Prostaglandin E\(_2\) and F\(_{2\alpha}\) activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element

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Abstract

In endometrial adenocarcinomas COX-2 and F-series prostanoid (FP) receptor expression and prostanoid biosynthesis (PGE\(_2\) and PGF\(_{2\alpha}\)) are elevated. In the present study, we investigated the effect of PGE\(_2\) and PGF\(_{2\alpha}\) on the expression of COX-2 via the FP receptor in endometrial adenocarcinoma cells stably expressing the FP receptor (FPS cells). Using chemical inhibitors of intracellular signaling pathways, reporter gene assays and quantitative RT-PCR analysis, we show that PGE\(_2\) and PGF\(_{2\alpha}\) can mobilize inositol 1,4,5-trisphosphate, induce ERK1/2 phosphorylation via the phospholipase C-protein kinase A-epidermal growth factor receptor pathway and induce cyclooxygenase-2 (COX-2) expression via the FP receptor. In addition we show that the PGE\(_2\) or PGF\(_{2\alpha}\)-regulation of COX-2 via the FP receptor is mediated via the cyclic AMP response element (CRE) binding site on the COX-2 promoter. These data indicate that PGE\(_2\) and PGF\(_{2\alpha}\) biosynthesized locally within endometrial adenocarcinomas can regulate tumor cell function in an autocrine/paracrine manner via the FP receptor.

Keywords: Cyclooxygenase; Prostaglandin; Signal transduction; Cancer; Receptor

1. Introduction

Prostaglandin endoperoxide (PGH) synthase or cyclooxygenase (COX) catalyses the committed step in the conversion of arachidonic acid to prostaglandins (PG) (Marnett et al., 1999). Two COX enzymes, COX-1 and COX-2, which are the targets for non-steroidal anti-inflammatory drug treatment have been characterised (Vane and Botting, 1998; Smith et al., 2000). A third COX enzyme (COX-3), a variant of COX-1 formed by retention of intron-1 and which is sensitive to acetaminophen, has been cloned more recently from canine cerebral cortex (Chandrasekharan et al., 2002).

COX-1 is constitutively expressed in many cell types and is overexpressed in certain cancers (Hwang et al., 1998; Bauer et al., 2000; Kirschenbaum et al., 2000; Sales et al., 2002). COX-2 is the more inducible form of the enzyme and is commonly associated with pathological conditions including tumorigene-
transactivation of the epidermal growth factor receptor (EGFR) (Sheng et al., 2001; Regan, 2003; Jabbour and Sales, 2004; Sales et al., 2004a,b; Jabbour et al., 2005; Smith et al., 2006).

We have previously demonstrated elevated expression and signaling of COX-2 and FP receptor in human endometrial adenocarcinomas and have ascertained a role for PGF2α–FP receptor interaction in enhancing the proliferation of endometrial epithelial cells (Jabbour and Sales, 2004; Jabbour et al., 2005; Sales et al., 2004b) and promoting the expression of pro-angiogenic and inflammatory genes in endometrial adenocarcinoma cells and adenocarcinoma biopsy explants via activation of ERK1/2 (Jabbour et al., 2005; Sales et al., 2005, 2007).

Although PGE2 and PGF2α are considered to be the endogenous ligands for EP and FP receptors, respectively, PGE2 can bind to the FP receptor with an affinity that is only 10–30-fold less than PGF2α (Abramovitz et al., 2000). The affinity of PGF2α for EP receptors is 100–300-fold less than it is for the FP receptor (Abramovitz et al., 2000).

Recently, we have shown that COX-2 and PGF2α biosynthesis can be autoregulated in endometrial adenocarcinoma cells via the FP receptor (Jabbour et al., 2005). Given that both PGE2 and PGF2α biosynthesis are elevated in endometrial pathologies (Lundstrom and Green, 1978; Lumsden et al., 1983; Sales and Jabbour, 2003) and since PGE2 can act as an agonist of the FP receptor, we investigated the effect of PGE2 and PGF2α on regulation of COX-2 via the FP receptor in Ishikawa endometrial epithelial cells stably transfected with the human isoform of the FP receptor.

2. Materials and methods

2.1. Reagents

| Compound | Target Description | Reference |
|----------|--------------------|-----------|
| AL8810   | FP receptor antagonist | Griffin et al. (1999) |
| AH6809   | EP2 receptor antagonist | Woodward et al. (1995) |
| ONOAE2227| EP4 receptor antagonist | Munoz et al. (2002) |
| U73122   | PLC beta inhibitor    | Bleasdale et al. (1990) |
| 4C3MQ    | Protein kinase A inhibitor | Lu et al. (1996) |
| GF109203X| Protein kinase C inhibitor | Touleec et al. (1991) |
| AG1478   | Epidermal growth factor tyrosine kinase inhibitor | Eguchi et al. (1998) |
| PD98059  | Extracellular signal-regulated kinase kinase (MEK) inhibitor | Alessi et al. (1995) |

2.2. Cell culture

Ishikawa endometrial adenocarcinoma cells were obtained from the European Collection of Cell Culture (Wiltshire, UK). Stable FP transfectant cells were constructed, characterised and maintained as described (Sales et al., 2005), with the addition of a maintenance dose of 200 μg/ml G418.

