E-Ring 8-Isoprostanes Are Agonists at EP$_{2}^{-}$ and EP$_{4}^{-}$ Prostanoid Receptors on Human Airway Smooth Muscle Cells and Regulate the Release of Colony-Stimulating Factors by Activating cAMP-Dependent Protein Kinase

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ABSTRACT

8-Isoprostanes are bioactive lipid mediators formed via the non-enzymatic peroxidation of arachidonic acid by free radicals and reactive oxygen species. However, their cognate receptors, biological actions, and signaling pathways are poorly studied. Here, we report the effect of a variety of E- and F$_{3}$-ring 8-isoprostanes on the release of granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) from human airway smooth muscle (HASM) cells stimulated with interleukin-1β (IL-1β). The elaboration of GM-CSF and G-CSF by IL-1β was inhibited and augmented, respectively, in a concentration-dependent manner by 8-isoprostaglandin (PG) E$_{1}$ and 8-isopGE$_{2}$, but not by 8-iso-PGF$_{1}^{-}$, 8-iso-PGF$_{2}^{-}$, and 8-iso-PGF$_{3}^{-}$. AH 6809 (6-isopropoxy-9-oxooxanthine-2-carboxylic acid), an EP$_{1}$-EP$_{2}^{-}$-DP-receptor blocking drug, antagonized the inhibitory effect of 8-iso-PGE$_{1}$ and 8-iso-PGE$_{2}$ on GM-CSF output with an affinity consistent with an interaction at prostanoid receptors of the EP$_{2}^{-}$ subtype. In contrast, the facilitation by 8-iso-PGE$_{1}$ and 8-iso-PGE$_{2}$ of G-CSF release was unaffected by AH 6809 and the selective EP$_{4}^{-}$-receptor antagonist L-161,982 [4'-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-di-hydro-[1,2,4]triazol-4-ylmethyl]-biphenyl-2-sulfonic acid (3-methyl-thiophene-2-carbonyl)-amide]. However, when used in combination, AH 6809 and L-161,982 displaced 5-fold to the right the 8-iso-PGE$_{1}$ and 8-iso-PGE$_{2}$ concentration-response curves. The opposing effect of E-ring 8-isoprostanes on GM-CSF and G-CSF release was mimicked by 8-bromo-cAMP and abolished in cells infected with an adenovirus vector encoding an inhibitor protein of cAMP-dependent protein kinase (PKA). Together, these data demonstrate that E-ring 8-isoprostanes regulate the secretion of GM-CSF and G-CSF from HASM cells by a cAMP- and PKA-dependent mechanism. Moreover, antagonist studies revealed that 8-iso-PGE$_{1}$ and 8-iso-PGE$_{2}$ act solely via EP$_{2}$-receptors to inhibit GM-CSF release, whereas both EP$_{2}^{-}$ and EP$_{4}^{-}$-receptor subtypes positively regulate G-CSF output.

The isoprostanes embody a vast family of novel prostanoid-like lipids that are produced by nonenzymatic peroxidation of arachidonic acid (AA) in response to free radicals and reactive oxygen species (Janssen, 2001; Morrow and Roberts, 2002). Although free AA is required for the formation of prostaglandins by cyclooxygenases, the isoprostanes can be generated nonenzymatically from esterified AA in membrane phospholipids before being released by a phospholipase(s) (Liu et al., 1999). Another dissimilarity is that isoprostanes feature side chains that are almost exclusively orientated cis relative to the cyclopentane ring and are therefore distinct from the prostaglandins, which always have side chains in the trans configuration (Morrow et al., 1990). Nevertheless, antagonist studies revealed that 8-iso-PGE$_{1}$ and 8-iso-PGE$_{2}$ act solely via EP$_{2}$-receptors to inhibit GM-CSF release, whereas both EP$_{2}$- and EP$_{4}$-receptor subtypes positively regulate G-CSF output.

ABBREVIATIONS: AA, arachidonic acid; PG, prostaglandin; HASM, human airway smooth muscle; CSF, colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; MOI, multiplicity of infection; PKI, heat-stable inhibitor of protein kinase A; CMV, cytomegalovirus; DAPI, 4’6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; 8-Br-cAMP, 8-bromo-cAMP; ELISA, enzyme-linked immunosorbent assay; TBS-T, Tris-buffered saline/Tween 20; IL, interleukin; CREB, cAMP response element-binding protein; ET, endothelin; ICI 192,605, 4(2)-[6-(2-oxo-phenyl)-1,3-dioxan-5-yl]hexenoic acid; BQ 788, N-cis-2,6-dimethylpyrrolidenocarboxyl-L-γ-methyleneacetyl-D-1-methoxycarbonyl tryptophanyl-o-norleucine.
ertheless, isoprostanes are isomeric with prostaglandins and have been given the prefix D-, E-, and Fα- to denote the 
prostane ring shared with PGD$_2$, PGE$_2$, and PGF$_2\alpha$, respectively (Janssen, 2001). An additional level of complexity is that 
peroxidation of AA can occur at one of any of four carbon 
atoms producing regioisomers, the so-called 5-, 12-, 8-, and 15-series isoprostanes, each consisting of eight racemic 
diastereomers (Taber et al., 1997). Thus, a total of 64 isomers can be 
generated for each of the D-, E-, and Fα-ring isoprostanes.

