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RESEARCH PAPER

Prostaglandin E_2 couples through EP_4 prostanoid receptors to induce IL-8 production in human colonic epithelial cell lines

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Background and purpose: Prostaglandin (PG) E_2 and interleukin (IL)-8 are simultaneously increased during the inflammation that characterizes numerous pathologies such as inflammatory bowel disease. IL-8 is a potent neutrophil chemo-attractant and activator, and can initiate and/or exacerbate tissue injury. PGE_2 signals principally through prostanoid receptors of the EP_2 and/or EP_4 subtypes to promote cAMP-dependent cellular functions. The aim of this study was to identify the role of the EP_2 and EP_4 receptor subtype(s) on two human colonic epithelial cell lines (Caco-2 and T84), in regulating PGE_2-induced IL-8 production.
Experimental approach: To identify the causative receptor, we knocked-down and over-expressed EP_2 and EP_4 receptor subtypes in colonic epithelial cells and studied the effect of several selective EP_2/EP_4 receptor agonists and antagonists. The inductions of IL-8 and EP receptor mRNA and protein expression were determined by real-time PCR and western blot analysis. The affinity of PGE_2 and Bmax values for the EP_2 and EP_4 receptor on colonic epithelial cells were determined by radioligand-binding assays with [^{3}H]PGE_2.
Key results: PGE_2 had the highest affinity for the EP_4 receptor subtype and promoted a robust stimulation of cAMP-dependent IL-8 synthesis. This effect was mimicked by a selective EP_4 receptor agonist, ONO-AE1-329, and abolished by silencing the EP_4 receptor gene by using siRNA techniques, a selective EP_4 receptor antagonist (ONO-AE3-208) and a selective inhibitor (Rp-cAMP) of cAMP-dependent protein kinase.
Conclusions and implications: These findings suggest that initiation and progression of colonic inflammation induced by IL-8 could be mediated, at least in part, by PGE_2 acting via the EP_4 receptor subtype.
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Keywords: PGE_2; IL-8; EP receptors; colonic epithelial cells

Abbreviations: CRE, cAMP responsive element; DSS, dextran sodium sulphate; GI, gastrointestinal; IBD, inflammatory bowel disease; IL, interleukin; PDE, phosphodiesterase; PG, prostaglandin; PMA, phorbol myristate acetate

Introduction

Prostaglandins (PGs) are a major group of biochemical mediators involved in numerous physiological reactions, including inflammation and cellular differentiation (Srinivasan and Kulkarni, 1989). The actions of PGE_2 are mediated by four G-protein-coupled (EP) receptors that are encoded by different genes and referred to as EP_1, EP_2, EP_3 and EP_4 receptors (Breyer *et al.*, 2001). Biochemical studies have established that EP_2 and EP_4 receptors can couple, via G_s, to adenylyl cyclase to produce an increase in cAMP. However, despite this common mode of signalling, agonists of EP_2 and EP_4 receptors can evoke, in the same cell, distinct functional responses. For example, in human airways smooth muscle cells, activation of the EP_2 receptor inhibits the secretion of granulocyte/macrophage colony-stimulating factor, whereas in the same tissue the elaboration of a related cytokine, granulocyte colony-stimulating factor, is markedly enhanced (Clarke *et al.*, 2004; 2005). In contrast, other studies using dendritic cells have shown that despite equal expression of the EP_2 and EP_4 receptors, PGE_2 regulates migration by activating solely the EP_4 subtype (Kabashima *et al.*, 2003).

There is strong evidence suggesting that a variety of mediators including arachidonic acid metabolites, cytokines and growth factors are involved in intestinal inflammation (Sartor, 1994). In fact, arachidonic acid metabolites, in

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particular PGE_2 as well as its receptors, are increasingly being implicated in the pathogenesis of inflammatory bowel disease (IBD). Currently, the role of EP_2 and EP_4 receptors in regulating functional responses in colonic epithelial cells is unknown although the activation of EP receptors by PGE_2 on cells within the intestinal mucosa may have a critical role in both homeostasis and the onset of gastrointestinal (GI) inflammation. Indeed, the limited data currently available suggest that signalling via EP receptors in specific colonic mucosal cell types can determine whether PGE_2 exerts a pro-inflammatory or an anti-inflammatory effect. For example, studies *in vitro*, addressing early responses induced by PGE_2 in a variety of colonic epithelial cell lines, clearly demonstrated up-regulation of interleukin (IL)-8 production, which is consistent with a pro-inflammatory role for this PG (Yu and Chadee, 1999). Indeed, IL-8 is a potent chemokine that can attract neutrophils to the gut where, upon their activation, they may cause non-specific tissue damage that precedes the onset of colonic inflammation. However, in stark contrast, dextran sodium sulphate (DSS)-induced colitis in EP_4 receptor knockout mice was exacerbated when compared with the severity of disease induced by DSS in wild-type animals (Kabashima *et al*., 2002). Clearly, these *in vivo* data suggest that signalling via EP_4 receptors is not pro-inflammatory and, in fact, plays a critical role in maintaining normal mucosal integrity and/or in promoting healing. Thus, the functional role of EP_4 receptors in the gastric mucosa is unclear.

In the present study we have investigated and report here on the role of the EP_2 and EP_4 receptor subtypes in up-regulating IL-8 release evoked by PGE_2. Specifically, we describe the results of studies in which we have both stably over-expressed and knocked-down the EP_2 and EP_4 receptors in Caco-2 and T84 human colonic epithelial cells to mimic the differential receptor expression that can occur in IBD or in acute intestinal inflammation. Our results show that PGE_2 promotes a cAMP-dependent generation of IL-8 from human colonic epithelial cells by activating, exclusively, high affinity prostanoid receptors of the EP_4 subtype. Moreover, we report that PGE_2 can also augment the ability of IL-1β, another cytokine that is up-regulated in colonic inflammation, to induce the IL-8 gene by activating the same mechanism.

Materials and methods

Cells and reagents

Caco-2 and T84 cells were obtained from ATCC and maintained in MEM medium containing 5% serum and 5% Pen Strep (Gibco/Invitrogen, Burlington, Ontario, Canada). Forskolin, AH23848 (a TP/EP_4 receptor antagonist), AH6809 (a DP_1, EP_1 and EP_2 receptor antagonist) and Rp-cAMP [an inhibitor of cAMP-dependent protein kinase (PKA)] were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). ONO-AE1-329 (a selective EP_4 receptor agonist) and ONO-AE3-208 (a selective EP_4 receptor antagonist) were from Ono Pharmaceutical Co. Ltd (Osaka, Japan). All other reagents were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

Real-time PCR and construction of sense and antisense EP receptor plasmids

Total RNA from Caco-2 cells was isolated with TRIzol. Full-length cDNA fragments of the EP_2 and EP_4 receptors were PCR amplified by using the following primers: gtcgacctcgagATGGGC AATGCCTCCAATG (forward) and gtcgacgatatcTCAAAGGTCAGCCAGTTTAC (reverse) for EP_2; and gtcgacctcgagATGTCCACTCCCGGGGTC (forward) and gtcgacgatatcTTATATACATTTTTCTGATAAGTTC (reverse) for EP_4 and were cloned in sense and antisense orientations in the pCI-neo vector (Promega Madison, WI, USA). Sense and antisense constructs were then verified by sequencing.