2.3. Total inositol phosphate (IP) assays

Total inositol phosphate (InsP) production was measured in Ishikawa WT and FFS cells and assayed as described previously (Berg et al., 1994; Sales et al., 2005). Cells were treated either with vehicle, PGE2 or PGF2α, in the absence or presence of receptor antagonist or chemical inhibitor as shown in the figure legends. Data are presented as mean ± S.E.M. from at least 3 independent experiments.

2.4. cAMP assay

cAMP accumulation was determined in response to administration of vehicle, PGE2 or PGF2α, in the absence or presence of receptor antagonist or chemical inhibitor as shown in the figure legend and was performed as described previously (Sales et al., 2002). Briefly, cells (2 × 10⁵) were seeded and allowed to attach overnight. The following day, the cells were serum starved by incubating with fresh serum-free medium containing 8.4 μM indomethacin for at least 16 h. Thereafter the culture medium was removed and replaced with serum-free medium containing 3-isobutyl-1-methyl xanthine (IBMX; Sigma) to a final concentration of 1 mM and receptor antagonist/chemical inhibitor for 30 min at 37 °C. Cells were then stimulated with ligand for the time indicated in the figure legend. Following stimulation, cells were lysed in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit (Biomed, Affiniti, Exeter, UK) according to the manufacturer’s protocol and normalised to protein concentration of the lysate. Protein concentrations were determined using protein assay kits (Bio-Rad Laboratories, Hemel Hamstead, UK). Data are presented as mean ± S.E.M. from at least 4 independent experiments.

2.5. In-cell Western detection

Cell signaling to ERK1/2 was investigated using an In-cell Western assay. Approximately, 20,000 cells were seeded per well in a 96-well microtiter plate and allowed to adhere overnight at 37 °C. The following day cells were starved by serum withdrawal in serum-free culture medium containing 8.4 μM indomethacin for at least 16 h at 37 °C. Cells were incubated for 30 min with vehicle, receptor antagonist or chemical inhibitor as described in the figure legend. Thereafter cells were stimulated with either vehicle PGE2 or PGF2α, in the absence or presence of receptor antagonist or chemical inhibitor for the
time indicated in the figure legend. Following stimulation, cells were washed with ice-cold PBS, fixed in 3.7% (v/v) Formaldehdye for 20 min at room temperature, and permeabilised with 0.1% Triton X-100 in PBS. Cells were then blocked for 45 min at room temperature with Odyssey Blocking buffer™ (LI-COR Biosciences, Cambridge, UK) before overnight incubation with primary rabbit phospho-p42/44 and goat p42/44 antibodies (diluted 1:100 in Odyssey blocking buffer) at 4 °C. The following day, cells were washed and incubated with the goat anti-rabbit Alexafluor 680 (1:200) and goat anti-mouse IRDye™ 800 (1:800) for 60 min at room temperature.

Immunoreactive proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). ERK1/2 phosphorylation was calculated by dividing the value obtained from the phosphorylated ERK1/2 channel (700 nm) by the value obtained from total ERK1/2 channel (800 nm) and expressed as fold above vehicle controls. Results are expressed as mean ± S.E.M. from at least 3 independent experiments performed in triplicate.

2.6. Taqman quantitative RT-PCR

COX-2 mRNA expression in FPS cells was measured by quantitative RT-PCR analysis. FPS cells were starved by serum withdrawal for at least 12 h in serum-free medium containing 8.4 μM indomethacin. Thereafter medium was removed and replaced with fresh medium containing indomethacin with either vehicle, PGE2 or PGF2α in the absence or presence of receptor antagonist or chemical inhibitor as described in the figure legend. RNA was extracted using Tri-reagent (Sigma) following the manufacturers guidelines. Once extracted and quantified, RNA samples were reverse transcribed and subjected to RT-PCR analysis using an ABI Prism 7900 (Jabbour et al., 2005). COX-2 primers and probe for quantitative PCR were designed using the PRIMER express program (PE Applied Biosystems, Warrington, UK) as described previously (Jabbour et al., 2005). Data were analysed and processed using Sequence Detector v1.6.3 (PE Applied Biosystems). Expression of COX-2 was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed as fold increase above vehicle treated from at least 4 independent experiments and represented as mean ± S.E.M.