Despite the bewildering number of isoprostanes that the-
eretically can be formed, the most extensively studied com-
ponents are the 8-isoprostanes, in particular, 8-iso-PGF$_{2\alpha}$, 
and 8-iso-PGE$_2$ (Janssen, 2001). With respect to respiratory dis-
eases associated with oxidative stress, 8-isoprostanes have been 
detected in the urine and plasma at significantly elevated 
levels in subjects with asthma, chronic obstructive pulmonary disease, cystic fibrosis, and interstitial lung dis-
ease compared with normal healthy subjects (Kharritonov and 
Barnes, 2002; Morrow and Roberts, 2002). Indeed, it has been proposed that 8-isoprostanes may be biomarkers of air-
way inflammation and that their level of expression relates to disease severity (Kharritonov and Barnes, 2002; Morrow 
and Roberts, 2002). However, these novel lipids also have direct effects in the lung. For example, 8-isoprostanes can 
contract and relax human airway smooth muscle (Kawikova 
et al., 1996; Janssen et al., 2000), modulate cholinergic neu-
rotransmission (Spicuzza et al., 2001; Clarke et al., 2004b), 
enhance neutrophil function (Zahler and Becker, 1999), and 
promote transepithelial anion secretion (Cowley, 2003). Thus, 
isoprostanes constitute yet another group of mediators that 
can regulate, positively or negatively, smooth muscle contractility, and, potentially, inflammatory responses in the 
airways.

Many of the effects of 8-isoprostanes are known to be 
mediated through prostanoid receptors, although evidence for a specific isoprostane binding site(s) has also been ad-
vanced (Janssen, 2001). Five classes of G protein-coupled 
receptor for the naturally occurring prostanoid agonists have 
advanced (Janssen, 2001). Five classes of G protein-coupled 
receptor subtypes, respectively.

To date, the majority of investigations have, almost exclu-
sively, examined the effect of isoprostanes on smooth muscle 
tone. Herein, we describe an anti- and proinflammatory ef-
effect of E-ring 8-isoprostanes on the synthetic capacity of 
HASM cells and have identified the receptors and signal 
transduction pathways involved. In particular, the effect of 
8-iso-PGE$_1$ and 8-iso-PGE$_2$ has been compared with Fα-ring 
isoprostanes on the secretion of two colony-stimulating fac-
tors (CSFs), G-CSF and GM-CSF, which have previously 
been shown to be modulated by E-series prostaglandins 
(Clarke et al., 2001, 2004a, 2005). These cytokines, discov-
ered in the 1960s, were selected for study because they are essential for the differentiation, proliferation, and survival of 
bone marrow-derived hematopoietic stem cells and have been implicated in the pathogenesis of asthma and chronic ob-
structive pulmonary disease where they enhance in the air-
ways the activation and survival of eosinophils, neutrophils, 
and cells of the monocyte/macrophage lineage (Metcalf, 
1985).

Materials and Methods

Patients providing human airway smooth muscle gave written 
informed consent. The Ethics Committee of the Royal Brompton 
and Harefield National Health Service Trust and National Heart 
and Lung Institute approved this study.

Isolation of HASM Cells. Tracheal rings from either lung 
or heart and lung transplantation donors (four female and seven male 
donor, aged 17–57 years) were dissected under sterile conditions in 
HBSS (136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO$_4$, 0.4 mM 
Na$_2$HPO$_4$·7H$_2$O, 1.3 mM CaCl$_2$·2H$_2$O, 4.2 mM NaHCO$_3$, and 5.6 mM 
glucose) supplemented with 100 U/ml penicillin, 100 μg/ml strepto-
mycin, and 2.5 μg/ml amphotericin B. The smooth muscle layer was 
disseminated free of adherent connective tissue and cartilage, and the 
epithelium was removed using a rounded scalpel blade. The smooth 
muscle was incubated for 30 min at 37°C in 5% CO$_2$/air in HBSS 
containing 10 mg/ml BSA, collagenase (type XI, 1 mg/ml), and elast-
tase (type I, 3.3 U/ml). After the removal of any remaining connective 
tissue, the smooth muscle was chopped finely and incubated for a 
Further 150 min in the enzyme solution described above with the 
esterase concentration increased to 15 U/ml. Dissociated cells 
were centrifuged (100g, 5 min, 4°C) and resuspended in DMEM containing 
10% (v/v) heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM L-
glutamine, 1x nonessential amino acids, and antimicrobial agents 
as detailed above.

Primary Culture of HASM Cells. HASM cells in suspension 
were placed in a tissue culture flask (75 cm$^2$) containing 6 ml of 
supplemented DMEM and allowed to adhere (-12 h) at 37°C in 5% 
CO$_2$/air. The culture medium was replaced after 4 to 5 days (12 ml) 
and thereafter every 3 to 4 days. When the cells reached confluence 
(-10–14 days) and demonstrated a typical “hill and valley” appear-
ance and positive immunostaining for α-actin (routinely >95%), they 
were seeded into either 96-well plates (Costar UK Ltd., High 
Wycombe, UK) at an initial density of 2000 cells per well or six-well 
plates (Costar UK Ltd.) at an initial density of 20,000 cell per well for 
cytokine release and Western blotting/immunocytochemistry exper-
iments, respectively. At subconfluence, the cells were growth ar-
rested by being placed in DMEM containing apotransferrin (5 μg/ 
ml), insulin (1 μM), ascorbate (100 μM), and BSA [0.1% (w/v)] for 
24 h. The medium was replaced with DMEM containing 3% FCS (v/v) 
and drugs or appropriate vehicles as described below.

