EP_2 and EP_4 receptor antagonists: impact on cytokine production and β_2-adrenergic receptor desensitization in human airway smooth muscle

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Abstract

Prostaglandin E_2 (PGE_2) is a key prostanoid known to have both pro-inflammatory and anti-inflammatory impact in the context of chronic respiratory diseases. We hypothesize that these opposing effects may be the result of different prostanoid E (EP) receptor-mediated signaling pathways. In this study, we focus on two of the four EP receptors, EP_2 and EP_4, as they are known to induce cAMP-dependent signaling pathways. Using primary human airway smooth muscle (ASM) cells, we first focussed on PGE_2-induced production of two, cAMP-dependent, pro-inflammatory mediators; interleukin 6 (IL-6) and cyclo-oxygenase 2 (COX-2) production. We show that PGE_2-induced interleukin 6 (IL-6) protein secretion occurs via an EP_2-mediated pathway, in a manner independent of receptor-mediated effects on mRNA expression and temporal activation kinetics of the transcription factor cAMP response element binding (CREB). Moreover, stimulation of ASM with PGE_2 did not establish a positive, receptor-mediated, feedback loop, as mRNA expression for EP_2 and EP_4 receptors was not upregulated, and receptor antagonists were without effect. Secondly, our studies revealed that the EP_2, but not the EP_4, receptor is responsible for β_2-adrenergic desensitization induced by PGE_2. We demonstrate that PGE_2-induced heterologous receptor desensitization responsible for tachyphylaxis to short- (salbutamol) or long- (formoterol) β_2-agonists (measured by cAMP release) can be reversed by the EP_2 receptor antagonist PF-04418948. Importantly, this study highlights that inhibiting the EP_2 receptor restores β_2-adrenergic receptor function *in vitro* and offers an attractive novel therapeutic target for treating infectious exacerbations in people suffering from chronic respiratory diseases in the future.

Keywords: prostaglandin E_2; CREB; airway smooth muscle; inflammation; desensitization; EP_2 receptor; EP_4 receptor.

Introduction

Prostaglandin E_2 (PGE_2) is a prostanoid with diverse actions in health and disease. In chronic respiratory diseases driven by inflammation, PGE_2 has both positive and negative effects that may emerge as being the consequence of different receptor-mediated cellular functions (see our recent review (Lebender et al., 2018)). Four receptors are responsible for inducing PGE_2-induced effects. Known as the prostanoid E (EP) receptors (EP_1- EP_4), EP_2 and EP_4 in particular exert their effects via cAMP-mediated signaling pathways. In the context of chronic respiratory diseases, our *in vitro* studies utilizing human airway smooth muscle (ASM) have confirmed that PGE_2 exerts both positive (production of anti-inflammatory molecules, i.e. mitogen activated protein kinase phosphatase 1 (MKP-1) (Rumzhum and Ammit, 2016b)) and negative effects (production of pro-inflammatory molecules (including interleukin 6 (IL-6) and cyclo-oxygenase 2 (COX-2)) as well as β_2-adrenergic desensitization (Alkhouri et al., 2014; Ammit et al., 2000; Rumzhum et al., 2016a; Rumzhum et al., 2016b)) in a manner linked with cAMP. However, as selective receptor antagonists continue to emerge (Lebender et al., 2018) it may prove possible in the future to separate the "good" from the "bad" effects of this important prostanoid. Accordingly, in this study, we focus on investigating the role of the EP_2 or EP_4 receptor on some of the PGE_2-induced negative outcomes in ASM cells (*viz* cytokine production and β_2-adrenergic desensitization) utilizing the selective receptor antagonists PF-04418948 and GW 627368X for the EP_2 and EP_4 receptors, respectively.

Herein, we show that PGE_2-induced IL-6 protein secretion occurs in an EP_2-mediated pathway in a manner independent of receptor-mediated effects on mRNA expression and cAMP response element binding (CREB) protein phosphorylation. Furthermore, blocking EP_2 receptor activation with PF-04418948 restores β_2-adrenergic receptor function. Notably, the EP_4 receptor antagonist,

GW 627368X, was without effect. Taken together, these *in vitro* studies indicate that targeting the EP_2 receptor with selective antagonists offer a potential *in vivo* strategy for minimizing the negative effects of PGE_2 in chronic respiratory disease.

Material and Methods

Cell culture

Human bronchi were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney. ASM cells were dissected and purified as previously described by Johnson *et al.* (Johnson et al., 1995). A minimum of three different ASM primary cell lines were used for each experiment.