2.7. Transfection of CRE and COX-2 promoter with deletions and mutations

Ishikawa FPS cells were transiently transfected for 6 h using a liposomal transfection system (Superfect, Qiagen, Crawley, UK). Transfections were performed with CRE-Luciferase a specific cis-acting DNA binding sequence of the cAMP response element ligated with a Luciferase reporter plasmid (Clontech Laboratories, BD Bioscience, Cowley, UK) or the COX-2 promoter, performed using C2.1 (~917 to +49) 966 base pair (bp) fragment of the COX-2 promoter and C2.1 with a series of deletions or site specific mutations ligated with a Luciferase reporter plasmid pGL3 basic (Promega, Southampton, UK) as described in Bradley et al. (2003) kindly supplied by Dr. Robert Newton, BioMedical Research Institute, Department of Biological Sciences, The University of Warwick, UK. Deletions consisted of Dra (~625/+49) 674 bp, Sty (~358/+49) 407 bp, Alu (190/+49) 239 bp and RSA (−86/+49) 135 bp fragments. The description of each clone is based on the restriction site used to generate the construct (Dra, Alu, Sty, RSA, HIN). Mutations consisted of CRE (~59−53), a mutation in the cAMP response element. The HIN (~79/+53) 200 bp and HIN CRE-mutation (HINcrem; ~79/+53, 200 bp) fragments were generated by polymerase chain reaction (PCR) using the Sty and Stycrem cDNA (Bradbury et al., 2003) as a template.

 Amplification of HIN and HINcrem was carried out using standard PCR mix containing forward 5′-AAGGCGGAAAAGACGCTCA-3′ and reverse 5′-AACAGTACCGGAAATGC-3′ primers containing the HindIII restriction site at the start for ease of cloning. To amplify by PCR, sample mix was denatured at 94 °C for 5 min and subjected to 40 cycles of 94 °C for 1 min, 58 °C for 1 min and 68 °C for 1 min, with a final extension step of 68 °C for 7 min. After amplification, samples were cooled to 4 °C and visualised on 1% agarose gels. The PCR product was ligated into the pCR®II-TOPO vector (Invitrogen, De Schelp, The Netherlands) followed by sequencing in both directions using a PE Applied Biosystems 373A automated sequencer. The HIN and HINcrem cDNA was ligated into the pGL3 basic (Promega) expression vector followed by sequencing.

2.8. Luciferase reporter assays

CRE or COX-2 promoter firefly Luciferase reporter vectors were co-transfected into Ishikawa FPS cells in triplicate with an internal control pRL-TK (containing the Renilla Luciferase coding sequence; Promega) as described (Jabbour et al., 2005). The following day the cells were serum-starved for at least 16 h 37 °C with 8.4 μM indomethacin prior to stimulation for 4 h with vehicle, PGE2 or PGF2α in the absence or presence of receptor antagonist or chemical inhibitor as described in the figure legend. The activity of both firefly and Renilla Luciferase was determined using the dual Luciferase assay kit (Promega) and total Luciferase activity was determined by dividing the relative light units generated by the firefly Luciferase by the relative light units generated by the Renilla Luciferase in the same reaction. Fold increase in Luciferase activity was calculated by dividing the total Luciferase activity in cells treated with PGE2 or PGF2α, in the absence or presence of receptor antagonist or chemical inhibitor by the total Luciferase activity in cells treated with vehicle. Data are presented as mean ± S.E.M. from at least 4 independent experiments.

2.9. Statistics

Data were subjected to statistical analysis with ANOVA and Fishers protected least significant difference tests (Statview 5.0; Abacus Concepts Inc., USA).

3. Results

3.1. PGE2 and PGF2α mobilize inositol 1,4,5-trisphosphate in FPS cells

We have previously reported elevated FP receptor expression in endometrial adenocarcinomas (Sales et al., 2004b) and constructed and characterized an endometrial adenocarcinoma (Ishikawa) cell line expressing FP receptor (FPS cells) to the levels observed in endometrial adenocarcinomas (Sales et al., 2005). In this latter study we showed that PGF2α-FP receptor interaction in FPS cells increases the hydrolysis of InsP3 in FPS cells via Gq/11 to a greater extent than in wild-type (WT) Ishikawa cells (Sales et al., 2005).

In the present study, we found that PGE2 could also dose-dependently mobilize InsP in FPS cells (Fig. 1A; P < 0.05). No such increase in InsP production was observed in WT cells in response to PGE2, but a modest increase was observed in response to PGF2α (Fig. 1A). Co-incubation of FPS cells with the specific FP receptor antagonist AL8810 abolished the PGE2 or PGF2α-mediated increase in InsP production at all concentrations of ligand administered (Fig. 1B, P < 0.05). These results suggest that the PGE2-mediated InsP hydrolysis in FPS cells was via the FP receptor.