Infection of HASM Cells with Ad5.CMV.PKIα. In some exper-
iments, subconfluent, growth-arrested HASM cells were infected 
(MOI = 100) with an E1/E3 replication-deficient adenovirus vec-
tor (Ad5.CMV.PKIα) containing a 251-base pair DNA fragment en-
coding the complete amino acid sequence of the α-isofrom of cAMP-
dependent protein kinase inhibitor (PKIα) downstream of the 
constitutively active CMV immediate early promoter (Meja et al., 
2004). After 48 h, cells were processed for immunocytochemistry, 
CSF release, and Western blotting as described below. To control 
for biological effects of the virus per se, the vector Ad5.CMV.Null, ex-
pressing no transgene, was used in parallel. Preliminary exper-
iments established that infection with Ad5.CMV.PKIα resulted in the 
expression of a completely functional transgene and that neither 
vector at an MOI of 100 had any effect on HASM cell viability (data 
not shown). Immunofluorescence microscopy established that >90% 
of cells expressed PKIα 48 h after infection. This was determined by

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were incubated for 1 h at room temperature in TBS-T containing 5% (v/v) Tween 20 containing 5% (w/v) nonfat dry milk. The filters were incubated overnight in TBB-T [25 mM Tris base, pH 7.4, 150 mM NaCl, and glycine, and 20% (v/v) methanol]. The nitrocellulose was incubated in Hampshire, UK) in Tris buffer [50 mM Tris base, pH 8.3, 192 mM deoxycholate, 0.025% (w/v) SDS, and 0.1% (v/v) Triton X-100 supplemented with phenylmethylsulfon fluoride (0.1 mg/ml), leupeptin (10 μg/ml), and aprotinin (25 μg/ml). Insoluble protein was removed by centrifugation (10,000g, 3 min), and aliquots of the resulting supernatant were diluted 1:4 in Laemmli buffer [62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, and 0.01% (v/v) bromphenol blue] and boiled for 5 min. Denatured proteins (25 μg) were separated by SDS-polyacrylamide gel electrophoresis using a 4 to 20% gradient gel (Bio-Rad, Hemel Hempstead, UK) and transferred to Hybond enhanced chemiluminescence membranes (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) in Tris buffer [50 mM Tris base, pH 8.3, 192 mM glycine, and 20% (v/v) methanol]. The nitrocellulose was incubated overnight in TBB-T [25 mM Tris base, pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween 20] containing 5% (w/v) nonfat dry milk. The filters were incubated for 1 h at room temperature in TBS-T containing 5% BSA plus either an anti-human pCREB, PKIα, or β-actin polyclonal antibody (diluted 1:500, 1:250, and 1:1000, respectively). Membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG (diluted 1:4000) in TBS-T/5% nonfat dry milk for 1 h at room temperature. The nitrocellulose was washed again and developed using enhanced chemiluminescence Western blotting detection reagents on Kodak X-OMAT-S film.

Cell Viability. At the end of each experiment, cell viability was determined colorimetrically by measuring the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium to formazan by mitochondrial dehydrogenases.

Protein Determination. The protein concentration was determined according to the method of Bradford using BSA as standard.

Drugs and Analytical Reagents. IL-1β was from R & D Systems; DMEM and HBSS were from Flow Laboratories (Rickmansworth, Hertfordshire, UK); nonessential amino acids were purchased from Invitrogen (Paisley, UK); and indomethacin, heat-inactivated FCS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium were from Sigma Chemical (Poole, Dorset, UK). AH 6809 (6-isopropoxy-9-oxoanthracene-2-carboxylic acid), SQ 29,548 (1S-[1α,2α(Z),3α,4α]-7-[3-[(phenylamino)carbonyl]hydrazinyl]methyl)-7-oxo-2,11-dihydroxy-1(2-cyclohexyl-2-hydroxyethylamino) hydantoin), PDB, PGE2, U-46619 (9,11-dideoxy-11α,9α-methanoepoxy-prostaglandin F2α), and all isoprostanes were obtained from Cayman Chemical (Ann Arbor, MI). L-161,982 ([4-[2-(cyclohexyl-2-hydroxyethylamino) hydantoin]-1,5-dihydro-(1,2,4) triazol-4-ylmethyl]-3-benzyl-2-carboxyl-2-aminic acid) was donated by Merck Frost (Montreal, Canada). Goat anti-human PKIα (code sc1944) and goat anti-human β-actin (code sc1615) were purchased from Autogen Bioleaf (Calne, Wiltshire, UK). Goat anti-human pCREB (code 9191S) was purchased from New England Biolabs (Hitchin, Hertfordshire, UK).

Statistical Analyses. Data points, and values in the text and figure legends, represent the mean ± S.E.M. of n independent determinations using tissue from different donors. Concentration-response curves were analyzed by least-squares, nonlinear iterative regression with the PRISM curve-fitting program (GraphPad Software Inc., San Diego, CA), and log EC50 or pIC50 values were subsequently interpolated from curves of best fit.

Effect of 8-Isoprostanes on IL-1β-Induced GM-CSF and G-CSF Release. None of the E- or F-ring 8-isoprostanes examined in the present study elaborated GM-CSF or G-CSF from HASM cells. However, pretreatment of cells with the highest concentration (10 μM) of an agonist in the presence of the antagonist divided by the EC50 of the agonist alone, K0 is the equilibrium dissociation constant, and [B] is the concentration of antagonist. In the experiments described herein, the term apparent pA2 is substituted for pK0 as antagonists were used at one concentration only, which precludes assumptions being made about the nature of the antagonist.

Where appropriate, data were analyzed statistically using Student’s paired t test or by one-way analysis of variance/Newman-Keuls multiple comparison test. The null hypothesis was rejected when P < 0.05.

Results
8-iso-PGF$_{3\alpha}$ were without significant effect on the release of CSFs from IL-1$\beta$-stimulated HASM cells at concentrations up to 10 $\mu$M (Fig. 1).