Chemicals

PGE_2, PF-04418948 and GW 627368X were purchased from the Cayman Chemical Company (Ann Arbor, MI). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcription performed by using the RevertAid First strand cDNA Synthesis kit (Fermentas Life Sciences, Hanover, MD) according to the manufacturer's protocol. Real-time RT-PCR was performed on an ABI Prism 7500 with IL-6 (Hs00174131_m1), COX-2 (Hs0015133_m1), EP_2 (Hs00168754_m1) and EP_4 (Hs00168761_m1) TaqMan gene expression assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems, Foster City, CA) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA expression quantified by delta delta Ct calculations.

ELISAs

IL-6 and IL-8 ELISAs were performed using kits from BD Biosciences Pharmingen, San Diego, CA.

Western blotting

COX-2 was detected using a mouse monoclonal antibody (29: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin as the loading control (DM1A: Santa Cruz). Phosphorylated (Ser^{133}) and total CREB were detected with rabbit monoclonal antibodies from Cell Signaling Technology, Danvers, MA. Primary antibodies were detected with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

cAMP assay

Desensitization of the β_2-adrenergic receptor was assessed by measuring production of cAMP in response to stimulation with the β_2-agonists salbutamol (10 μM) and formoterol (10 nM) for 15 min, in the presence of the pan-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX: 30 min pretreatment at 10 μM), in accordance with previously published methods (Alkhouri et al., 2014). cAMP was measured by enzyme immunoassay (cAMP EIA 581001: Cayman Chemical Company) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using one-way ANOVA then Fisher's PLSD post-test or two-way ANOVA with Dunnett's multiple comparison test. P values < 0.05 were sufficient to reject the null hypothesis for all analyses.

Results

PGE_2 induces IL-6 and COX-2 mRNA expression and protein upregulation, but not IL-8 protein secretion

We showed some time ago (Ammit et al., 2000) that the prostanoid PGE_2 dose-dependently increases cAMP production in ASM cells. A number of mediators with pro-inflammatory roles in respiratory disease can be upregulated in a cAMP-dependent manner, including the cytokine IL-6 (Ammit et al., 2000) and the enzyme cyclooxygenase-2 (COX-2) (Rumzhum and Ammit, 2016a). Herein, using ASM cells we demonstrate that PGE_2 (100 nM) induced IL-6 mRNA expression and protein secretion in a time-dependent manner (Figures 1A and 1B: $P<0.05$). Similarly, COX-2 mRNA and protein was also upregulated following PGE_2 stimulation. COX-2 mRNA was significantly increased by approximately 6-fold at both 1 h and 2 h post stimulation (Figure 1C: $P<0.05$) resulting in increased COX-2 protein expression at 3 h (Figure 1D). In parallel studies, the cAMP dependence of PGE_2-induced synthetic responses in ASM cells was supported by the lack of significant secretion of IL-8 over the same time course (Figures 1E); IL-8 is a chemokine that is not upregulated by cAMP (Manetsch et al., 2013).

PGE_2-induced IL-6 protein secretion occurs via the EP_2 receptor

We then examined whether the EP_2 or EP_4 receptor was responsible for the PGE_2-induced effects. We utilized the selective EP_2 and EP_4 receptor antagonists PF-04418948 and GW 627368X, respectively, and used IL-6 secretion as an exemplar functional outcome. Firstly, we performed a dose-response analysis by pretreating growth-arrested ASM cells for 30 min with a range of concentrations of PF-04418948 or GW 627368X (0, 0.25, 0.5, 1, 2.5, 5 and 10 μM), before stimulation with 100 nM PGE_2 for 24 h. As shown in Figure 2A, IL-6 secretion was

significantly reduced in a dose-dependent manner for those cells treated with EP_2 receptor antagonist, PF-04418948. ASM cells treated with 0.25 μM and 0.5 μM PF-04418948 had no inhibitory impact on PGE_2-induced IL-6 protein secretion, however, significant repression was observed at 1 μM PF-04418948 (P<0.05). Interestingly, the level of repression did not increase with higher concentrations of receptor antagonist (2.5-10 μM), thus, all future experiments were carried out using 1 μM PF-04418948. Notably, ASM cells treated with GW 627368X did not significantly inhibit IL-6 secretion regardless of concentration (Figure 2A). Secondly, we performed a time course and pretreated ASM cells with either 1 μM PF-04418948 or GW 627368X before stimulating with PGE_2 (100 nM) for up to 24 h. We observed significant repression of PGE_2-induced IL-6 protein secretion with 1 μM PF-04418948, but not GW 627368X, as early as 4 h (P<0.05). Taken together, these results show that PGE_2-induced IL-6 protein secretion was mediated via the EP_2, not the EP_4, receptor.