Development of stable sense and antisense cell lines

Sense and antisense EP receptor plasmids were used to transfect cells ($1–2 \times 10^5$) to obtain stable clones for each receptor subtype by using Fugene-6 (Roche Diagnostics, details) according to the manufacturer's instructions. The empty vector (pCI-neo) was used as a negative control. Using green fluorescent protein as control, the transfection efficiency was routinely found to be between 65% and 75%. Cells stably expressing full-length human EP prostanoid receptors (sense or antisense) were selected with Geneticin (G-418, 1 $mg \cdot mL^{-1}$, Invitrogen, Burlington, Ontario, Canada). Henceforth, Caco-2 cells stably expressing EP_2 and EP_4 sense mRNA are referred to as EP2S-C and EP4S-C respectively. Similarly, Caco-2 cells stably over-expressing EP_2 and EP_4 antisense mRNA are referred to as EP2A-C and EP4A-C respectively. T84 cells stably over-expressing EP_2 and EP_4 receptors are termed EP2S-T and EP4S-T respectively.

Stimulation of cells with agonists, antagonists and inhibitors

Cells (10^6 $well^{-1}$) were fasted in serum-free medium overnight and then stimulated for the indicated times with IL-1β (100 $U \cdot mL^{-1}$), PGE_2 (1 $\mu mol \cdot L^{-1}$), forskolin (10 $\mu mol \cdot L^{-1}$), ONO-AE1-329 (1 $\mu mol \cdot L^{-1}$), 1-hydroxy prostaglandin E_1 (PGE_1-OH; 1 $\mu mol \cdot L^{-1}$; a moderately selective EP_4 receptor agonist) or butaprost (1 $\mu mol \cdot L^{-1}$; an EP_2 receptor agonist) in the manner described in the text, tables and figure legends. We have previously shown that the concentration of PGE_2 used in these experiments approximates to the EC_{70} for the induction of the IL-8 gene in colonic epithelial cells (Yu and Chadee, 1999). In some experiments, cells were pretreated with ONO-AE3-208 (1 $\mu mol \cdot L^{-1}$, p*K*i = 8.9 at the human EP_4 receptor; Kabashima *et al*., 2002), AH6809 (50 $\mu mol \cdot L^{-1}$, $pA_2 = 5.9–6.5$ at the human EP_2 receptor; Woodward *et al*., 1995; Abramovitz *et al*., 2000), AH23848 (1 $\mu mol \cdot L^{-1}$, $pA_2 = 5.6$ at the human EP_4 receptor; Davis and Sharif, 2000) or Rp-cAMP (10 $\mu mol \cdot L^{-1}$) for 60 min prior to being stimulated with PGE_2. Forskolin (10 $\mu mol \cdot L^{-1}$) and phorbol myristate acetate (PMA; 1 $\mu mol \cdot L^{-1}$; a phorbol ester and inducer of IL-8) were used in the absence of antagonists as positive controls for all induction studies.

Luciferase and IL-8 assays

Wild-type Caco-2 and Caco-2 cells (10^5) stably expressing human EP receptors in 6-well plates were transfected with

2 μg pCRE-Luc plasmid containing two copies of cAMP responsive elements (CREs) by using Fugene-6. Cells transfected with pFC-PKA plasmid were used as a positive control. Path Detect CRE cis–reporting systems (pCRE-Luc and pFC-PKA plasmids) were obtained from Stratagene (La Jolla, CA, USA). IL-8 production was measured by a Titerzyme enzyme immunoassay kit (Assay designs Inc, Ann Arbor, MI) according to the manufacturer's instructions.

Membrane preparation and radioligand-binding assays

Caco-2 cell membrane preparation and saturation isotherms of the specific binding of $[^3H]PGE_2$ to membrane fractions from wild-type Caco-2 and stably transfected with EP receptor cell lines were performed essentially as previously reported (Belley and Chadee, 1999). Affinity constants (K_d) and Bmax values of $[^3H]PGE_2$ binding to the EP_2 and EP_4 receptors were determined by using PRISM (GraphPad software, San Diego, USA).

Real-time PCR

Total RNA was extracted with TriZol reagent (Invitrogen), quantified and 5 μg of RNA was reverse transcribed by using M-MLV reverse transcriptase (Invitrogen) and oligo d(T) as per manufacturer's instructions. Two microlitre of cDNA was used for real-time PCR. Real-time primers used are forward: GATCTCAGTGCAGAGGCTCG, reverse: TGCTTGTCCAGGTGGTC CAT for MCP-1, forward: GAAGATGGTGATGGGATTTC, reverse: GAAGGTGAAGGTCGGAGT for GAPD. Amplifications were carried out with Qiagen's Quantitect SYBR Green PCR kit by using the following cycling conditions: 94°C hold for 15 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s and extension at 72°C for 30 s by using EP_2 forward GACCGCTTACCTGCAGCTGTAC, EP_2 reverse TGAAGTTGCAGGCGAGCA, EP_4 forward ACGCC GCCTACTCCTACATG and EP_4 reverse AGAGGACGGTGGC GAGAAT following the manufacturer's instructions. Specificity of amplification was checked by melt curve analysis. IL-8 mRNA expression was normalized against GAPDH, and fold change over control was determined according to the Ct method (Bas *et al.*, 2004). Qualitatively identical data were obtained when β-actin and 18S rRNA were used as denominators (data not shown) indicating that under our experimental conditions the expression of GAPDH was not affected.

RNA interference

Wild-type Caco-2, EP2S-C, EP2S-T, EP4S-C and EP4S-T cells were transfected with EP2 siRNA (Smartpool M-005712-00), EP4 siRNA (Smartpool M-005714-00) or control siRNA (D-001210-02, D-001140-01) from Dharmacon, Inc. (Lafayette, CO, USA) as described by the manufacturer. Briefly, subconfluent (50–60%) cells were transfected by using X-Treme Gene siRNA transfection reagent (Roche) for 36 h at a siRNA concentration of 40 $nmol \cdot L^{-1}$. Cells were immediately used for subsequent experiments.

Western blotting

Cell lysates (50 μg) were denatured by boiling in sample buffer (64 $mmol \cdot L^{-1}$ Tris-Cl pH 6.8, 10.3% glycerol, 2% SDS and 5.1% β-mercaptoethanol) containing protease inhibitors (1.5 $mmol \cdot L^{-1}$ EDTA, 23 $\mu mol \cdot L^{-1}$ leupeptin, 14.5 $\mu mol \cdot L^{-1}$ pepstatin, 1.5 $\mu mol \cdot L^{-1}$ aprotinin, 30 $\mu mol \cdot L^{-1}$ TLCK, 1 $mmol \cdot L^{-1}$ PMSF). Proteins were run on 10% SDS-PAGE gels, and Western blot analyses were performed by using rabbit polyclonal antibodies (1:1000) raised against peptides within unique regions of the EP_2 and EP_4 receptors (Cayman Chemicals, Ann Arbor, MI, USA). Mouse anti-β-actin antibody (Santa Cruz Biotechnology, CA, USA) was used as a loading control. Immunoreactive bands were visualized by chemiluminescence (ECL, Amersham, Buckinghamshire, England).

Statistical analyses and nomenclature

Data in the text, tables and figure legends refer to the mean ± SEM of n independent determinations. When required, data were analysed by using either Student's t-test for unpaired data or one-way ANOVA using Prism software with appropriate post hoc test. Significance was assumed when $P \leq 0.05$. All drug and molecular target nomenclature used herein conforms to the *British Journal of Pharmacology's* guidelines (Alexander *et al.*, 2008).