**Effect of AH 6809 on the Modulation of CSF Release by E-ring 8-Isoprostanes.** AH 6809 is a competitive antagonist at the human EP$_1$, EP$_2$, and DP-receptor subtypes (Coleman et al., 1987; Keery and Lumley, 1988; Woodward et al., 1995). Pretreatment of cells with AH 6809 (10 $\mu$M) produced a parallel $\sim$10-fold shift to the right of the mean 8-iso-PGE$_1$ and 8-iso-PGE$_2$ concentration-response curves that described the inhibition of GM-CSF release from which apparent $K_B$ values of 5.73 $\pm$ 0.3 and 5.67 $\pm$ 0.14 were derived, respectively (Fig. 2, a and b). In contrast, AH 6809 failed to antagonize the ability of E-ring 8-isoprostanes to augment the elaboration of G-CSF under identical experimental conditions (Fig. 2, c and d; Table 1).

**Effect of a Selective EP$_4$-Receptor Antagonist on the Modulation of CSF Release by E-Ring 8-Isoprostanes.** Pretreatment of HASM cells with the selective EP$_4$-receptor antagonist L-161,982 (10 $\mu$M) significantly suppressed IL-1$\beta$-induced GM-CSF and G-CSF release, respectively (Fig. 7, a and b). On the other hand, the mean 8-iso-PGE$_1$ and 8-iso-PGE$_2$ concentration-response curves constructed in the presence of L-161,982 were also displaced to the right and in a parallel manner in the presence of AH 6809 (Fig. 6, c and d; Table 2). There was no significant difference in the degree of antagonism between the four experiments.

**Effect of 8-Br-cAMP on the Modulation of CSF Release by E-Ring Isoprostanes.** Pretreatment of HASM cells with 8-Br-cAMP (1 $\mu$M) inhibited the ability of IL-1$\beta$ to promote GM-CSF release from HASM cells under experimental conditions where PGD$_2$ and the thromboxane mimetic U-46619 were inactive (Fig. 4a). In contrast, all three prostanoids at 10 $\mu$M significantly augmented IL-1$\beta$-induced G-CSF output, although PGE$_2$ was the most effective (Fig. 4b).

**Effect of Selective TP- and DP$_1$-Receptor Antagonists on the Modulation of CSF Release by E-Ring Isoprostanes.** Pretreatment (30 min) of HASM cells with SQ 29,548 (1 $\mu$M) or BW A868C (1 $\mu$M), at concentrations that are $\sim$500 and 2000 times greater than their affinity at TP- and DP$_1$-receptors, respectively (Swayne et al., 1988; Giles et al., 1989), failed to antagonize the effect of 8-iso-isoprostanes [at 100 nM ($\sim$EC$_{40}$) and 10 $\mu$M ($\sim$EC$_{95}$)] on CSF secretion (Fig. 5; Table 1). The enhancement by PGD$_2$ (10 $\mu$M) of G-CSF release similarly was insensitive to BW A868C (Table 1).

**Effect of Concurrent Antagonism of EP$_2$- and EP$_4$-Receptors on PGE$_2$-Induced G-CSF Release.** Consistent with the data presented in Figs. 2 and 3, pretreatment (30 min) of HASM cells with AH 6809 (10 $\mu$M) or L-161,982 (2 $\mu$M) failed to antagonize the enhancement by 8-iso-isoprostanes of G-CSF release (data not shown for clarity). In contrast, in AH 6809-treated tissues L-161,982 produced a parallel rightwards shift ($\sim$5-fold) of the mean 8-iso-PGE$_1$ and 8-iso-PGE$_2$ concentration-response curves that described the augmentation of G-CSF release relative to the effect of AH 6809 alone (Fig. 6, a and b). On the other hand, the mean 8-iso-PGE$_1$ and 8-iso-PGE$_2$ concentration-response curves constructed in the presence of L-161,982 were also displaced to the right and in a parallel manner in the presence of AH 6809 (Fig. 6, c and d; Table 2). There was no significant difference in the degree of antagonism between the four experiments.

**Role of PKA in the Action of 8-Br-cAMP and E-Ring 8-Isoprostanes on CSF Release.** To determine the role of PKA in mediating the effect of 8-iso-PGE$_1$, 8-iso-PGE$_2$, and 8-Br-cAMP on CSF release from HASM cells, a virus vector, Ad$_5$CMV.PKI$\alpha$, encoding an endogenous, potent, and highly selective inhibitor of PKA (PKI$\alpha$) was used (Glass et al., 1986; Olsen and Uhler, 1991). In uninfected cells, PKI$\alpha$ was not

![Fig. 1. Effect of E- and F$_\alpha$-ring 8-isoprostanes on IL-1$\beta$-induced GM-CSF and G-CSF release. Adherent HASM cells were pretreated for 5 min with 8-isoprostane before being exposed to IL-1$\beta$. Cells were maintained at 37°C in a thermostatically controlled incubator under a 5% CO$_2$ atmosphere, and the amount of GM-CSF (a) and G-CSF (b) released into the culture supernatant was quantified at 24 h by a sandwich ELISA. Each data point represents the mean $\pm$ S.E.M. of 19 to 29 determinations using tissue from different donors. Indomethacin (10 $\mu$M) was present in the culture medium throughout the experiment.](image-url)
detected by Western blotting in any experiment. However, 48 h after infection with Ad5.CMV.PKIa (MOI = 100), a single peptide was labeled by the anti-PKIα antibody that migrated as a 12-kDa band on SDS polyacrylamide gels (Fig. 8a). Confocal microscopy confirmed that at this time the number of PKIα cells expressed as a percentage of cells staining for the nuclear marker DAPI was greater than 90% (Fig. 8b).