We further confirmed that the InsP hydrolysis in response to 100 nM PGE2 or 100 nM PGF2α was not mediated via activation of either of the endogenous EP2 or EP4 receptors or intracellular signaling cascades downstream of phospholipase C8 (PLC) as neither the specific EP2 receptor antagonist (AH6809), the EP4 receptor antagonist (ONOAE2227; Fig. 1C, P > 0.05) nor the chemical inhibitors of the protein kinase A (PKA; 4-cyano-3-methylisouquinoline; 4C3MQ), protein kinase C (PKC; GF109203x) and ERK1/2 (PD98059) signaling path-
Fig. 1. (A) Total inositol phosphate (InsP) production was assessed in Ishikawa WT or FPS cells treated with increasing doses of PGE$_2$ or PGF$_{2\alpha}$ for 1 h at 37 °C.

(B) Total InsP production in FPS cells treated with increasing doses of PGE$_2$ or PGF$_{2\alpha}$ in the absence or presence of 50 μM of the specific FP receptor antagonist AL8810 for 1 h at 37 °C.

(C) Total InsP production in FPS cells treated with 100 nM PGE$_2$ or 100 nM PGF$_{2\alpha}$ in the absence/presence of the FP receptor antagonist AL8810 (50 μM), EP2 receptor antagonist (AH6809; 10 μM), EP4 receptor antagonist (ONOAE2227; 1 μM) or chemical inhibitors of phospholipase C (U73122, 10 μM), protein kinase A (4C3MQ, 1 μM), protein kinase C (GF109203x, 10 μM) or ERK1/2 kinase (PD98059, 50 μM) for 1 h at 37 °C. Data are presented as mean ± S.E.M. b is significantly different from a, c is significantly different from a and b; $P < 0.05$.

ways (Fig. 1C, $P < 0.05$, $P < 0.05$) inhibited the PGE$_2$ or PGF$_{2\alpha}$-mediated InsP production. As shown for the specific FP receptor antagonist (AL8810), the PLC inhibitor (U73122) also abolished the InsP produced in response to treatment of FPS cells with 100nM PGE$_2$ or 100nM PGF$_{2\alpha}$ (Fig. 1C, $P < 0.05$) further demonstrating the InsP production in response to ligand activation was mediated via the FP receptor-PLC pathway.
Fig. 2. (A) cAMP accumulation in FPS cells in response to treatment with 100 nM PGE\(_2\) or 100 nM PGF\(_2\alpha\) for 0, 5 and 10 min. (B) cAMP accumulation in FPS cells in response to treatment with 100 nM PGE\(_2\) or 100 nM PGF\(_2\alpha\) for 10 min in the presence/absence of the FP receptor antagonist AL8810 (50\(\mu\)M), EP\(_2\) receptor antagonist (AH6809; 10 \(\mu\)M), EP\(_4\) receptor antagonist (ONOAE2227; 1 \(\mu\)M) or chemical inhibitors of phospholipase C\(_\beta\) (U73122, 10 \(\mu\)M), protein kinase A (4C3MQ, 1 \(\mu\)M), protein kinase C (GF109203x, 10 \(\mu\)M) or ERK1/2 kinase (PD98059, 50 \(\mu\)M). Data are presented as mean ± S.E.M. b is significantly different from a and c is significantly different from a and b; \(P < 0.05\).

### 3.2. PGE\(_2\) and PGF\(_2\alpha\) promote cyclic adenosine-3,5-monophosphate (cAMP) in FPS cells

In addition to elevated FP receptor, Ishikawa FPS cells also express basal levels of EP2 and EP4 receptor, but not detectable EP1 receptor (data not shown), which couple to \(G_s\) and mobilize intracellular cAMP. We investigated cAMP accumulation in FPS cells in response to treatment with 100 nM PGE\(_2\) or 100 nM PGF\(_2\alpha\) for 0, 5 or 10 min (Fig. 2A). We found that 100 nM PGE\(_2\) rapidly mobilized intracellular cAMP at 5 and 10 min (Fig. 2A; \(P < 0.05\)). By contrast 100 nM PGF\(_2\alpha\) modestly increased cAMP following 10 minutes of treatment only (Fig. 2A; \(P < 0.05\)).

