

Prostaglandin E₂ induces expression of MAPK phosphatase 1 (MKP-1) in airway smooth muscle cells

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Abstract

Prostaglandin E₂ (PGE₂) is a prostanoid with diverse actions in health and disease. In chronic respiratory diseases driven by inflammation, PGE₂ has both positive and negative effects. An enhanced understanding of the receptor-mediated cellular signalling pathways induced by PGE₂ may help us separate the beneficial properties from unwanted actions of this important prostaglandin. PGE₂ is known to exert anti-inflammatory and bronchoprotective actions in human airways. To date however, whether PGE₂ increases production of the anti-inflammatory protein MAPK phosphatase 1 (MKP-1) was unknown. We address this herein and use primary cultures of human airway smooth muscle (ASM) cells to show that PGE₂ increases MKP-1 mRNA and protein upregulation in a concentration-dependent manner. We explore the signalling pathways responsible and show that PGE₂-induces CREB phosphorylation, not p38 MAPK activation, in ASM cells. Moreover, we utilize selective antagonists of EP2 (PF-04418948) and EP4 receptors (GW 627368X) to begin to identify EP-mediated functional outcomes in ASM cells *in vitro*. Taken together with earlier studies, our data suggest that PGE₂ increases production of the anti-inflammatory protein MKP-1 via cAMP/CREB-mediated cellular signalling in ASM cells and demonstrates that EP2 may, in part, be involved.

Keywords: prostaglandins; cell signalling; CREB; MKP-1; airway smooth muscle; inflammation

1. Introduction

The prostanoid prostaglandin E₂ (PGE₂) is recognised as playing a number of important physiological and pathophysiological roles in health and disease. In respiratory conditions driven by inflammation, such as asthma and chronic obstructive pulmonary disease (COPD), PGE₂ can exert both beneficial and detrimental effects (reviewed in (Rumzhum and Ammit, 2015)). The myriad actions of PGE₂ occur because the prostanoid activates diverse intracellular signalling pathways via interaction with a family of G protein-coupled receptors (EP1-4) and receptor redundancy and cross-talk exists (reviewed in (Claar et al., 2015)).

We are interested in understanding the molecular mechanisms responsible for the action/s of PGE₂ in airway cells in order to inform future development of pharmacotherapeutic strategies to combat inflammation in chronic lung disease. Some of the more well-established positive impacts of PGE₂ include reduction of inflammation in human airways and bronchoprotective effects (reviewed in (Rumzhum and Ammit, 2015)). This is due to the ability of PGE₂ to induce cAMP production in airway cells. We and others have reported that PGE₂ (and other cAMP-elevating agents) repress cytokine production in the pivotal airway cell type - airway smooth muscle (ASM) (Ammit et al., 2000; Billington et al., 2013; Kaur et al., 2008b; Lazzeri et al., 2001). We have also shown that cAMP can increase production of the anti-inflammatory protein, mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) in ASM cells (Che et al., 2012; Manetsch et al., 2013; Manetsch et al., 2012; Patel et al., 2015). To date however, whether PGE₂ increases production of the MAPK deactivator MKP-1 in ASM cells was unknown. This is the aim of the current study.

2. Material and Methods

2.1 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). In brief, ASM cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated foetal bovine serum, 5 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Confluent ASM cells were growth-arrested for 48 h in DMEM with 0.1% BSA. A minimum of three different ASM primary cell lines were used for each experiment.

2.2 Chemicals

PGE₂, 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).

2.3 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 MKP-1 (DUSP1: Hs00610256_g1) 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.

2.4 Western blotting

MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19: Santa Cruz Biotechnology, Santa Cruz, CA). α -tubulin was used as the loading control (mouse monoclonal IgG₁, DM1A: Santa Cruz Biotechnology). Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) and total p38 MAPK, phosphorylated (Ser¹³³) and total cAMP response element binding protein (CREB) (all from Cell Signaling Technology, Danvers, MA). Primary antibodies were detected with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

2.5 Statistical analysis

Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (Fig. 1-3) or two-way ANOVA then Bonferroni's post-hoc multiple comparison test (Fig. 4). *P* values < 0.05 were sufficient to reject the null hypothesis for all analyses.