HASM cells exposed to IL-1β elaborated GM-CSF and G-CSF in an amount that was not significantly altered after infection with Ad5.CMV.PKIa or Ad5.CMV.Null (MOI = 100, 48 h) (Fig. 7, a and b). 8-iso-PGE1 (10 μM) and 8-iso-PGE2 (10 μM) inhibited and enhanced, respectively, IL-1β-stimulated GM-CSF and G-CSF release by a mechanism that was prevented in cells expressing the PKIα transgene but not those infected with the empty virus (Fig. 7, a and b). Identical results were obtained with 8-Br-cAMP (Fig. 7, a and b). Furthermore, 8-iso-PGE1 and 8-iso-PGE2 promoted the phosphorylation of CREB, a well established substrate of PKA, which was prevented in HASM cells infected with Ad5.CMV.PKIa, indicating that PKA was inhibited (Fig. 9). Similar results were obtained with 8-Br-cAMP (data not shown).

Discussion

It is well established that isoprostanes can directly regulate airway smooth muscle contractility. However, only a paucity of research has focused on the possible pro- and/or anti-inflammatory potential of these novel lipids, and their

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<th>TABLE 1</th>
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<td>Effect of BW A868C on the augmentation of IL-1β-induced G-CSF release by E-ring 8-isoprostanes</td>
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<td>Values in parentheses indicate number of determinations. Adherent HASM cells were pretreated (30 min) with BW A868C (1 μM) and then for a further 5 min with 8-iso-PGE1, 8-iso-PGE2, or vehicle. IL-1β (10 pg/ml) was then added to the cells and the G-CSF released into the culture medium was quantified at 24 h by a sandwich ELISA. See text for further details.</td>
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<td>G-CSF Released (ng/ml)</td>
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<td>Treatment</td>
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<td>IL-1β</td>
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<td>IL-1β + PGD2 (10 μM)</td>
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<td>IL-1β + 8-iso-PGE1 (100 nM)</td>
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<td>IL-1β + 8-iso-PGE2 (10 μM)</td>
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* P < 0.05, significant augmentation of IL-1β-induced G-CSF release.
ability to modify the synthetic capacity of HASM muscle is unexplored. In the present study, we describe the effect of certain E- and Fα-ring 8-isoprostanes on the secretion of GM-CSF and G-CSF from HASM cells in culture. In addition, we have also assessed the extent to which isoprostanes use prostanoid receptors to regulate CSF secretion and the possible signaling pathway(s) involved.

Two E-ring and three Fα-ring 8-isoprostanes were examined. Without exception, 8-iso-PGF1α, 8-iso-PGF2α, and 8-iso-PGF3α were without effect on the release of CSFs. In contrast, the secretion from IL-1β-stimulated HASM cells of GM-CSF and G-CSF was inhibited and enhanced, respectively, by both 8-iso-PGE1 and 8-iso-PGE2. These results were identical to those evoked by PGE2, implicating an interaction of 8-isoprostanes with prostanoid receptors of the EP-subtype. Indeed, although the two E-ring 8-isoprostanes were equipotent at enhancing G-CSF release, 8-iso-PGE1 was 10-fold less potent than 8-iso-PGE2 in suppressing the elaboration of GM-CSF. These data could relate to agonism of multiple receptor subtypes, and this possibility was addressed empirically using selective agonists and antagonists.

**GM-CSF Release**

To determine whether the EP2-receptor subtype mediated the inhibitory effect of E-ring 8-isoprostanes on GM-CSF release, AH 6809, an EP1-/EP2-/DP-receptor antagonist, was used. Clarke et al. (2004a) have established previously that neither PGD2 nor 17-phenyl-trinor PGE2 (a selective EP1-receptor agonist) has any effect on IL-1β-induced GM-CSF release from HASM cells. Therefore, AH 6809 was used in the present study as a selective EP2-receptor antagonist and produced a rightwards shift of the mean 8-iso-PGE1 and 8-iso-PGE2 concentration-response curves from which apparent pA2 values of 5.73 and 5.67 were derived, respectively. These values agree with the affinity of AH 6809 in other EP2-containing preparations, including human myometrial smooth muscle (Hillock and Crankshaw, 1999) and Chinese hamster ovary cells expressing the recombinant human form of the receptor (Woodward et al., 1995), indicating that these E-ring 8-isoprostanes suppress GM-CSF release by activating the EP2-receptor subtype. Two further pieces of evidence support this conclusion. First, 8-Br-cAMP mimicked the effect of 8-iso-PGE1 and 8-iso-PGE2, which is consistent with the positive coupling of the EP2-receptors to adenylyl cyclase. Second, L-161,982, a highly selective antagonist of the EP4-receptor subtype (Machwate et al., 2001) that can also couple to adenylyl cyclase and the activation of the cAMP/PKA cascade, was inactive. Many actions of the E-ring 8-isoprostanes have been found to be mediated by TP-receptors (Janssen, 2001; Cowley, 2003). Indeed, the spasmodic activity of 8-iso-PGE2 on HASM is insensitive to AH 6809 but blocked by the TP-receptor antagonist ICI 192,605 (Janssen et al., 2000). In the present study, SQ 29,548 at a concentration that is known to abolish TP-receptor-mediated signaling (Swayne et al., 1988), did not antagonize the inhibitory effect of 8-iso-PGE1 or 8-iso-PGE2 on GM-CSF (or G-CSF) release. Thus, in human airways, E-ring 8-isoprostanes are agonists at multiple prostanoid receptors where they can influence independently both tone and the synthetic capacity of the smooth muscle.

