunoblotted as described in Materials and Methods using anti-phospho-ERK 1/2 and anti-total-ERK 1/2 antibodies. EP_{3-1a} autoradiographs were exposed for 3–5 mins, EP_{3-II} and EP_{3-III} autoradiographs exposed 15–30 sec. Blots are representative of three independent experiments.

Figure 3. Effects of various signaling pathway inhibitors on the PGE₂ induced phosphorylation of ERK 1/2 in HEK cells expressing either the EP_{3-II} or EP_{3-III} isoforms

Cells were untreated or pre-treated with either 100 nM bisindolylmaleimide I (BIM I), 100 nM wortmannin (WORT), 100 nM genistein, 100 nM AG1478, or 10 μ M PP2 for 30 mins, or with 100 ng/ml pertussis toxin (PTX) for 16 hrs, followed by stimulation with 50 nM PGE₂ for 10 mins then lysed on ice. Samples of the cell lysates were immunoblotted as described in Materials and Methods using anti-phospho-ERK 1/2 and anti-total-ERK 1/2 antibodies. The autoradiographs were also scanned by densitometry and the results presented as bar graphs

normalized to PGE₂ stimulated cells that were not treated with inhibitors. Blots are representative and the bar graphs are the mean $\pm$ SEM of the three experiments; levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Figure 4. PGE₂ stimulated ELK1 and AP-1 luciferase reporter gene activity in HEK cells stably expressing either the EP_{3-1a}, EP_{3-II} or EP_{3-III} receptor isoforms (A) or following pre-treatment of EP_{3-II} or EP_{3-III} expressing cells with various signaling pathway inhibitors (B)

HEK cells stably expressing either EP_{3-1a}, EP_{3-II} or EP_{3-III} isoforms were transfected with the ELK1 and AP-1 reporter plasmids and luciferase activity was determined as described in Materials and Methods. (A) After transfection, cells were treated with either vehicle (V) or 50 nM PGE₂ (PG) for 16–24 hrs, then were lysed and samples analyzed for luciferase activity. (B) EP_{3-II} or EP_{3-III} expressing cells were untreated or pre-treated with either 1 μ M PD98059 (PD), 100 nM bisindolylmaleimide I (BIM I), 100 nM wortmannin (Wort), 100 nM AG1478 (AG) or 10 μ M PP2 for 30 mins, or 100 ng/ml pertussis toxin (PTX) for 16 hrs, followed by

stimulation with either vehicle (V) or 50 nM PGE₂ (PG) for 16–24 hrs. The cells were then lysed and samples analyzed for luciferase activity. Data are from 3–6 experiments and are normalized to vehicle control. Data in panel A for EP_{3-II} and EP_{3-III} are the same as in panel B for cells that were not treated with inhibitors. Levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ as compared to PGE₂ treated cells.