Results

Knock-down or over-expression of EP receptors and their localization

EP_2 and EP_4 receptor expression was assessed in cells stably transfected with over-expression plasmids encoding the full-length EP_2 and EP_4 receptors in the sense or antisense orientations. Real-time PCR data showed that EP_2 and EP_4 receptor mRNA expression was up-regulated 17- and 14-fold in EP2S-C and EP4S-C cells respectively over control Caco-2 cells (Figure 1A). In contrast, EP_2 and EP_4 receptor mRNA expression was reduced by ~75% in both EP2A-C and EP4A-C cells, relative to untransfected controls. The level of EP_2 receptor mRNA was virtually unchanged in EP4A-C and EP4S-C cells. Similarly, the expression level of EP_4 receptor mRNA was not changed in EP2A-C and EP2S-C cells (Figure 1A).

Consistent with the mRNA results, immunofluorescence microscopy showed increased EP_2 and EP_4 receptor immunoreactivity localized to the basolateral membrane in EP2S-C and EP4S-C cells when compared with wild-type cells (Figure 1B). Conversely, in EP2A-C and EP4A-C cells, reduced EP_2 and EP_4 receptor immunoreactivity was observed compared with their non-transfected counterparts. Western blot analysis (Figure 1C) performed on cellular extracts from wild-type cells and cells expressing the two EP receptors in the sense (EP2S-C and EP4S-C) and antisense (EP2A-C and EP4A-C) orientations confirmed increased and reduced EP_2 and EP_4 receptor expression when compared with wild-type Caco-2 cells. Indeed, the expression of the EP_2 receptor subtype was 10- and 48-fold greater in EP2S-C cells compared with wild-type and EP2A-C cells respectively (Figure 1C) whereas in cells expressing the antisense plasmid EP_2 receptor levels were reduced by ~80% versus controls (Figure 1C). Similarly, the expression level of EP_4 receptors was 10- and 65-fold greater in EP4S-C cells compared with wild-type and EP4A-C cells respectively whereas EP_4 receptor mRNA levels were

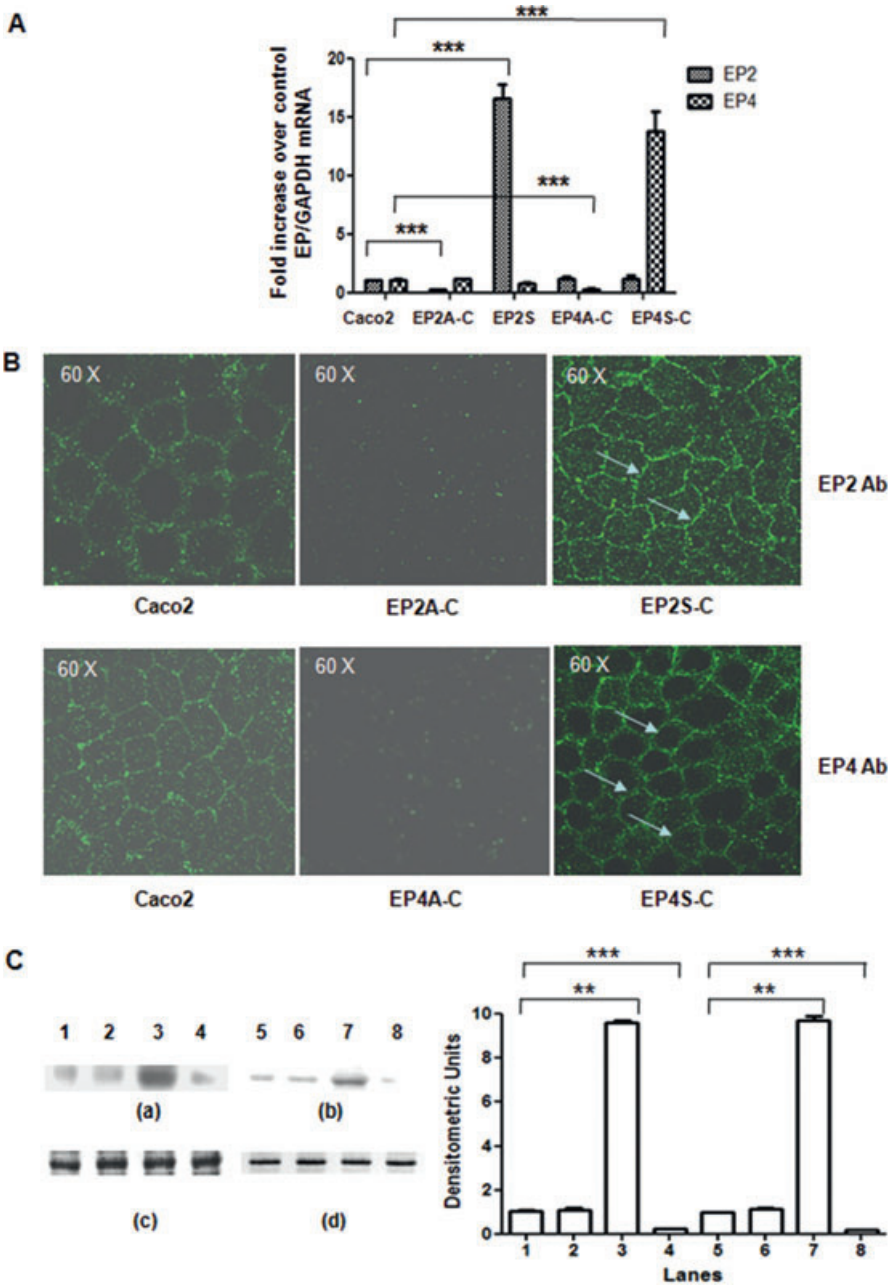

Figure 1 Differential expression of sense and antisense EP receptors in stably transfected cells. (A) Total RNA was extracted from 10^6 wild-type Caco-2, EP2S-C and EP4S-C stably transfected cells. Real-time PCR was performed by using primers specific for the EP_2 and EP_4 receptor mRNAs. Data represent fold increase of mRNA expression over controls (Caco-2) and are expressed as the mean ± SEM of three different experiments repeated three times. ***$P < 0.001$ over control. (B) Representative immunofluorescence localization as detected by confocal microscopy (original magnification 60×) of EP_2 and EP_4 receptors in wild-type Caco-2 cells stably expressing sense or antisense EP receptor mRNA. Experiments were repeated three times with similar results. Arrows show the expression of EP_2 and EP_4 receptors on basolateral membranes. (C) Immunoblot analysis of Caco-2 cells stably transfected with a plasmid encoding the full-length (a) EP_2 or (b) EP_4 receptor. (a) Lane 1, control cells; lane 2, cells transfected with empty vector; lane 3, cells transfected with sense EP_2 and lane 4, cells transfected with antisense EP_2. Lanes 5, 6, 7 and 8 in (b) are the same as panel (a) but refer to the EP_4 receptor subtype. (c) and (d) are β-actin controls for Western blot (a) and (b) respectively. Densitometric analysis was normalized against β-actin. ***$P < 0.001$; **$P < 0.05$ respectively.

reduced (by 86%) in EP4A-C cells relative to wild-type control cells (Figure 1C).