PGE_2-induced IL-6 and COX-2 mRNA expression is not repressed by EP_2/EP_4 receptor antagonists

We were then interested in examining whether inhibition of PGE_2-induced IL-6 by EP_2 receptor antagonism was observed at the mRNA expression level. Growth-arrested ASM cells were pretreated with either vehicle, 1 μM PF-04418948, or 1 μM GW 627368X before stimulating with PGE_2 (100 nM) for 0, 1, 2, 4, 8 and 24 h. As shown in Figure 3A, there was no significant effect of PF-04418948 or GW 627368X on PGE_2-induced IL-6 mRNA expression at any time point. We extended these studies further and also observed that PGE_2-induced COX-2 mRNA was also unaffected (Figure 3B). Thus, EP receptor antagonists were without effect on mRNA expression on key PGE_2-induced messages.

PGE_2 does not induce EP_2 or EP_4 receptor mRNA expression

We then examined whether the prostanoid PGE_2 might exert a positive feedback loop by increasing mRNA expression of the EP_2 or EP_4 receptor, as there is the potential that differential regulation by EP receptor antagonism might exert confounding effects. Accordingly, we measured the temporal kinetics of EP_2 (Figure 4A) or EP_4 receptor (Figure 4B) mRNA expression induced by PGE_2. As shown in Figure 4A and 4B, PGE_2 did not induce significant upregulation of EP_2 or EP_4 receptor expression. In fact, it appeared that EP_2 receptor mRNA levels at 8 h were significantly reduced (Figure 4A: $P<0.05$) and may represent a down-regulation of EP_2 expression. However, the time course was unaffected by antagonists of the EP_2 or EP_4 receptors, as PF-04418948 or GW 627368X were without effect on PGE_2-induced EP_2 (Figure 4A) or EP_4 receptor (Figure 4B) mRNA expression.

EP_2/EP_4 receptor antagonists do inhibit PGE_2-induced CREB phosphorylation

As stimulation of EP_2 and EP_4 receptors by PGE_2 activates adenylate cyclase, resulting in cAMP production and phosphorylation of the transcription factor cAMP response element binding protein (CREB) at Ser^{133} (Lebender et al., 2018), we were interested to examine the effect of PF-04418948 or GW 627368X on PGE_2-induced CREB phosphorylation in ASM cells. As shown in Figures 5A and 5B, PGE_2 significantly induced phosphorylation of CREB at Ser^{133} at 15 min and 30 min in ASM cells ($P<0.05$), however pretreatment with PF-04418948 or GW 627368X was without effect. The cell permeable cAMP analogue, dibutyryl cAMP, was used a positive control and was shown to induce CREB phosphorylation in ASM cells in accordance with our earlier publication (Che et al., 2012). Collectively, these studies reveal that while the EP_2 receptor, but

not the EP_4 receptor, was involved in PGE_2-induced IL-6 protein secretion, this repression was not mediated via effects on CREB phosphorylation, nor mRNA expression.

EP_2 receptor antagonism restores β_2-adrenergic receptor function

As we have previously shown, PGE_2 induces heterologous desensitization of the β_2-adrenergic receptor (Alkhouri et al., 2014; Rumzhum et al., 2016a; Rumzhum et al., 2016b) thus, we hypothesized that EP_2 or EP_4 receptors may be involved in regulating β_2-adrenergic receptor function in ASM cells. In accordance with our previous publication (Alkhouri et al., 2014), PGE_2-induced tachyphylaxis to short- (salbutamol) or long- (formoterol) β_2-agonists (measured by cAMP release). Because of β_2-adrenergic receptor hyporesponsiveness, the levels of bronchodilatory mediator cAMP induced by short and long β_2-agonists (salbutamol (Figure 6A) and formoterol (Figure 6B), respectively) are significantly reduced. Since β_2-adrenergic receptor sensitization *in vivo* limits the efficacy of bronchodilator treatment in chronic respiratory disease, it was of interest to examine whether EP_2 and EP_4 prostanoid receptor antagonists could restore β_2-adrenergic receptor function. Importantly, our data showed that the EP_2 receptor antagonist PF-04418948 ameliorated the effects of PGE_2 (at all concentrations tested; 1-1000 nM) and restored the effect of both salbutamol and formoterol on cAMP production in ASM cells (Figures 6A and 6B: $P<0.05$). In contrast, the EP_4 receptor antagonist was without effect (except at very low concentrations of PGE_2 in Figure 6A). Taken together, these results demonstrate that PGE_2-induced β_2-adrenergic desensitization occurs via the EP_2 receptors and suggests that targeting EP_2 with receptor antagonists may be an effective pharmacotherapeutic strategy to restore β_2-adrenergic receptor function.