![Fig. 3. Effect of an EP4-receptor antagonist, L-161,982, on the inhibition and augmentation, respectively, of G-CSF and GM-CSF release from HASM cells by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with L-161,982 (2 μM) and then for a further 5 min with 8-iso-PGE1 or 8-iso-PGE2. IL-1β was then added to the cells and the GM-CSF (a and b) and G-CSF (c and d) released into the culture medium was quantified at 24 h by a sandwich ELISA. Each data point represents the mean ± S.E.M. of eight determinations using tissue from different donors. Indomethacin (10 μM) was present throughout the experiment.](molpharm.aspetjournals.org)
G-CSF Release

AH 6809 was also used to assess the role of EP<sub>2</sub>-receptors in the enhancement by E-ring 8-isoprostanes of G-CSF secretion from HASM cells. However, on this functional output, AH 6809 was inactive. Because 8-Br-cAMP mimicked the behavior of 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub>, it was reasoned that these isoprostanes may also be agonists at additional prostanoid receptors that can couple positively to adenyl cyclase. Of the four known EP-receptor subtypes, agonism of the EP<sub>2</sub>- and certain splice variants of the EP<sub>3</sub>-receptor can enhance cAMP synthesis (Breyer et al., 2001). Sulprostone was very weak at enhancing G-CSF secretion from IL-1β-stimulated HASM cells, suggesting that the EP<sub>3</sub>-subtype is unlikely to mediate the actions of 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub> reported herein. Likewise, despite coupling to adenyl cyclase in many circumstances, the EP<sub>2</sub>-receptor subtype was also excluded on the basis that a highly selective antagonist L-161,982 (Machwate et al., 2001) did not suppress the facilitation by 8-iso-PGE<sub>1</sub> or 8-iso-PGE<sub>2</sub> of G-CSF release. However, given that 8-Br-cAMP enhanced G-CSF output, the role of adenylyl cyclase-coupled EP-receptors was investigated in more detail. In particular, the effect of blocking EP<sub>2</sub>- and EP<sub>4</sub>-receptors concurrently was investigated. Precedents for performing this experiment were derived from several studies, including those of Fukuroda and colleagues, who demonstrated that endothelin (ET)-1-induced contractions of human bronchus and rabbit pulmonary artery were insensitive to selective ET<sub>A</sub>- and ET<sub>B</sub>-receptor antagonists, whereas a significant inhibition was seen when the antagonists were used in combination (Fukuroda et al., 1994, 1996). In the present investigation, the mean 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub> concentration-response curves that described the augmentation of G-CSF release from HASM cells were not significantly affected by either AH 6809 or L-161,982 (although G-CSF output evoked by an EP<sub>4</sub>-receptor agonist, PGE<sub>2</sub>-OH, was antagonized indicating that L-161,982 is active in this preparation). However, the addition of L-161,982 or AH 6809, respectively, to EP<sub>2</sub>- and EP<sub>4</sub>-blocked tissues displaced to the right the mean 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub> concentration-response curves approximately 5-fold. Thus, selective antagonism of EP<sub>2</sub>- or EP<sub>4</sub>-receptors is insufficient to suppress, detectably, the enhancement of G-CSF release evoked by E-ring 8-isoprostanes, and both subtypes need to be blocked to compromise signaling. It is noteworthy that these results are not peculiar to 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub>. On HASM cells, we have found that PGE<sub>2</sub>, butaprost (EP<sub>2</sub>-receptor agonist), and PGE<sub>2</sub>-OH also augment G-CSF release together with a number of other ligands (e.g., 16,16-dimethyl-PGE<sub>2</sub>, ONO-AE1–259, and misoprostol) that have selectivity for the EP<sub>2</sub>- and EP<sub>4</sub>-receptor subtypes (Clarke et al., 2005). Furthermore, although butaprost- and PGE<sub>2</sub>-OH-induced G-CSF secretion was antagonized by AH 6809 and L-161,982, respectively, with the expected affinity, the same response evoked by PGE<sub>2</sub>-OH was insensitive to these antagonists unless they were deployed simultaneously (Clarke et al., 2005).

In terms of receptor theory, these data may indicate that there exists on HASM cells a sufficient EP<sub>2</sub>- and EP<sub>4</sub>-receptor reserve for 8-isoprostanes at maximal response such that in the presence of AH 6809 or L-161,982 the antagonist-free prostanoid receptor population can still efficiently mediate the full effect of 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub>. However, even in the presence of “spare receptors” for E-ring 8-isoprostanes, a parallel, rightward shift of their concentration-response curves would be expected unless the receptor reserve is so large at maximal response that the antagonism cannot be

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**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>8-iso-PGE&lt;sub&gt;1&lt;/sub&gt;</th>
<th>8-iso-PGE&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (M)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (M)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6.53 ± 0.19 (9)</td>
<td>6.89 ± 0.51 (6)</td>
</tr>
<tr>
<td>AH 6809 (10 μM)</td>
<td>6.67 ± 0.24 (9)</td>
<td>7.27 ± 0.49 (6)</td>
</tr>
<tr>
<td>L-161,982 (2 μM)</td>
<td>6.30 ± 0.13 (8)</td>
<td>6.37 ± 0.16 (8)</td>
</tr>
<tr>
<td>AH 6809 + L-161,982</td>
<td>6.11 ± 0.21 (8)</td>
<td>6.27 ± 0.21 (8)</td>
</tr>
<tr>
<td>AH 6809</td>
<td>6.48 ± 0.14 (8)</td>
<td>6.58 ± 0.12 (7)</td>
</tr>
<tr>
<td>AH 6809 + L-161,982</td>
<td>5.98 ± 0.11* (8)</td>
<td>6.04 ± 0.09* (7)</td>
</tr>
<tr>
<td>L-161,982</td>
<td>6.57 ± 0.11 (8)</td>
<td>6.51 ± 0.11 (7)</td>
</tr>
<tr>
<td>L-161,982 + AH 6809</td>
<td>5.98 ± 0.10* (8)</td>
<td>6.13 ± 0.12* (7)</td>
</tr>
</tbody>
</table>

* P < 0.05, significant difference in EC<sub>50</sub> values with respect to vehicle or single antagonist-treated cells.