3. Results

3.1 PGE₂ induces MKP-1 mRNA expression

In early studies (Ammit et al., 2000) we showed that the prostanoid PGE₂ dose-dependently increased cAMP production in ASM cells. We also showed that MKP-1 expression in ASM cells is cAMP-dependent (Che et al., 2012; Manetsch et al., 2013; Manetsch et al., 2012; Patel et al., 2015). This led us to hypothesize that PGE₂ will increase MKP-1 expression. We test this herein by treating growth-arrested ASM cells with increasing concentrations of PGE₂ (0.1-1000 nM) and examining MKP-1 mRNA expression at 1 h, compared to untreated controls. As shown in Fig. 1, PGE₂ dose-dependently increased MKP-1 mRNA expression with significant increases observed at 100 nM and 1000 nM PGE₂ ($P<0.05$).

3.2 PGE₂ increases MKP-1 protein upregulation

MKP-1 is an immediate early gene (Sun et al., 1993) that can be rapidly translated into protein. We observe this in Fig. 2, where we show MKP-1 protein upregulation in ASM cells that had been treated for 1 h with increasing concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls. In the representative Western blot shown in Fig. 2A, detectable amounts of MKP-1 protein were first detected in concentrations of PGE₂ as low as 1 nM, which were then augmented with increasing concentrations of PGE₂. Densitometric analysis of the extent of MKP-1 protein upregulation was in accord with MKP-1 mRNA expression, with statistically significant levels of MKP-1 protein detected at 100 nM and 1000 nM PGE₂ (Fig. 2B: $P<0.05$).

3.3 PGE₂ increases CREB phosphorylation, rather than p38 MAPK phosphorylation, in ASM cells

We previously demonstrated that MKP-1 expression is mediated via activation of p38 MAPK and CREB-mediated pathways in ASM cells (Che et al., 2012). To explore whether these pathways were responsible for MKP-1 expression induced by PGE₂ ASM cells were treated with increasing concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls, and p38 MAPK and CREB phosphorylation at 15 min measured by Western blotting. As shown in Fig. 3A, p38 MAPK phosphorylation was not enhanced by PGE₂ treatment. This was confirmed by densitometric analysis, where PGE₂ did not significantly increase p38 MAPK phosphorylation compared to untreated controls (Fig. 3B). In contrast, PGE₂ robustly increased CREB phosphorylation as detected by Western blotting (Fig. 3C); with significantly augmented CREB phosphorylation demonstrated after treatment with 10, 100 and 1000 nM PGE₂ (Fig. 3D: $P<0.05$).

3. 4 Effect of EP2/EP4 receptor antagonists on PGE₂-induced MKP-1 mRNA expression

Taken together with previous studies (Ammit et al., 2000), these results suggest that PGE₂ increases MKP-1 expression in ASM cells via cAMP/CREB-mediated pathways. Notably, two of the EP receptor family members – EP2 and EP4 – are known to activate cAMP-mediated signalling pathways, although receptor redundancy exists (Claar et al., 2015). Thus, in order to examine whether PGE₂ signals via these receptors to increase MKP-1 expression, ASM cells were pretreated for 30 min with PF-04418948 (EP2 receptor antagonist) or GW 627368X (EP4 receptor antagonist) (both at 1 μ M) for 30 min, prior to 1 h treatment with a range of concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls. As shown in Fig. 4, pretreatment with the EP4 receptor antagonists GW 627368X had no significant effect on PGE₂-induced MKP-1 mRNA expression. In contrast, there was a small, but significant effect of

blocking the EP2 receptor with PF-04418948 on MKP-1 expression induced by 1000 nM PGE₂ (Fig. 4: $P<0.05$).