PGE_2 couples through EP_4 receptors to stimulate luciferase expression and IL-8 production

The EP_2 and EP_4 receptor, classically, couple to adenylyl cyclase via G_s resulting in increased formation of cAMP (Breyer *et al.*, 2001). To determine whether this signal transduction pathway is also activated by PGE_2, colonic epithelial cells, Caco-2 and T84 cells stably over-expressing sense or antisense EP receptors were transfected with a plasmid containing a CRE-driven luciferase reporter gene (pCRE-Luc). Both PGE_2 and PGE_1-OH (each at 1 $\mu mol \cdot L^{-1}$) stimulated luciferase activity and IL-8 production in a time-dependent

Table 1 Luciferase activity in EP sense and antisense cells following stimulation with EP receptor agonists and antagonists

Cells	*PGE_2 (1 μmol·L^{-1})*	*PGE_1-OH (1 μmol·L^{-1})*	*ONO-AE1-329 (1 μmol·L^{-1})*	*Butaprost (1 μmol·L^{-1})*	*PGE_2 + AH6809 (50 μmol·L^{-1})*	*PGE_2 + AH23848 (1 μmol·L^{-1})*	*PGE_2 + ONO-AE3-208 (1 μmol·L^{-1})*
Caco-2	1	1	1	1	1	1	1
EP4S-C	47.6 ± 0.3***	58.4 ± 0.4***	61.4 ± 1.6***	2.4 ± 0.5**	54.1 ± 1.5**	6.3 ± 1.3**	3.0 ± 0.2***
EP4A-C	0.5 ± 0.4**	–0.3 ± 0.1*	1.2 ± 0.3***	–0.6 ± 1.0**	–0.5 ± 0.8*	–0.1 ± 0.9**	0.1 ± 0.1**
EP2S-C	14.0 ± 0.4***	13.1 ± 0.6***	9.7 ± 2.1**	12.2 ± 0.9***	–0.6 ± 0.5***	9.9 ± 0.1***	11.2 ± 0.3***
EP2A-C	0.6 ± 0.6**	0.7 ± 0.2***	1.3 ± 0.7***	0.3 ± 0.5**	–0.4 ± 0.2**	–0.2 ± 0.7*	0.5 ± 0.4***

Caco-2 cells stably expressing EP sense (S) and antisense (A) receptors were treated with PGE_2, or other EP receptor agonists or antagonists for 6 h, and luciferase activity was quantified, as described in *Methods*. Data represent the mean ± SEM of the fold change in luciferase activity compared with their respective mock-transfected wild-type control Caco-2 cells from three different experiments. Treatment of cells with AH6809 and AH23848 alone did not have any significant effect on luciferase production. Caco-2 cells stably over-expressing EP_2 and EP_4 sense mRNA are referred to as EP2S-C and EP4S-C respectively, whereas, cells stably over-expressing EP_2 and EP_4 antisense mRNA are referred to as EP2A-C and EP4A-C respectively.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$, over control.

Table 2 IL-8 production in EP sense and antisense cells following stimulation with EP receptor agonists and antagonists

Cells	*PGE_2 (1 μmol·L^{-1})*	*PGE_1-OH (1 μmol·L^{-1})*	*ONO-AE1-329 (1 μmol·L^{-1})*	*Butaprost (1 μmol·L^{-1})*	*PGE_2 + AH6809 (50 μmol·L^{-1})*	*PGE_2 + AH23848 (1 μmol·L^{-1})*	*PGE_2 + ONO-AE3-208 (1 μmol·L^{-1})*
Caco-2	1	1	1	1	1	1	1
EP4S-C	17.4 ± 3.3***	11.4 ± 4.4**	21.3 ± 6.1**	0.2 ± 1***	10.4 ± 3.4***	1.2 ± 1.4***	0.2 ± 0.1***
EP4A-C	1.0 ± 1**	1 ± 1.1***	2.3 ± 0.3***	1.0 ± 1.3**	0.8 ± 0.1***	1.2 ± 0.1*	1.4 ± 0.3**
EP2S-C	1.7 ± 1.6**	1.4 ± 1.4***	0.9 ± 0.2**	1.9 ± 1.2**	4.4 ± 0.2**	1.4 ± 1.4***	1.2 ± 0.5**
EP2A-C	0.3 ± 0.4**	0.3 ± 0.3**	1.5 ± 0.4***	0.3 ± 0.6**	0.3 ± 0.2*	0.9 ± 0.2*	0.2 ± 0.1*

Caco-2 cells stably expressing EP sense (S) and antisense (A) receptors were treated with PGE_2, other EP receptor agonist/antagonist for 16 h, and IL-8 production was measured by enzyme immunoassay, as described in *Methods*. Data represent the mean ± SEM of the fold increase of IL-8 production from three different experiments over their respective mock-transfected wild-type control Caco-2 cells. Treatment with AH6809 and AH23848 alone did not have any significant effect on IL-8 production. Caco-2 cells stably over-expressing EP_2 and EP_4 sense mRNA are referred to as EP2S and EP4S respectively, whereas, cells stably over-expressing EP_2 and EP4 antisense mRNA are referred to as EP2A and EP4A respectively.
IL, interleukin; PG, prostaglandin.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$, over control.

manner, which peaked at 6 h and 16 h respectively (data not shown). Therefore, these time points were selected in all further experiments in which cells were treated with PGE_2 or other selective EP receptor agonists for the measurement of IL-8 mRNA and protein production respectively. In EP4S-C cells, PGE_2 (1 μmol·L^{-1}), PGE_1-OH (1 μmol·L^{-1}) and ONO-AE1-329 (1 μmol·L^{-1}) induced robust luciferase activity (>48-fold) and IL-8 production (>11-fold) when compared with wild-type, mock-transfected controls, whereas butaprost (1 μmol·L^{-1}) was much less effective on luciferase (2.4-fold induction) and inactive on IL-8 generation (Tables 1 and 2). Similarly, in EP2S-C cells, PGE_2, PGE_1-OH, butaprost and ONO-AE1-329 (each at 1 μmol·L^{-1}) also induced luciferase activity (>12-fold) although IL-8 secretion was unaffected (Tables 1 and 2). It should be noted that the activity of butaprost and ONO-AE1-329 in EP4S-C and EP2S-C cells respectively was due to basal expression in Caco-2 cells of the native EP_2 and EP_4 receptor subtypes (data not shown). Pretreatment of EP4S-C cells with the EP_4 receptor antagonists ONO-AE3-208 and AH23848 (each at 1 μmol·L^{-1}) inhibited by >90% PGE_2-induced luciferase activity and IL-8 production whereas responses in EP2S-C cells were unaffected.

However, studies with AH6809 (50 μmol·L^{-1}), a non-selective EP_2 receptor antagonist, yielded data that were not always consistent with EP_2 receptor blockade. For example, AH6809 was without effect on the induction of luciferase by PGE_2 in EP4S-C cells whereas IL-8 generation was suppressed by ~40% (Tables 1 and 2). It has been reported that AH6809 is a weak inhibitor of cAMP phosphodiesterase (PDE; Keery and Lumley, 1988) and, conceivably, this additional action could explain its ability to attenuate IL-8 generation, independently of receptor blockade. Indeed, support for a PDE-dependent mechanism was the finding that AH6809 increased IL-8 production (4.4-fold) in EP2S-C cells under conditions where PGE_2 was inactive (Table 2). To explore this possibility, experiments were planned in which AH6809 was to be used at a lower concentration (5 μmol·L^{-1}) that should be devoid of PDE-inhibitory activity (Keery and Lumley, 1988). Because the pA_2 of AH6809 for the human EP_2 receptor subtype lies between 5.9 and 6.5 (Woodward *et al*., 1995; Abramovitz *et al*., 2000), significant antagonism of EP_2 receptor-mediated responses still should be seen at this lower concentration. However, this experimental approach was not adopted because AH6809 significantly augmented luciferase activity in epithelial cells expressing the CRE reporter at concentrations as a low as 1 μmol·L^{-1} (Figure S1).