Discussion

Our study has revealed that EP_2 receptor is involved in PGE_2-induced IL-6 cytokine secretion and β_2-adrenergic receptor function. These studies add to the growing realization that EP receptor subtypes may be targeted to separate the "good" from the "bad" impacts of PGE_2 (Lebender et al., 2018).

We showed that PGE_2-induced IL-6 protein secretion was repressed in a time- and dose-dependent manner by EP_2, but not EP_4, receptor antagonism. While we are yet to fully understand the mechanism responsible for repressive effects of PF-04418948 on PGE_2-induced IL-6 protein secretion, we have ruled out a number of possible explanations: inhibition is not at the level of CREB signalling; nor does PF-04418948 repress PGE_2-induced IL-6 mRNA expression. Importantly, stimulation of ASM cells with PGE_2 did not upregulate EP_2 and EP_4 mRNA expression, suggesting that PGE_2 does not exert a receptor-mediated positive feedback loop. It was interesting to note however that there was down-regulation of the EP_2 receptor after 8 hours stimulation with PGE_2, suggesting potential biphasic regulation of this EP receptor sub-type. Moreover, pretreatment of ASM cells with PF-04418948 or GW 627368X did not increase EP_2 and EP_4 mRNA expression. The possibility remains that inhibition may occur at the translational level, as recent reports have implicated inhibition of protein translation as a novel mechanism for PGE_2 regulation of cellular functions in other cell types (Degraaf et al., 2014; Okunishi et al., 2014). Future studies examining whether PGE_2 controls protein translation in ASM cells are warranted. Additionally, it would be of interest to examine the impact of antagonising both the EP_2 and EP_4 receptor at the same time by pretreating ASM cells with PF-04418948 and GW 627368X concomitantly.

There are broad-ranging implications of our *in vitro* studies targeting PGE_2-mediated functions. Prostanoids, such as PGE_2, have been linked with infectious exacerbation of chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD). When a person with asthma or COPD experiences a respiratory infection their lung function worsens as a result of inadequate respiratory medicines. Therapeutics such as bronchodilatory relievers (i.e. β_2-agonists such as salmeterol and formoterol) lose their effectiveness because viral or bacterial infections change the molecular pathways by which respiratory medicines act, making them less sensitive or resistant. A body of evidence (reviewed in (Rumzhum and Ammit, 2015)) has demonstrated that respiratory infections induce heterologous desensitization of the β_2-adrenergic receptor in a COX-2/prostanoid (especially PGE_2)-dependent manner. Infections ultimately induce PGE_2 and cause β_2-adrenergic desensitization, severely curtailing the beneficial bronchodilatory actions of β_2-agonists. Our current study has enabled us to learn more about the receptor-mediated pathway responsible for prostanoid PGE_2 effects on β_2-adrenergic receptor desensitization in ASM cells. Our data clearly shows that blocking the EP_2 receptor with PF-04418948 reverses β_2-adrenergic hyporesponsiveness to both short- and long-acting β_2-agonists *in vitro.* These data demonstrate the potential of the selective EP_2 receptor antagonist PF-04418948 to restore β_2-agonist efficacy *in vitro* and supports the prevailing view (reviewed in (Lebender et al., 2018; Rumzhum and Ammit, 2015)) that targeting prostanoid receptors, rather than prostanoids themselves, or their upstream enzymatic pathways (such as COX-2), offers the exciting potential of future therapeutics.

Treating exacerbation in chronic respiratory disease is clinically challenging. Given that hyporesponsiveness to bronchodilators is a hallmark feature of acute exacerbation in respiratory disease, an important clinical goal is to restore β_2-agonist efficacy. Prostanoid receptor selective

antagonists that target EP_2 receptors offer great potential and may advance better treatment options to treat infectious exacerbation in asthma and COPD in the future.

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Figure Legends

Figure 1. PGE_2 induces IL-6 and COX-2 mRNA expression and protein upregulation, but not IL-8 protein secretion. Growth-arrested ASM cells were treated with PGE_2 (100 nM) for 0, 1, 2, 4, 8, and 24 h. (A, C) IL-6 and COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to 0 h). (B, E) IL-6 and IL-8 protein secretion (pg/mL) was detected by ELISA. (D) COX-2 protein was measured by Western blotting (compared to α-tubulin as a loading control). Statistical analysis was performed using one-way ANOVA then Fisher's PLSD post-test (where * denotes a significant effect of PGE_2 compared to 0 h (P<0.05)). (A, B, C, E) Data are mean+SEM values from n=6 primary ASM cell cultures and (D) representative blots from n=4 primary ASM cell cultures.