We confirmed that the cAMP produced in response to 100 nM PGE\(_2\) or 100 nM PGF\(_2\alpha\) was not mediated by an intracellular mechanism via activation of the FP receptor or PLC, PKA, PKC or ERK1/2 signaling cascades as neither the specific FP receptor antagonist (AL8810; Fig. 2B; \(P < 0.05\)) nor the chemical inhibitors of the PKA (4C3MQ), PKC (GF109203x) or ERK1/2 (PD98059) signaling pathways (Fig. 2B; \(P < 0.05\)) inhibited the PGE\(_2\) or PGF\(_2\alpha\)-mediated cAMP production following ligand stimulation for 10 min. However, co-treatment of FPS cells with the EP2 receptor antagonist (AH6809) or EP4 receptor antagonist (ONOAE2227) significantly reduced the PGE\(_2\) or PGF\(_2\alpha\)-mediated cAMP response. Moreover, the EP2 (AH6809) and EP4 (ONOAE2227) receptor antagonist in combination totally abolished the PGE\(_2\), or PGF\(_2\alpha\)-mediated cAMP response following 10 min of ligand stimulation (Fig. 2B; \(P < 0.05\)).

### 3.3. PGE\(_2\) and PGF\(_2\alpha\) signaling cascades converge on ERK1/2

The effect of PGE\(_2\) or PGF\(_2\alpha\) on the activation of the downstream extracellular signal-regulated kinase (ERK1/2) signaling
pathway was determined after treatment of FPS cells with vehicle, 100 nM PGE2 or 100 nM PGF2α for 0, 5, 10 and 20 min (Fig. 3A; \( P < 0.05 \)). Stimulation of FPS cells with PGE2 or PGF2α caused a rapid time-dependent activation of ERK1/2 (Fig. 3A; \( P < 0.05 \)). The peak of ERK1/2 activation was observed after 5 min in FPS cells treated with 100 nM PGE2 and 10 min in cells treated with 100 nM PGF2α (Fig. 3A; \( P < 0.05 \)).

We next evaluated the effect of the FP receptor antagonist (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227) and chemical inhibitors of PLC (U73122), PKC (GF109203x), PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059) on the PGE2 or PGF2α-induced activation of ERK1/2 signaling. As observed in Fig. 3A, ERK1/2 phosphorylation was significantly elevated in FPS cells treated for 10 min with PGE2 (Fig. 3B; \( P < 0.05 \)). The PGE2-induced elevation in ERK1/2 activation was significantly inhibited by co-treatment of FPS cells with FP receptor antagonist (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227) and PLC inhibitor (U73122) and abolished by treatment of cells with the PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059) inhibitors, but not the PKC inhibitor (GF109203x; Fig. 3B, \( P < 0.05 \)). The PGF2α-induced elevation in ERK1/2 activation was significantly inhibited by co-treatment of cells with the EP2 antagonist (AH6809), but not the EP4 receptor antagonist (ONOAE2227) or PKC inhibitor (GF109203x), and was abolished by treatment with the FP receptor antagonist (AL8810), and inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059; Fig. 3B, \( P < 0.05 \)).

3.4. Activation of COX-2 Luciferase reporter and mRNA by PGE2 and PGF2α

The role of PGE2 or PGF2α on the activation of COX-2 in FPS cells was investigated by Luciferase reporter gene assay (Fig. 4A) and quantitative RT-PCR analysis (Fig. 4B). Treatment of FPS cells with PGE2 or PGF2α caused a significant time-dependent increase in COX-2 Luciferase reporter activity (Fig. 4A; \( P < 0.05 \)) and mRNA expression (Fig. 4B; \( P < 0.05 \)) which peaked at 4–6 h. The PGF2α-induced increase in COX-2 reporter gene activation (Fig. 4A) and mRNA expression (Fig. 4B) was greater than that induced by PGE2 (\( P < 0.05 \)).

3.5. COX-2 expression is mediated via activation of the ERK pathway

We set out to determine the signaling pathways mediating COX-2 expression in FPS cells. Cells were treated with vehicle, 100 nM PGE2 or 100 nM PGF2α, in the presence/absence of the FP receptor antagonist (AL8810), EP2 receptor antag-
Fig. 4. COX-2 Luciferase activity (A) and mRNA expression (B) in FPS cells in response to treatment with 100 nM PGE$_2$ or 100 nM PGF$_{2\alpha}$ for 0, 2, 4, 6, 8 and 24 h. Data are presented as mean ± S.E.M. b is significantly different from a, c is significantly different from a and b; $P < 0.05$.

The PGF$_{2\alpha}$-induced elevation in COX-2 Luciferase (Fig. 5C) and mRNA expression (Fig. 5D) was significantly inhibited by co-treatment of cells with the EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227), and chemical inhibitors of PLC (U73122), PKA (4C3MQ), PKC (GF109203x), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059), but not the PKC inhibitor (GF109203x; $P < 0.05$).

The PGF$_{2\alpha}$-induced elevation in COX-2 Luciferase (Fig. 5C) and mRNA expression (Fig. 5D) was significantly inhibited by co-treatment of cells with the FP receptor antagonist (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227), and chemical inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059), but not the PKC inhibitor (GF109203x; $P < 0.05$).