4. Discussion

In this study we show for the first time that MKP-1 mRNA expression and protein levels are increased by PGE₂ in ASM cells in a concentration-dependent manner. We explore the signalling pathways responsible and show that PGE₂-induces CREB phosphorylation, not p38 MAPK activation, in ASM cells. Moreover, we utilize selective antagonists of EP2 and EP4 receptors and begin to identify EP-mediated functional outcomes. Taken together with earlier studies, these data suggest that PGE₂ increases production of the anti-inflammatory protein MKP-1 via cAMP/CREB-mediated cellular signalling and demonstrates that EP2 may, in part, be involved. MKP-1 is the founding member of the family of dual-specificity phosphatases (DUSPs) that dephosphorylate and inactivates MAPKs superfamily members (p38 MAPK, ERK and JNK). Also referred to as DUSP1, MKP-1 is negative regulator of innate immune responses and has been demonstrated to robustly repress inflammation in *in vivo* and *in vitro* models of human disease, including arthritis, asthma and COPD (Doddareddy et al., 2012; Issa et al., 2007; Quante et al., 2008; Smallie et al., 2015). In inflammatory lung diseases, MKP-1 upregulation is considered an important way in which medicines such as corticosteroids and β_2 -agonists have clinical benefit (Che et al., 2012; Giembycz et al., 2008; Giembycz and Newton, 2015; Kaur et al., 2008a; Newton, 2014; Shah et al., 2014). More recently, we have explored the cAMP dependence of MKP-1 upregulation because MKP-1 is a cAMP responsive gene (Kwak et al., 1994; Sommer et al., 2000). We have shown that a number of compounds known to elevate cAMP in ASM cells, including: sphingosine 1-phosphate (Che et al., 2012), forskolin (Manetsch et al., 2013), cell-permeable dibutyl cAMP (Manetsch et al., 2013), β_2 -agonists (Manetsch et al., 2013; Manetsch et al., 2012; Patel et al., 2015), phosphodiesterase 4 inhibitors (Patel et al., 2015), can all increase MKP-1 via CREB-mediated pathways. PGE₂ is known to increase cAMP

in ASM cells (Ammit et al., 2000) and in this study we show that PGE₂ induces CREB phosphorylation at 15 min, and consequently MKP-1 mRNA expression and protein upregulation ensue after 1 h. The unifying hypothesis stemming from these studies is that compounds that increase cAMP in ASM cells may result in anti-inflammatory capability via MKP-1 upregulation.

As recently reviewed by Claar *et al.* (Claar et al., 2015), the actions of PGE₂ occur via receptor-mediated cell signalling interaction. The prostanoid PGE₂ interacts with a family of G protein-coupled receptors - EP1-EP4 - that are known to vary in their tissue distribution and downstream signalling effectors (Woodward et al., 2011). The EP receptors known to increase cAMP are the EP2 and EP4 receptors (Coleman et al., 1994) and they functionally couple to CREB (Fujino et al., 2005a). In some notable examples, EP receptor ligation is can result in opposing effects on downstream second messenger production; that is, EP2 and EP4 are G_s-coupled and both ultimately increase cAMP, however this is opposed by EP3 receptor ligation that can inhibit cAMP production. Differences in the signalling intermediates stimulated by EP2 or EP4 activation exist and differential regulation has been reported (Fujino et al., 2005b; Woodward et al., 2011). Moreover, the EP2 and EP4 receptor subtypes demonstrate different rates of desensitization and internalization. EP2 appears to be more resistant to PGE₂-mediated downregulation compared to EP4 (Desai et al., 2000; Nishigaki et al., 1996).