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**Fig. 4.** Effect of PGD<sub>2</sub>, PGE<sub>2</sub>, and the TP-receptor agonist U-46619 on CSF release from IL-1β-stimulated HASM cells. Adherent HASM cells were pretreated for 5 min with prostanoid before being exposed to IL-1β. Cells were maintained at 37°C in a thermostatically controlled incubator under a 5% CO<sub>2</sub> atmosphere, and the amount of GM-CSF (a) and G-CSF (b) released into the culture supernatant was quantified at 24 h by a sandwich ELISA. Each bar represents the mean ± S.E.M. of four determinations using tissue from different donors. Indomethacin (10 μM) was present in the culture medium throughout the experiment.
visualized. Three additional explanations could therefore account for these results.

**Intramolecular Cross-Talk between EP<sub>2</sub>- and EP<sub>4</sub>-Receptors.** Agonism of EP<sub>2</sub>-receptors may reduce the affinity of L-161,982 for the EP<sub>4</sub>-receptor subtype. This phenomenon has been demonstrated for endothelin receptor subtypes in Girardi cardiac myocytes where endothelin-1, acting through the ET<sub>α</sub>-receptor, reduces the affinity of BQ788, a selective ET<sub>β</sub>-receptor antagonist (Ozaki et al., 1997). Whether analogous signaling can occur in tissues that express multiple EP-receptor subtypes is unexplored. However, from an empirical standpoint, intramolecular cross-talk could accommodate our G-CSF data more readily given that the antagonism effected by AH 6809 and L-161,982 in combination was considerably less than would be predicted from their affinity for the human EP<sub>2</sub>- (p<sub>A2</sub> = 6) and EP<sub>4</sub>-receptor subtypes (p<sub>A2</sub> = 8.4) (Woodward et al., 1995; Machwate et al., 2001).

**Agonism of DP<sub>1</sub>- and/or IP-Receptors.** Selective antagonists at the IP-receptor have not yet been described, and so it is difficult to ascertain whether they contributed to the regulation by E-ring 8-isoprostanes of CSF output. Nonetheless, to our knowledge there are no reports that 8-iso-PGE<sub>1</sub> or 8-iso-PGE<sub>2</sub> are IP-receptor agonists in any tissue. In fact, in the GM-CSF experiments AH 6809 antagonized the inhibition by E-ring 8-isoprostanes of GM-CSF output with an apparent p<sub>A2</sub> of ~6, which is consistent with an EP-receptor-mediated process. Furthermore, the K<sub>i</sub> of PGE<sub>2</sub>, which is isomeric with 8-iso-PGE<sub>2</sub>, for the human cloned IP-receptor is approximately 2000 and 12,500 times lower than its affinity at the EP<sub>2</sub>- and EP<sub>4</sub>-receptor subtypes, respectively (Breyer et al., 2001), and it is known that chiral inversion at the C8 position can further reduce potency of PGE<sub>2</sub> for prostanoid receptors (Ungrin et al., 2001). Thus, on balance, modulation of CSF release by E-ring 8-isoprostanes is unlikely to involve agonism of the IP-receptor subtype.

A role for Gs-coupled DP<sub>1</sub>-receptors in mediating the excitatory actions of E-ring 8-isoprostanes was also excluded on the basis that 8-iso-PGE<sub>1</sub>, 8-iso-PGE<sub>2</sub> as well as PGE<sub>2</sub> enhanced IL-1β-induced G-CSF release by a mechanism that was insensitive to the highly selective DP<sub>1</sub>-receptor antagonist BW A868C.

**Agonism of Novel “Isoprostane” Receptors.** 8-iso-PGE<sub>1</sub> and 8-iso-PGE<sub>2</sub> may act, at least in part, through a novel isoprostane receptor for which some evidence is available (Janssen, 2001). Again, this explanation could account for the weaker than expected antagonism of isoprostanes-induced G-CSF release produced by the combination of AH 6809 and L-161,982. However, as butaprost- and PGE<sub>1</sub>-OH-induced G-CSF output is antagonized by AH 6809 and L-161,982, respectively, with the expected affinity, this explanation seems unlikely (Clarke et al., 2005).

**Role of the cAMP/PKA Cascade**

Agonism of EP<sub>2</sub>- and EP<sub>4</sub>-receptors evokes many responses that are thought to rely exclusively on the activation of the cAMP/PKA cascade (Breyer et al., 2001). However, in many biological systems persuasive evidence implicating this signaling pathway is difficult to obtain because several commonly used compounds marketed as PKA inhibitors such as H89 (N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline) are isoquinoelinesulfonamides and are nonselective (Davies et al., 2000; Meja et al., 2004), presumably because they block a conserved ATP-binding site found among many protein kinases (Engh et al., 1996). The limitations of small molecule kinase inhibitors prompted us to establish the role of PKA in the regulation of CSF generation by E-ring 8-isoprostanes using PKI, an endog-

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**Fig. 5.** Effect of a TP-receptor antagonist, SQ 29,548, on the inhibition and augmentation, respectively, of G-CSF and GM-CSF release from HASM cells by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with SQ 29,548 (1 μM) and then for a further 5 min with 8-iso-PGE<sub>1</sub> or 8-iso-PGE<sub>2</sub>. IL-1β was then added to the cells and the GM-CSF (a and b) and G-CSF (c and d) released into the culture medium was quantified at 24 h by a sandwich ELISA. Each data point represents the mean ± S.E.M. of three determinations using tissue from different donors. Indomethacin (10 μM) was present throughout the experiment.
Fig. 6. Effect of dual blockade of EP2- and EP4-receptors on the augmentation of IL-1β-induced G-CSF evoked by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with AH 6809 (10 μM) or L-191,982 (2 μM) alone and in combination and then for a further 5 min with 8-iso-PGE1 (a and b) and 8-iso-PGE2 (c and d). IL-1β (10 pg/ml) was then added to the cells and the G-CSF released into the culture medium was quantified at 24 h by a sandwich ELISA. Each data point represents the mean ± S.E.M. of eight and seven determinations for using tissue from with 8-iso-PGE1 and 8-iso-PGE2, respectively. Indomethacin (10 μM) was present throughout the experiment.