The PGF$_{2\alpha}$-induced elevation in COX-2 Luciferase (Fig. 5C) and mRNA expression (Fig. 5D) was significantly inhibited by co-treatment of cells with the FP receptor antagonist (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227), and chemical inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059), but not the EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOAE2227) or PKC inhibitor (GF109203x; Fig. 5C and D, $P < 0.05$).
3.6. Mutation of the CRE-binding site in the COX-2 promoter inhibits PGE2 and PGF2α-mediated Luciferase activity

To determine which transcription factors were involved in mediating COX-2 expression in response to PGE2 or PGF2α, FPS cells were transiently transfected with the 966 bp full length COX-2 promoter (C2.1) or COX-2 promoter containing a series of deletions as described in Bradbury et al. (2003). PGE2 or PGF2α increased COX-2 Luciferase activity by 2.2 ± 0.1 and 2.6 ± 0.1-fold, respectively (Fig. 6A; P < 0.05). There was no significant reduction in Luciferase activity with any of the sequential 5′ deletions compared with the C2.1, suggesting that even the smallest HIN fragment of the COX-2 promoter, which contains only the CRE, was able to induce COX-2 Luciferase activity (Fig. 6A). Transfection of FPS cells with the HIN COX-2 promoter fragment with a mutated CRE (HINcrem; −59/−53) abolished the COX-2 activity induced by PGE2 or PGF2α (Fig. 6A; P < 0.05), suggesting that this factor is necessary for COX-2 induction by prostanoids.

3.7. CRE is activated by PGE2 and PGF2α signaling to ERK

We further confirmed the signaling pathways activating the CRE in FPS cells transfected with cDNA construct containing a specific cis-acting DNA binding sequence of the cAMP response element ligated with a Luciferase reporter plasmid. Cells were treated with vehicle, 100 nM PGE2 or 100 nM PGF2α in the presence/absence of the FP receptor antagonist (AL8810), EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOE2227), PLC inhibitor (U73122), PKA inhibitor (4C3MQ), PKC inhibitor (GF109203x), EGFR kinase inhibitor (AG1478) or ERK1/2 inhibitor (PD98059) for 4 h. CRE Luciferase activity in FPS cells was significantly elevated in response to PGE2 (Fig. 6B) or PGF2α (Fig. 6C) treatment for 4 h (P < 0.05).

The PGE2 (Fig. 6B) -induced activation of CRE Luciferase was significantly reduced by treatment of FPS cells with the FP receptor (AL8810), EP2 receptor (AH6809), or EP4 receptor (ONOE2227) antagonists or chemical inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase (AG1478) or ERK1/2 kinase (PD98059), but not the PKC inhibitor (GF109203x; Fig. 6B P < 0.05). The PGF2α (Fig. 6C) -induced activation of CRE Luciferase was significantly inhibited by co-treatment of cells with the FP receptor antagonist (AL8810) or chemical inhibitors of PLC (U73122), PKA (4C3MQ), EGFR kinase (AG1478) and ERK1/2 kinase (PD98059), but not the EP2 receptor antagonist (AH6809), EP4 receptor antagonist (ONOE2227) or PKC inhibitor (GF109203x; Fig. 6C; P < 0.05).

4. Discussion

COX-2 expression is up-regulated in numerous pathologies including those of the reproductive tract such as ovarian carcinoma, cervical carcinoma and endometrial adenocarcinoma.
Since endometrial pathologies biosynthesize PGE2 and PGF2\(_\alpha\) in endometrial adenocarcinomas (Sales et al., 2004b, 2005). Jabbour et al., 2005).

We and others’ have shown that PGE\(_2\)-EP receptor interaction can down regulate the expression of tumour suppressor genes (Sales et al., 2004c), increase cellular growth, migration and invasiveness (Sheng et al., 2001) and promote angiogenesis in in vitro and in vivo model systems (Watanabe et al., 2000; Sonoshita et al., 2001; Seno et al., 2002; Buchanan et al., 2003; Fujino et al., 2003; Sales et al., 2004a). Similarly PGF\(_{2\alpha}\) can also enhance cell growth rate and induce the expression of inflammatory and angiogenic genes, in Ishikawa cells stably expressing the FP receptor and endometrial adenocarcinoma explants, via the FP receptor (Sales et al., 2004b, 2005; Jabbour et al., 2005).

We have recently reported elevated expression of FP receptor in endometrial adenocarcinomas (Sales et al., 2004b, 2005). Since endometrial pathologies biosynthesize PGE\(_2\) and PGF\(_{2\alpha}\) in the micromolar range (Lundstrom and Green, 1978; Smith et al., 1981; Rees et al., 1984; Adelantado et al., 1988) which can act locally at the site of production on prostaglandin receptors and since PGE\(_2\) can bind to the FP receptor with an affinity that is only 10–30-fold less than PGF\(_{2\alpha}\) (Abramovitz et al., 2000), we investigated the effect of PGE\(_2\) and PGF\(_{2\alpha}\) on regulation of COX-2 via the FP receptor.