In an attempt to identify the EP receptor involved in PGE₂-induced MKP-1 expression, we pretreated cells with the receptor antagonists PF-04418948 or GW 627368X, before stimulation with PGE₂. These antagonists are considered to be selective for EP2 (af Forselles et al., 2011; Birrell et al., 2013) and EP4 receptors (Birrell et al., 2015), respectively. In our experiments, it appears that blocking EP4 with GW 627368X had no impact on MKP-1 upregulation. However,

pretreatment with the EP2 receptor antagonist PF-04418948 does significantly repress MKP-1 mRNA expression induced by 1000 nM PGE₂. Whether these differences are due to the fact that there is greater expression of EP2 on ASM cells (Burgess et al., 2004) or variance in the functional coupling of the EP2/EP4 receptor to adenylate cyclase/cAMP are unknown at present. However, it is important to note that the EP2 receptor antagonist only showed a small inhibitory effect on MKP-1 upregulation induced by PGE₂ at the highest concentration tested. In contrast, CREB phosphorylation, MKP-1 mRNA expression and protein upregulation all occurred at lower concentrations of PGE₂. A number of possible explanations exist and warrant further investigation. These include receptor redundancy (when EP2 is blocked, EP4 can signal), differential rates of cAMP second messenger generation by the EP2 compared to the EP4 receptor, or perhaps EP2 receptor upregulation. The latter suggestion is supported by evidence that suggest that PGE₂ itself can induce EP2 receptor upregulation (Sagana et al., 2009); however the short time-frames utilized in this experimental context argue against this possibility. Further investigations into the functional outcome of activating individual EP receptors on the hallmark features of chronic inflammatory lung disease, perhaps with knock-out mice, are warranted.

In summary, we have demonstrated that PGE₂ increases MKP-1 expression in ASM cells and have begun to explore the EP receptor-mediated cellular signalling pathways involved. These studies improve our understanding of the mechanism of action of prostanoids in health and disease.

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Figure Legends

Fig. 1. PGE₂ induces MKP-1 mRNA expression. Growth-arrested ASM cells were treated with increasing concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls. MKP-1 mRNA expression at 1 h was measured by RT-PCR (results expressed as fold increase compared to untreated controls). Data are mean+S.E.M. values from n=3 primary ASM cell cultures (* $P<0.05$).

Fig. 2. PGE₂ increases MKP-1 protein upregulation. Growth-arrested ASM cells were stimulated with increasing concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls. MKP-1 protein was quantified at 1 h by Western blotting, using α -tubulin as the loading control. Results are shown as (A) representative Western blots and (B) densitometric analysis (results expressed as fold increase compared to untreated controls). Data are mean+S.E.M. values from n=3 primary ASM cell cultures (* $P<0.05$).

Fig. 3. PGE₂ increases CREB phosphorylation, rather than p38 MAPK phosphorylation, in ASM cells. Growth-arrested ASM cells were stimulated for 15 min with increasing concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls. Western blotting was performed to demonstrate the impact of PGE₂ on p38 MAPK (A, B) or CREB (C, D) phosphorylation measured at 15 min (with total p38 MAPK or CREB as loading controls, respectively). (A, C) Results are representative Western blots, while (B, D) demonstrates densitometric analysis of the effect of PGE₂ on p38 MAPK or CREB phosphorylation (results expressed as fold increase compared to untreated controls). Data are mean+S.E.M. values from n=3 primary ASM cell cultures (* $P<0.05$).

Fig. 4. Effect of EP2/EP4 receptor antagonists on PGE₂-induced MKP-1 mRNA expression.

Growth-arrested ASM cells were pretreated for 30 min with PF-04418948 (EP2 receptor antagonist) or GW 627368X (EP4 receptor antagonist) (both at 1 μ M) for 30 min, prior to 1 h treatment with a range of concentrations of PGE₂ (0.1-1000 nM), compared to untreated controls. MKP-1 mRNA expression at 1 h was measured by RT-PCR (results expressed as a percentage of MKP-1 mRNA expression induced by 1000 nM PGE₂). Data are mean+S.E.M. values from n=3 primary ASM cell cultures (* P <0.05).