Forskolin (10 μmol·L^{-1}; a direct activator of adenylyl cyclase) and PMA (1 μmol·L^{-1}) were used as positive controls in these experiments, and these stimuli alone markedly induced luciferase activity and IL-8 secretion (195- to 223-fold and 70- to 95-fold respectively relative to untreated cells; data not shown). At the transcriptional level, the IL-8 gene was also significantly induced (~25-fold) by PGE_2 and PGE_1-OH (each at 1 μmol·L^{-1}) in EP4S-C but not EP2S-C cells (Figure 2A) and this effect was abolished by AH23848 (1 μmol·L^{-1}).

To confirm that these effects of PGE_2 were not peculiar to Caco-2 cells, the same experiments described in Tables 1 and

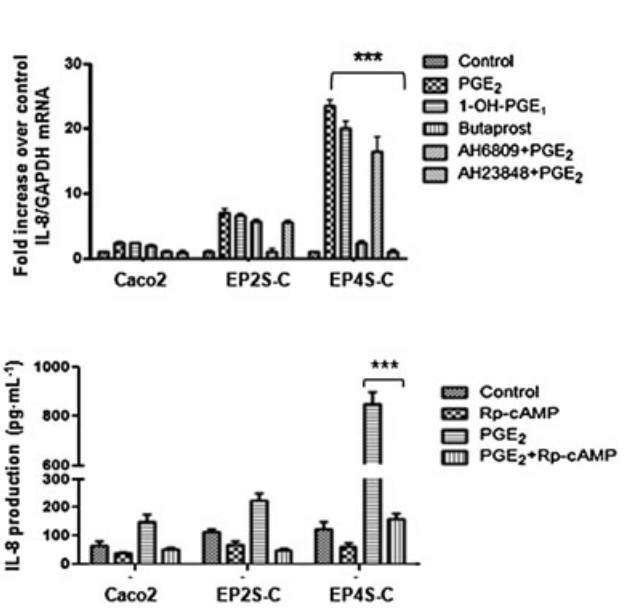

Figure 2 Prostaglandin (PG) E_2 induces interleukin (IL)-8 mRNA expression through activation of the EP_4 receptor. (A) Wild-type Caco-2, EP2S-C and EP4S-C cells were treated with or without PGE_2, PGE_1-OH and butaprost (each at 1 μmol·L^{-1}) for 6 h. In some experiments cells were first pretreated with AH6809 (50 μmol·L^{-1}) or AH23848 (1 μmol·L^{-1}) for 60 min prior to PGE_2. Total RNA was extracted, and real-time PCR was performed. (B) Cells were pretreated with or without Rp-cAMP for 1 h prior to stimulation with PGE_2 treatment for 16 h. IL-8 production was quantified by enzyme immunoassay as described before. ***$P < 0.001$ (with respect to PGE_2-treated EP4S cells). In both panels data are expressed as the mean ± SEM of three experiments.

2 were performed in stable EP2S-T and stable EP4S-T cells with qualitatively identical results except that AH6809 did not suppress PGE_2-induced IL-8 generation in EP2S-T cells (cf. EP2S-Cs; see Tables S1 and S2).

Effect of EP receptor agonists and antagonists on cAMP levels in Caco-2 cells

To confirm the data derived from the CRE reporter, cAMP levels were measured under similar experimental conditions. In EP4S-C cells, PGE_2 and PGE_1-OH significantly increased cAMP mass when compared with wild-type, mock-transfected controls, whereas butaprost was inactive (Table 3). For PGE_2, this effect was abolished by AH23848 (1 μmol·L^{-1}) and suppressed (77%) by AH6809 (50 μmol·L^{-1}), which may reflect, at least in part, the antagonism of PGE_2 at the endogenous EP_2 receptor population. In EP2S-C cells, PGE_2, PGE_1-OH similarly increased the cAMP content via a mechanism that was blocked by AH6809 (50 μmol·L^{-1}) but not by AH23848 (1 μmol·L^{-1}; Table 3). Although the specificity of AH6809 cannot be guaranteed for the reasons described above, butaprost also increased the cAMP content in Caco-2 and EP2S-C cells, indicating the presence of functional EP_2 receptors (Table 3).

Effect of an inhibitor of PKA

Pretreatment of EP4S-C cells with a PKA inhibitor, Rp-cAMP, abolished PGE_2-induced IL-8 production (Figure 2B) demonstrating that activation of the EP_4 receptor is responsible for cAMP accumulation, luciferase induction and IL-8 production.

Effect of PGE_2 on IL-1β-induced IL-8 production

During colonic inflammation, several pro-inflammatory cytokines, including IL-1β, are up-regulated. In wild-type Caco-2 cells, IL-1β alone and IL-1β and PGE_2 in combination induced IL-8 production by 4.5- and 8.2-fold respectively relative to the amount generated spontaneously (Figure 3). Similarly, in EP4S-C cells that over-express the EP_4 receptor, IL-1β alone, and IL-1β and PGE_2 in combination induced a robust release of IL-8 (10- and 21-fold respectively) that was significantly greater (2.3- and 3-fold respectively) than that seen in wild-type Caco-2 cells (Figure 3).

In EP2S-C, EP2A-C and EP4A-C cells neither IL-1β nor IL-1β and PGE_2 in combination had any significant effect on IL-8 production (data not shown).

Identification of EP receptors by competitive radioligand binding

To establish the affinity (K_d) of PGE_2 for the EP_2 and EP_4 receptor subtypes, radioligand binding was performed (Figure 4; Table 4). With Caco-2 cell membranes, EP receptor agonists competitively and completely inhibited the specific binding of [^{3}H]PGE_2 with a rank order of potency of $PGE_2 \geq PGE_1$-OH >> sulprostone > butaprost. This ranking was same in membranes prepared from all other cells except EP2S-Cs where the potency of sulprostone and butaprost was reversed. However, depending on the agonist, the degree of displacement of [^{3}H]PGE_2 varied between wild-type and transfected Caco-2 cells. Thus, PGE_2 and PGE_1-OH completely displaced [^{3}H]PGE_2 in membranes prepared from all cell types (Figure 4B–E). Similarly, the EP_2 receptor agonist, butaprost, effectively displaced [^{3}H]PGE_2 from membranes prepared from EP2S-C and EP4A-C cells, although complete displacement of the radiolabel was not achieved (Figure 4B,E). In contrast, butaprost weakly displaced [^{3}H]PGE_2 in EP2A-C and EP4S-C cells in which the EP_2 and EP_4 receptor was silenced and over-expressed respectively (Figure 4C,D). For sulprostone, a selective EP_3 receptor agonist, the maximum degree of displacement of [^{3}H]PGE_2 was reasonably consistent across all transfected cells although its IC_{50} value was ~10-fold lower in cells in which the EP_2 and EP_4 receptor was silenced. This, presumably, is due to a proportional increase in EP_3 receptor number as well as the EP_1 receptor population at which sulprostone also has moderate affinity (K_d = 110 nmol·L^{-1}; Abramovitz *et al.*, 2000).