Fig. 7. Effect of PKIα on the regulation by 8-Br-cAMP and E-ring 8-isoprostanes of GM-CSF and G-CSF secretion from HASM cells. Adherent cells were infected with Ad5.CMV.Null or Ad5.CMV.PKIα (MOI = 100, 48 h). 8-Br-cAMP (1 mM), 8-iso-PGE1 (10 μM), or 8-iso-PGE2 (10 μM) was then added for 30 min. At this point, cells were exposed to IL-1β for 24 h to promote GM-CSF (a) and G-CSF (b) release, which was measured by ELISA. Each bar represents the mean ± S.E.M. of four independent determinations using tissue from different donors. Indomethacin (10 μM) was present throughout the experiment. *, P < 0.05, significant inhibition of IL-1β-stimulated GM-CSF release. **, P < 0.05, significant augmentation in G-CSF release over the effect of IL-1β alone.
enous, potent ($K_i = 50–100$ pM) and highly selective inhibitor of PKA (Olsen and Uhler, 1991; Scarpetta and Uhler, 1993; Collins and Uhler, 1997). Indeed, PKIα at a concentration $\sim 10^6$ times higher than its affinity for PKA fails to inhibit the highly homologous cGMP-dependent protein kinase (Glass et al., 1986).

Methods

Fig. 8. Expression of PKIα in HASM cells infected with Ad5.CMV.PKIα. Adherent cells were cultured until 50% confluent and then infected with Ad5.CMV.PKIα (MOI = 100) or left untreated (naive) for 48 h at 37°C. Cells were growth arrested in serum-free medium and processed for PKIα expression by Western blotting (a) and confocal microscopy (b). Data are representative of three independent determinations. See Materials and Methods for further details.

Fig. 9. Effect of PKIα on 8-Br-cAMP- and E-ring 8-isoprostane-induced CREB phosphorylation in HASM cells. Adherent cells were infected with Ad5.CMV.Null or Ad5.CVM.PKIα (MOI = 100, 48 h). 8-Br-cAMP (1 mM), 8-iso-PGE1, (10 μM), or 8-iso-PGE2 (10 μM) was added for 30 min, and the cells were processed for CREB phosphorylation and β-actin content (to confirm equal loading) by Western blotting. Data are representative of three independent determinations. Indomethacin (10 μM) was present throughout the experiment. Lanes 1 and 2, naive; lanes 3 and 4, Ad5.CMV.Null; and lanes 5 and 6, Ad5.CMV.PKIα.

to which it is most closely related (Takio et al., 1984). To this end, HASM cells were infected with an adenovirus vector encoding the complete amino acid sequence of PKIα. Under these conditions, CREB phosphorylation and the modulation CSF release evoked by 8-iso-PGE1 and 8-iso-PGE2 were abolished in HASM cells expressing PKIα, indicating that the CSF2 and CSF3 genes are both regulated, directly or indirectly, by PKA. It is noteworthy that the EP4 receptor can also couple via Gα to the activation of the phosphatidylinositol 3-kinase/protein kinase Bα pathway independently of cAMP and PKA (Fujino and Regan, 2003). However, as discussed above, the participation of this novel signaling pathway seems unlikely given that the modulation of CSF output elicited by the two E-ring isoprostanes was mimicked by 8-Br-cAMP and abolished by PKIα (Glass et al., 1986; Scarpetta and Uhler, 1993; Collins and Uhler, 1997).

Divergent Signaling

IL-1β is known to activate multiple mitogen-activated protein kinase signaling cascades and transcription factors in HASM cells. However, the critical elements required for CSF release and the divergent modulation of those responses by the cAMP/PKA pathway are not fully understood. cAMP can reduce GM-CSF output from many cells by repressing the CSF2 gene, and this mechanism could satisfactorily account for the data reported herein. In contrast, the enhancement of G-CSF output by cAMP has not been studied and the mechanism is unknown. Nevertheless, our data are reminiscent of the ability of cAMP-elevating agents to enhance IL-6 generation from tumor necrosis factor-α-stimulated HASM cells (Ammit et al., 2000). In that investigation, elevation of the cAMP content was associated with an increase in IL-6 mRNA transcripts over that produced by tumor necrosis factor-α alone (Ammit et al., 2000), indicating that PKA may enhance the expression of this and possibly other genes, such as CSF3, through stabilization of existing mRNA transcripts. Indeed, the inability of E-ring 8-isoprostanes to promote G-CSF release in the absence of IL-1β is consistent with post-transcriptional mechanisms involving mRNA stabilization.

Summary and Conclusions.

The results of the present investigation demonstrate that E-ring 8-isoprostanes regulate positively and negatively the secretion of G-CSF and GM-CSF, respectively, from HASM cells by mechanisms that rely upon the activation of the cAMP/PKA cascade. However, although suppression of IL-1β-induced GM-CSF secretion by 8-iso-PGE1 and 8-iso-PGE2 was mediated exclusively by prostanoid receptors of the EP3 subtype, studies with selective antagonists revealed that blockade of both the EP2- and EP4-receptors concurrently was necessary to antagonize the facilitation by E-series isoprostanes of G-CSF output. The biological significance of this divergent regulation of CSF release by cAMP remains to be established.

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References


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