Figure 2. PGE_2-induced IL-6 protein secretion occurs via the EP_2 receptor. (A) Growth-arrested ASM cells were pretreated for 30 min with a range of concentrations (0, 0.25, 0.5, 1, 2.5, 5, 10 μM) of PF-04418948 (EP_2 receptor antagonist) or GW 627368X (EP_4 receptor antagonist) or vehicle controls, prior to 24 h treatment with PGE_2 (100 nM). (B) Growth-arrested ASM cells were pretreated for 30 min with 1 μM PF-04418948 (EP_2 receptor antagonist) or GW 627368X (EP_4 receptor antagonist), then treated with PGE_2 (100 nM) for 0, 1, 2, 4, 8, and 24 h. IL-6 protein secretion was measured by ELISA. Statistical analysis was performed using two-way ANOVA with Dunnett's multiple comparison test (where * denotes significant repression by the EP_2 receptor antagonist PF-04418948 (P<0.05)). Data are mean+SEM values from (A) n=5 and (B) n=6 primary ASM cell cultures.

Figure 3. PGE_2-induced IL-6 and COX-2 mRNA expression is not repressed by EP_2/EP_4 receptor antagonists. (A) Growth-arrested ASM cells were pretreated for 30 min with vehicle, 1 μM PF-04418948 (EP_2 receptor antagonist) or GW 627368X (EP_4 receptor antagonist), then treated with PGE_2 (100 nM) for 0, 1, 2, 4, 8, and 24 h. (A) IL-6 and (B) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold difference to vehicle control at 0 h). Data are mean+SEM values from n=6 primary ASM cell cultures.

Figure 4. PGE_2 does not induce EP_2 or EP_4 receptor mRNA expression. Growth-arrested ASM cells were pretreated for 30 min with vehicle, 1 μM PF-04418948 (EP_2 receptor antagonist) or GW 627368X (EP_4 receptor antagonist), then treated with PGE_2 (100 nM) for 0, 1, 2, 4, 8, and 24 h. (A) EP_2 and (B) EP_4 mRNA expression was quantified by real-time RT-PCR (results expressed as fold difference to vehicle control at 0 h). Statistical analysis was performed using one-way ANOVA then Fisher's PLSD post-test (where * denotes a significant reduction in EP_2 mRNA expression compared to 0 h ($P<0.05$)). Data are mean+SEM values from n=4 primary ASM cell cultures.

Figure 5. EP_2/EP_4 receptor antagonists do inhibit PGE_2-induced CREB phosphorylation. Growth-arrested ASM cells were pretreated for 30 min with vehicle, 1 μM PF-04418948 (EP_2 receptor antagonist), GW 627368X (EP_4 receptor antagonist), then treated with PGE_2 (100 nM) for 0, 15, and 30 min. The cell permeable cAMP analogue, dibutyryl cAMP (1 mM), was used a positive control over the same time course. Western blotting to detect CREB phosphorylation (Ser^{133}) was performed with total CREB as loading controls. Results are (A) representative Western blots and (B) densitometric analysis (results expressed as CREB phosphorylation (fold

increase over vehicle control at 0 min)) from n=3 primary ASM cell cultures. Statistical analysis was performed using one-way ANOVA then Fisher's PLSD post-test (where * denotes a significant increase in CREB phosphorylation induced by PGE_2, compared to vehicle control at 0 min (P<0.05)).

Figure 6. EP_2 receptor antagonism restores β_2-adrenergic receptor function. ASM cells were pretreated for 30 min with vehicle, 1 μM PF-04418948 (EP_2 receptor antagonist), GW 627368X (EP_4 receptor antagonist), then treated for 24 h with vehicle, or a range of PGE_2 concentrations (1-1000 nM). Desensitization of the β_2-adrenergic receptor was assessed by measuring production of cAMP in response to stimulation with (A) 10 μM salbutamol or (B) 0.01 μM formoterol for 15 min compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor IBMX. Results are expressed as a percentage of β_2-agonist-induced cAMP. Statistical analysis was performed using two-way ANOVA with Dunnett's multiple comparison test (where * denotes significant repression by the EP receptor antagonists (P<0.05)). Data are mean±SEM values from n=4 primary ASM cell cultures.