Prostaglandin E_2 bound to EP4S-C cell membranes with the highest affinity (K_d = 0.2 nmol·L^{-1}) but also labelled EP2S-C cells (K_d = 1.2 nmol·L^{-1}). However, in wild-type Caco-2 cells the affinity of PGE_2 was 7.5-fold lower than that in cells over-expressing the EP_4 receptor subtype. To check the affinity of PGE_2 for the two subtypes, radioligand binding was performed by using HEK293 cells over-expressing the EP_2 or EP_4 subtypes as they do not express either receptor endogenously. As shown in Table 4, K_d values were 5- and 4.3-fold lower than those found in the Caco-2 cell membranes. Membranes from wild-type, mock-transfected and EP2A, EP4A cells showed no specific binding of [^{3}H]PGE_2.

Table 3 Effect of prostanoid receptor agonists and antagonists on cAMP levels in EP sense and antisense cells

Cells	*Control*	*PGE_2 (1 μmol·L^{-1})*	*PGE_1-OH (1 μmol·L^{-1})*	*Butaprost (1 μmol·L^{-1})*	*PGE_2 + AH6809 (50 μmol·L^{-1})*	*PGE_2 + AH23848 (1 μmol·L^{-1})*	*Forskolin (10 μmol·L^{-1})*
EP4S-C	32 ± 4	460 ± 31***	479 ± 45***	51 ± 11**	129 ± 6**	21 ± 2***	896 ± 39**
EP4A-C	26 ± 3	82 ± 10**	65 ± 2**	31 ± 8*	34 ± 3*	24 ± 4**	750 ± 40*
EP2S-C	25 ± 6	194 ± 11***	213 ± 19**	180 ± 10***	60 ± 8**	151 ± 10**	845 ± 56**
EP2A-C	21 ± 4	111 ± 8**	102 ± 11**	26 ± 2*	22 ± 9**	30 ± 12**	839 ± 25*
Caco-2	13 ± 5	116 ± 13***	132 ± 9***	22 ± 10**	28 ± 1***	44 ± 2**	791 ± 22**

Cells were pretreated with 1 mmol·L^{-1} 3-isobutyl-1-methylxanthine for 15 min and then challenged with PGE_2, forskolin and other EP receptor agonists and antagonists for 20 min. cAMP mass was then was quantified by enzyme immunoassay as described in *Methods* and expressed as pmol (10^6 cells)$^{-1}$. Forskolin was used as non-receptor-mediated positive control. Data represent the mean ± SEM of three different experiments. Cells stably over-expressing EP_2 and EP_4 sense mRNA are referred to as EP2S-C and EP4S-C respectively, whereas, cells stably over-expressing EP_2 and EP_4 antisense mRNA are referred to as EP2A-C and EP4A-C respectively.
PG, prostaglandin.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$, over control.

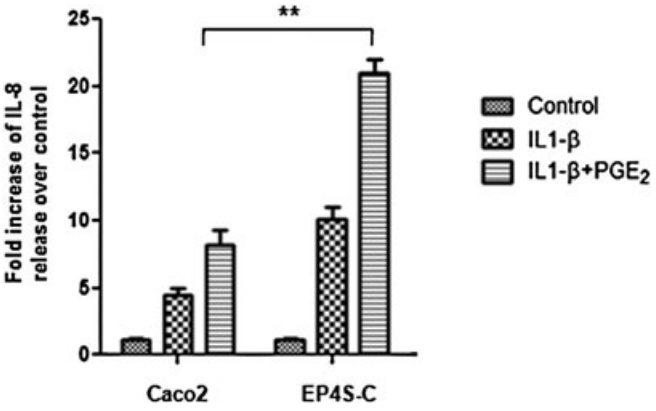

Figure 3 Effect of interleukin (IL)-1β on prostaglandin (PG) E_2-induced IL-8 production. Wild-type Caco-2 and EP4S-C cells were treated with or without IL-1β (100 U·mL^{-1}) and PGE_2 (1 μmol·L^{-1}) for 16 h, and IL-8 production was measured by enzyme immunoassay, as described in *Methods*. Data represent the mean ± SEM of three experiments. **$P < 0.05$.

Inhibition of EP receptor expression by RNA interference prevents PGE_2-induced IL-8 production

To confirm that PGE_2 was coupling exclusively through the EP_4 receptor to up-regulate IL-8 gene expression and protein production, we used siRNAs to silence the genes that encode the EP_2 and EP_4 receptor subtypes (Figure 5). Real-time PCR experiments showed that in EP2S-C and EP4S-C cells, EP_2 and EP_4 receptor mRNA expression was significantly reduced (>95%) by EP2 siRNA and EP4 siRNA. Similarly, in EP2S-T and EP4S-T cells, EP_2 and EP_4 receptor mRNA expression was suppressed by >93% (Figure 5A). EP4 siRNA treatment of EP4S-C and EP4S-T cells abolished PGE_2-induced IL-8 production as compared with untransfected control cells (Figure 5B). In contrast, silencing of EP_2 receptor in EP2S-C and EP2S-T cells did not have any effect on IL-8 production (Figure 5B).

Discussion

Prostaglandin E_2 is considered to play an important role in the GI tract. In particular, PGE_2 exerts beneficial physiological effects on epithelial cytoprotection including enhancement of GI motility and of mucosal barrier functions that include mucus secretion and bicarbonate output as well as reducing gastric acid secretion. However, paradoxically, PGE_2 is also implicated in several GI pathologies including enteroinvasive bacterial diseases, colorectal cancers and IBD (Ahrenstedt *et al.*, 1994). A major finding of the present investigation is that PGE_2 couples through high affinity prostanoid receptors of the EP_4 subtype expressed by human colonic epithelial cells to stimulate the output of IL-8 by a cAMP-dependent mechanism (Tables 1 and 2; Figure 2). These experiments logically extend the results of our previous study (Yu and Chadee, 1999) and suggest that PGE_2 can theoretically function as a major pro-inflammatory mediator in the gut. By radioligand binding, the K_d of PGE_2 for EP receptor subtypes in homologous (Caco-2) and heterologous (HEK293) cell systems was found, generally, to be consistent with the results of other studies (Abramovitz *et al.*, 2000; Davis and Sharif, 2000). Moreover, competition-binding experiments clearly showed that PGE_2 had the highest affinity for the EP_4 receptor on EP4S cell membranes (K_d = 0.2–1.1 nmol·L^{-1}). However, one discrepancy that merits discussion is that that affinity of PGE_2 for the wild-type EP_4 receptor expressed in Caco-2 cells was 1.5 nmol·L^{-1}. This value is 7.5-fold lower than that the affinity of PGE_2 obtained in EP4S-C cells (K_d = 0.1 nmol·L^{-1}), which express a composite of wild-type receptor and the EP_4 receptor transgene product. Perhaps the most likely explanation relates to the fact that PGE_2 does not have equal affinity for each human EP receptor (K_d varies ~28-fold; Abramovitz *et al.*, 2000). Thus, because Caco-2 cells express multiple EP receptors (Shoji *et al.*, 2004), the affinity of PGE_2 determined from saturation binding isotherms will be influenced by the relative proportions of each EP receptor subtype expressed in wild-type and transfected cells. However, additional, ill-defined, factors are also likely to contribute to this discrepancy in affinity because the K_d of PGE_2 for the EP_4 receptor expressed in HEK293 cells was the same as that found in wild-type Caco-2 cells.