Using endometrial adenocarcinoma cells (Ishikawa cells) stably expressing the FP receptor to the levels observed in endometrial adenocarcinomas (FPS cells), we found that PGE\(_2\) dose-dependently mobilized InsP hydrolysis in FPS cells in a similar manner to that observed for PGF\(_{2\alpha}\) via the FP receptor, since the specific FP receptor antagonist AL8810 abolished the PGE\(_2\) and PGF\(_{2\alpha}\)-mediated increase in InsP production. Although the PGE\(_2\)-induced mobilization of InsP via the FP receptor was less than that observed for PGF\(_{2\alpha}\), which we postulated was due to the reduced affinity of PGE\(_2\) for the FP receptor compared with the native ligand PGF\(_{2\alpha}\), these data nevertheless demonstrate that elevated levels of PGF\(_{2\alpha}\) as well PGE\(_2\) as can activate FP receptor signaling in tumors expressing elevated levels of FP receptor.

As FPS cells also express basal levels of EP2 and EP4 receptor (but not detectable EP1 or EP3 receptor), we found that PGE\(_2\) and to a much lesser extent PGF\(_{2\alpha}\) could also mobilize intracellular cAMP. This effect of PGE\(_2\) on intracellular cAMP accumulation could be inhibited with the selective EP2 or EP4 receptor antagonists AH6809 and ONOAE2227 and abolished with the combination of EP2 and EP4 receptor antagonists. Interestingly only the EP2 receptor antagonist, but not the EP4 receptor antagonist abolished the PGF\(_{2\alpha}\)-mediated increase in cAMP. We found that neither the PGE\(_2\) nor PGF\(_{2\alpha}\) effects on inositol phosphate hydrolysis and intracellular cAMP release was mediated by an intracellular mechanism involving activation of the downstream PKA, PKC or ERK1/2 pathways, as the chemical inhibitors of PKA, PKC and ERK1/2 failed to reduced the prostanoid mediated effects on InsP and cAMP accumulation.

The integrated response to GPCR coupling and second messenger activation results in phosphorylation of numerous effector signaling pathways, including the MAPK pathway (Naor et al., 2000), to regulate gene transcription. The MAPK pathway is a key signaling mechanism that regulates many cellular functions such as growth, differentiation and transformation (Lewis et al., 1998; Naor et al., 2000). We investigated the signaling pathways mediating the effect of PGE\(_2\) or PGF\(_{2\alpha}\) on ERK1/2 in FPS cells. We found that PGE\(_2\) and PGF\(_{2\alpha}\) stimulation of FPS cells induces ERK1/2 phosphorylation via identical intracellular signaling pathways via the PLC-PKA-mediated activation of the EGFR since co-treatment of cells with the PLC, PKA or EGFR kinase inhibitors significantly inhibited the PGE\(_2\) or PGF\(_{2\alpha}\)-induced ERK1/2 phosphorylation. We previously reported that PGF\(_{2\alpha}\) signaling to ERK1/2 in FPS cells is mediated via the PKA and not PKC pathway (Sales et al., 2005). In the present study we demonstrate that the PGE\(_2\)-mediated signaling to ERK1/2 in FPS cells is also PKA-dependent and PKC-independent, since the PKC inhibitor GF109203x failed to inhibit either the PGE\(_2\) or PGF\(_{2\alpha}\)-mediated signaling to ERK1/2. Moreover, we found that the PGE\(_2\)-mediated effects on ERK1/2 are mediated largely in FPS cells via the FP receptor as the FP receptor antagonist inhibited the PGE\(_2\)-mediated ERK1/2 phosphorylation to a greater extent than the EP2 or EP4 receptor antagonists. These data suggest that the cAMP pathway activated by the endogenous EP2/EP4 receptors acts synergistically with the InsP pathway to augment the signaling of PGE\(_2\) to ERK1/2 via the PKA-EGFR pathway.