Recent studies have shown that expression of EP receptors changes dynamically during different stages of intestinal inflammation. In particular, EP_4 receptor density is increased during chronic inflammation of the human colonic mucosa (Dey *et al.*, 2006). It is well established that during colonic inflammation or colon cancer, PGE_2 production from

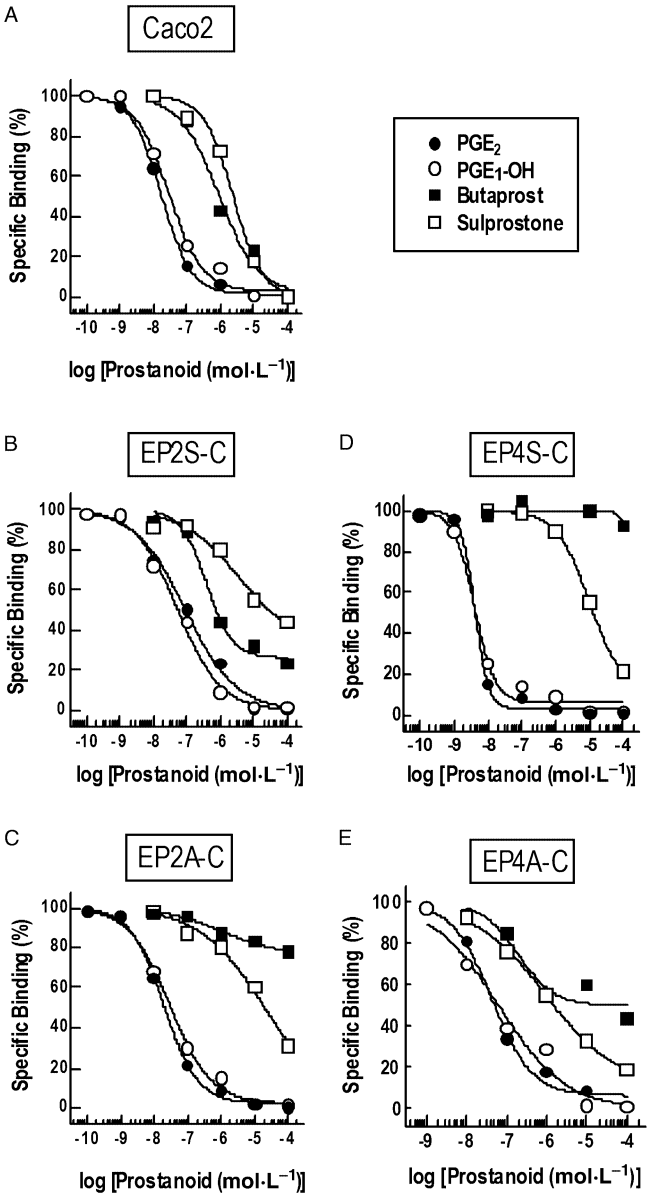

Figure 4 Displacement of [^{3}H]PGE_2 (specific binding) in cell membranes by PGE_2 and EP receptor agonists and antagonists. Membranes (25 μg) from mock-transfected Caco-2 cells and sense and antisense cells were incubated with 3 nmol·L^{-1} [^{3}H]PGE_2 and other EP receptor agonists and antagonists for 1 h. Binding was quantified by rapid vacuum filtration followed by liquid scintillation counting. Representative graphs are shown of three different experiments with similar results. PG, prostaglandin.

pro-inflammatory cells is high due to induction of the *COX-2* gene by various inflammatory stimuli (Vane *et al.*, 1998). In fact, COX-2 expression and increased PGE_2 production in the colonic mucosa is a hallmark of IBD (Takafuji *et al.*, 2000) as well as colorectal cancers and colitis caused by enteric pathogens. Moreover, Chell *et al* (2006) reported that EP_4 receptors were up-regulated in the colon of colorectal cancer patients and that an EP_4 receptor antagonist suppressed carcinogenesis. It was also recently reported that IL-8 could act as an autocrine growth factor in colon carcinoma (Brew *et al.*, 2000; Mutoh *et al.*, 2002), which could be driven by enhanced PGE_2 production and activation of the EP_4 receptor subtype.

In the present study, the potential pro-inflammatory role of EP_4 receptor agonism was explored in more detail using pharmacological and molecular approaches. Initially, the effect of a selective EP_4 receptor antagonist, ONO-AE3-208, was examined and found to abolish PGE_2-induced IL-8 generation. An important role for the EP_4 receptor was also suggested from other studies in which ONO-AE1-329, a selective EP_4 receptor agonist, but not butaprost (a selective EP_2 receptor agonist) mimicked the effect of PGE_2. This finding was endorsed in a complementary series of experiments using RNA interference techniques, which have become powerful and widely used tools for the analysis of gene function in mammalian cells (Sharp, 2001; Meister and Tuschi, 2004; Mello and Conte, 2004). Specifically, we adopted a siRNA strategy to silence the genes that encode the EP_2 and EP_4 receptor subtypes in EP2S-C, EP2-T, EP4S-C and EP4S-T cells respectively, and then assessed the ability of PGE_2 to generate IL-8 relative to control cells. Our results indicate that transfection with EP4 siRNA in EP4S-C or EP4S-T cells decreased EP_4 receptor expression through silencing of the *PTGER4* gene, and this intervention significantly reduced IL-8 production induced by PGE_2. In contrast, silencing of *PTGER2* in EP2S-C or EP2S-T cells, which encodes the EP_2 receptor, was without effect on PGE_2-induced IL-8 synthesis. Collectively, these pharmacological and gene silencing data clearly demonstrate that PGE_2 couples exclusively through the EP_4 receptor subtype to induce IL-8 production in human colonic epithelial cells. While this finding is not remarkable, the fact that activation of the EP_2 receptor with butaprost failed to promote IL-8 release from Caco-2 cells under conditions where the cAMP level was nevertheless increased is intriguing (Table 3). The explanation for this discrepancy is unknown. However, it is tempting to speculate that EP_2 receptor activation results in the accumulation of cAMP in a discrete locus within the cell that is spatially segregated from those cAMP-dependent processes to which the EP_4 receptor is coupled. Indeed, evidence for such compartmentalization of cAMP signalling is now well established (see Lynch *et al.*, 2006).

Selective and potent EP_2 receptor antagonists with which to unambiguously define EP_2 receptor-mediated responses currently are unavailable. The only compound described with moderate EP_2 receptor affinity is AH6809, but generally it is of limited value in receptor classification. Indeed, AH6809 also blocks the EP_1 and DP_1 receptor subtypes at similar concentrations (Abramovitz *et al.*, 2000) and is a weak inhibitor of cAMP PDE (Keery and Lumley, 1988). It is this latter property that may explain the ability of AH6809 to suppress PGE_2-induced IL-8 generation in EP4S-C cells. Moreover, our data suggest that this 'off-target' effect may be more prominent than was originally reported given that concentrations of AH6809 as low as 1 μmol·L^{-1} activated CRE-dependent transcription in epithelial cells (Figure S1). However, the finding that AH6809 failed to suppress IL-8 generation from PGE_2-stimulated EP4S-T cells suggests that the PDE profile between these two colonic epithelial cells lines might be different (11 distinct PDE families have been clearly defined; Bender and Beavo, 2006) and that AH6809 exhibits some degree of PDE isoenzyme selectivity.

A surprising pharmacological observation was the ability of AH23848 to abolish PGE_2-induced luciferase induction and

Table 4 Saturation analysis of [^{3}H]PGE_2 binding to cell membrane expressing native and recombinant prostanoid receptors

Caco-2			*HEK293*		
EP receptors in:	*p*K_d	*Bmax*	*EP receptors in:*	*p*K_d	*Bmax*
EP4S-C	9.70 ± 0.12	48.9 ± 6.3	EP4S-H	8.97 ± 0.05	37.3 ± 2.7
EP4A-C	8.58 ± 0.02	15.0 ± 3.3			
EP2S-C	8.92 ± 0.02	42.4 ± 5.7	EP2S-H	8.28 ± 0.02	33.2 ± 2.4
EP2A-C	8.76 ± 0.05	12.4 ± 1.5			
Caco-2	8.82 ± 0.04	12.5 ± 0.9			

Cell membranes were incubated with increasing concentrations of [^{3}H]PGE_2 as described in Belley and Chadee (1999). K_d and Bmax are expressed as the negative log molar concentration and fmole·mg^{-1} protein respectively. Non-specific binding was determined with 1000-fold excess of unlabelled PGE_2. Data represent the mean ± SEM of three different experiments. Caco-2 or HEK293 cells stably over-expressing EP_2 and EP_4 sense mRNA are referred to as EP2S-C or EP2S-H and EP4S-C or EP4S-H respectively, whereas, Caco-2 cells stably over-expressing EP_2 and EP_4 antisense mRNA are referred to as EP2A-C and EP4A-C respectively.
PG, prostaglandin.

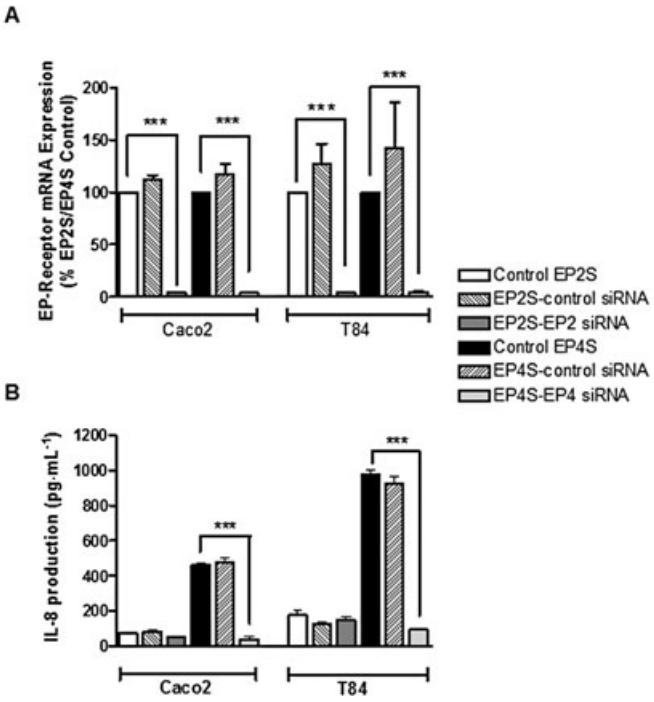

Figure 5 Effect of siRNAs on interleukin (IL)-8 production. Caco-2 and T84 cells stably expressing EP_2 and EP_4 sense receptors were transfected with or without EP_2 and EP_4 siRNAs respectively. (A) Expression of EP_2 and EP_4 receptor mRNA was determined by real-time PCR after 6 h of prostaglandin (PG) E_2 treatment. ***$P < 0.001$ relative to respective untransfected control cells using untransformed data. (B) Colonic cells were treated with PGE_2 for 16 h, and IL-8 production was measured by enzyme immunoassay. Data represent mean ± SEM of three experiments. ***$P < 0.001$ relative to untransfected cells.

IL-8 generation. Although AH23848 can block the human cloned EP_4 receptor subtype, it was active at a concentration of 1 μmol·L^{-1}, which is slightly below its reported affinity ($pA_2 = 5.6$; Davis and Sharif, 2000). Why AH23848 showed unusually high potency in Caco-2 cells currently is unclear although 'off-target' activity cannot be excluded. Nevertheless, in light of the results obtained with ONO-AE3-208 and ONO-AE1-329 (see *Results*), these anomalous data with AH23848 do not challenge, materially, our conclusion that the EP_4 receptor mediates PGE_2-induced IL-8 generation from Caco-2 cells.

It is noteworthy that the ability of PGE_2 acting via the EP_4 receptor to promote IL-8 generation in colonic epithelia is contrary to what is seen in many other cell types (e.g. macrophages – Takayama *et al*., 2002; Xu *et al*., 2008). Indeed, agents that increase cAMP, including PGE_2, generally suppress the generation of cytokines and chemokines induced by pro-inflammatory stimuli such as IL-1β and tumour necrosis factor α. However, in human gut epithelial cells, it is significant that PGE_2 powerfully induced IL-8 generation. Moreover, in contrast to many other cells, PGE_2 further increased IL-8 output above that promoted by IL-1β, another pro-inflammatory cytokine that is increased in colitis (see Figure 3). Thus, our data imply that the signalling pathways leading to IL-8 generation by PGE_2 in colonic epithelial cells are very different from those used by this PG in many other cell types.

A consistent observation seen in colonic inflammation is that PGE_2 and EP_4 receptor levels are significantly increased although the functional significance of these changes in the pathogenesis of IBD and colitis remains undefined. Indeed, although the aetiology of DSS-induced colitis in laboratory animals is not completely elucidated, it has been shown that EP_4 receptor agonists are beneficial in these model systems, while COX inhibitors and PGE_2 exacerbate the colitis, either by down-regulating the immune system or by enhancing the survival and/or the regeneration of the epithelium (Kabashima *et al*., 2002; Jiang *et al*., 2007). Clearly our *in vitro* data using immortalized adenocarcinoma cell lines (Caco-2 and T84), which show the induction of the IL-8 gene by PGE_2 cannot easily be reconciled with those *in vivo* findings. Currently the explanation for these diametrically opposite results is unknown but several possibilities merit consideration including species differences (rats/mice vs. humans), the fact that *in vitro* data may not be reproduced *in vivo*, anomalies resulting from the use of EP_4 receptor knockout mice and the means by which experimental colitis in laboratory animals is induced. We also acknowledge that Caco-2 and T84 cells may not be representative of primary colonic epithelial cells with respect to the regulation by PGE_2 of IL-8 gene expression.

In conclusion, IL-8 is believed to be an important mediator of colonic inflammation and is produced in response to viral, bacterial and parasitic infections. Using predominantly Caco-2 cells, our study demonstrates that PGE_2 generates IL-8 by activating a cAMP-dependent mechanism that is mediated

exclusively by activation of the EP_4 receptor. We suggest that this might be an important observation as expression of EP receptors, particularly those of the EP_4 subtype, is dynamically regulated in several GI disorders and may play a major pro-inflammatory role (Dey *et al.*, 2006). Thus, it is possible the EP_4 receptor could be exploited to therapeutic advantage with small molecule antagonists that could effectively suppress PGE_2-induced IL-8 production and the subsequent neutrophilic inflammation that is a defining pathological characteristic of colitis.

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Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of AH6809 on luciferase activity in epithelial cells expressing the cAMP responsive element reporter construct. Wild-type epithelial cells were exposed to AH6809

(1–30 μmol·L^{-1}) for 6 h, lysed and luciferase activity determined as described in *Materials and Methods*. Data represent the mean ± SEM of three experiments. *$P < 0.05$ significant augmentation of luciferase activity relative to unstimulated cells determined by using untransformed data.

Table S1 Luciferase activity in EP sense (S) and antisense (A) cells following stimulation with EP receptor agonists and antagonists

Table S2 Interleukin-8 production in EP sense (S) and antisense (A) cells following with EP receptor agonists and antagonists

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