We previously reported that PGF\(_{2\alpha}\) could regulate COX-2 expression in an autocrine/paracrine manner to establish a positive feedback system for regulating endometrial tumorigenesis (Jabbour et al., 2005). In the present study we have shown that PGE\(_2\) can regulate COX-2 promoter activity and mRNA expression in a similar manner via the FP receptor as observed for PGF\(_{2\alpha}\) via the PLC-PKA-EGFR-ERK1/2 signaling cascade. However, as observed for the PGE\(_2\)-mediated effects on second messenger production and intracellular signaling reported herein, the PGE\(_2\)-mediated increase in COX-2 expression via the FP receptor is also less than that produced by the native ligand PGF\(_{2\alpha}\). We believe this difference is due to the lower binding affinity of PGE\(_2\) for the FP receptor compared with PGF\(_{2\alpha}\) (Abramovitz et al., 2000). In addition, we found that the PGE\(_2\) activation of COX-2 promoter and mRNA expression was significantly reduced by the EP2 and EP4 receptor antagonists. This effect was not observed for PGF\(_{2\alpha}\). Thus it would appear that unlike the PGF\(_{2\alpha}\)-mediated activation of COX-2 that occurs solely via the G\(_q\) activation of InsP, the PGE\(_2\)-mediated increase in COX-2 expression via the FP receptor is also less than that produced by the native ligand PGF\(_{2\alpha}\). We believe this difference is due to the lower binding affinity of PGE\(_2\) for the FP receptor compared with PGF\(_{2\alpha}\) (Abramovitz et al., 2000). In addition, we found that the PGE\(_2\) activation of COX-2 promoter and mRNA expression was significantly reduced by the EP2 and EP4 receptor antagonists. This effect was not observed for PGF\(_{2\alpha}\). Thus it would appear that unlike the PGF\(_{2\alpha}\)-mediated activation of COX-2 that occurs solely via the G\(_q\) activation of InsP, the PGE\(_2\)-regulated COX-2 expression in FPS cells is mediated by the synergistic effects of the cAMP and InsP second messenger systems via the PKA-EGFR-ERK1/2 pathway.

We next investigated the transcription regulatory regions within the COX-2 promoter activated by PGE\(_2\) and PGF\(_{2\alpha}\) in FPS cells. The COX-2 promoter has binding sites for a number of transcription factors including nuclear factor (NF)-κB,
CCAAT/enhancer binding protein (C/EBP), AP-2 and cAMP response element (CRE) (Lukuk et al., 1998). Transfection studies with the cis-acting DNA binding sequence of the CRE or the full length C2.1 COX-2 promoter and a series of deletions containing key transcription factor-binding sites showed that promoter activity was maintained with a construct that had a CRE region only (RSA/HIN). Mutation of the CRE region of this construct resulted in complete loss of COX-2 promoter activity in response to administration of either PGE2 or PGF2α, indicating that the CRE is essential for transcriptional activation of the COX-2 gene by prostaglandins. The regulation of the CRE by PGE2 and PGF2α was further investigated by transient transfection studies using a cDNA construct containing the cis-acting DNA binding sequence of the CRE fused to a Luciferase reporter system. These studies showed that CRE activation in FPS cells is mediated by PGE2 and PGF2α via the same mechanisms regulating COX-2, namely by activation of the PKA-EGFR-ERK1/2 pathways further confirming the importance of the CRE in the regulation of COX-2 activity in FPS cells.

Because PGE2 and PGF2α mobilize intracellular cAMP and can activate protein kinase A (PKA) and since the PKA inhibitor 4C3MQ inhibited both the PGE2 and PGF2α-mediated signaling to ERK1/2 and COX-2 it is plausible that the mode of action of prostanooid signaling on activation of the CRE was by phosphorylation of CRE binding protein (CREB) at SER133, which can in turn bind the CRE and activate gene transcription. In the present study, we did not observe any significant phosphorylation of CREB at SER133 in response to agonist treatment by either PGE2 or PGF2α by Western blot analysis (data not shown). Although activation of COX-2 by CREB binding to the CRE has been shown by Bradbury et al. (2003), Subbaramaiah et al. (2002a,b) have shown that COX-2 transcriptional activation via the CRE binding site can be mediated by activator protein (AP)-1. It is feasible that COX-2 transcriptional activation via the CRE in our study may be regulated via the binding of an alternative transcription factor or transcription factor complex to CREB, such as AP1.

In conclusion, our data provide strong evidence that the elevated biosynthesis of PGE2 and PGF2α produced locally within endometrial adenocarcinomas can act in an autocrine/paracrine manner to enhance the expression of COX-2 via the CRE by means of their integrative actions on intracellular signaling pathways such as the ERK1/2 pathway. Moreover, we believe that these data have implications for the use of prostaglandin synthase inhibitors targeted against PGF synthase as therapeutic intervention strategies as suggested for PGE synthase (Murakami and Kudo, 2006; Iachak, 2007; Wang et al., 2006; Cheng et al., 2006). Our data highlight that in tumours expressing elevated levels of FP receptor, elevated biosynthesis of other prostanooids such as PGE2 can in the absence of the native ligand PGF2α activate tumorigenic genes via the FP receptor. It is thus feasible that signaling pathways such as the EGFR or ERK pathways, which integrate the signaling from second messenger systems to target genes, may offer a better therapeutic target to reverse the adverse effects of prostanooid signaling, or indeed signaling in response to multiple prostanooids, in cancer.

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