

E-Ring 8-Isoprostanes Are Agonists at EP₂- and EP₄-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 α -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-1 β (IL-1 β). The elaboration of GM-CSF and G-CSF by IL-1 β was inhibited and augmented, respectively, in a concentration-dependent manner by 8-iso-prostaglandin (PG) E₁ and 8-iso-PGE₂, but not by 8-iso-PGF_{1 α} , 8-iso-PGF_{2 α} , and 8-iso-PGF_{3 α} . AH 6809 (6-isopropoxy-9-oxoxanthine-2-carboxylic acid), an EP₁-/EP₂-/DP-receptor blocking drug, antagonized the inhibitory effect of 8-iso-PGE₁ and 8-iso-PGE₂ on GM-CSF output with an affinity consistent with an interaction at prostanoid receptors of the EP₂-subtype. In contrast, the facilitation by 8-iso-PGE₁ and 8-iso-

PGE₂ of G-CSF release was unaffected by AH 6809 and the selective EP₄-receptor antagonist L-161,982 [4'-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-dihydro-[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-PGE₁ and 8-iso-PGE₂ 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-PGE₁ and 8-iso-PGE₂ act solely via EP₂-receptors to inhibit GM-CSF release, whereas both EP₂- and EP₄-receptor subtypes positively regulate G-CSF output.

The isoprostanes embody a vast family of novel prostaglandin-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 forma-

tion 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). Nev-

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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(Z)-6-(2-o-chlorophenyl-4-o-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid; BQ 788, *N-cis*-2,6-dimethylpi-peridinocarbonyl-L- γ -methylleucyl-D-1-methoxycarbonyl tryptophanyl-D-norleucine.

ertheless, isoprostanes are isomeric with prostaglandins and have been given the prefix D-, E-, and F α - to denote the prostane ring shared with PGD₂, PGE₂, and PGF_{2 α} 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 F α -ring isoprostanes.

Despite the bewildering number of isoprostanes that theoretically can be formed, the most extensively studied compounds are the 8-isoprostanes, in particular, 8-iso-PGF_{2 α} and 8-iso-PGE₂ (Janssen, 2001). With respect to respiratory diseases 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 disease compared with normal healthy subjects (Kharitonov and Barnes, 2002; Morrow and Roberts, 2002). Indeed, it has been proposed that 8-isoprostanes may be biomarkers of airway inflammation and that their level of expression relates to disease severity (Kharitonov 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 neurotransmission (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 advanced (Janssen, 2001). Five classes of G protein-coupled receptor for the naturally occurring prostanoid agonists have been defined and given the prefix DP, EP, FP, IP, and TP, where the first letter refers to the natural ligand most selective for that receptor. Molecular biological techniques subsequently confirmed this pharmacological classification with the cloning and expression of cDNAs for representatives of the five prostanoid receptors in a number of species, including humans (Coleman et al., 1994; Breyer et al., 2001). In addition, four subtypes of the EP-receptor, denoted EP₁₋₄, and multiple spliced variants of the EP₃-subtype can also be derived (Breyer et al., 2001). Despite being isomeric with the prostaglandins, D-, E-, and F α -ring 8-isoprostanes are not invariably agonists at prostanoid receptors of the DP-, EP-, or FP-receptor subtypes, respectively.

To date, the majority of investigations have, almost exclusively, examined the effect of isoprostanes on smooth muscle tone. Herein, we describe an anti- and proinflammatory 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-PGE₁ and 8-iso-PGE₂ has been compared with F α -ring isoprostanes on the secretion of two colony-stimulating factors (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, discovered 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 obstructive pulmonary disease where they enhance in the airways 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 MgSO₄, 0.4 mM NaH₂PO₄·7H₂O, 1.3 mM CaCl₂·2H₂O, 4.2 mM NaHCO₃, and 5.6 mM glucose) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B. The smooth muscle layer was dissected 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% CO₂/air in HBSS containing 10 mg/ml BSA, collagenase (type XI, 1 mg/ml), and elastase (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 elastase 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, 1 \times 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 cm²) containing 6 ml of supplemented DMEM and allowed to adhere (~12 h) at 37°C in 5% CO₂/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” appearance 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 experiments, respectively. At subconfluence, the cells were growth arrested by being placed in DMEM containing apotransferrin (5 μ g/ml), insulin (1 μ M), ascorbate (100 μ M), 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.PKI α . In some experiments, subconfluent, growth-arrested HASM cells were infected (MOI = 100) with an E1[−]/E3[−] replication-deficient adenovirus vector (Ad5.CMV.PKI α) containing a 251-base pair DNA fragment encoding the complete amino acid sequence of the α -isoform of cAMP-dependent protein kinase inhibitor (PKI α) 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, expressing no transgene, was used in parallel. Preliminary experiments established that infection with Ad5.CMV.PKI α 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 PKI α 48 h after infection. This was determined by

enumerating the number of HASM cells expressing the transgene as a percentage of total number of cells that counterstained with the nuclear marker DAPI.

Staining and Visualization of PKI α . Naive and Ad5-infected HASM cells were grown on glass coverslips placed in six-well tissue culture plates containing supplemented DMEM and kept at 37°C until semiconfluent. Cells were washed free of drug and fixed in formaldehyde [4% (v/v) in PBS]. The fixative was decanted and cells were permeabilized with Nonidet P-40 [0.5% (v/v) in PBS]. After rehydration in glycine (100 mM in PBS), HASM cells were "blocked" by immersion in buffer [0.5% (v/v) BSA, 0.1% (v/v) gelatin in PBS] followed by overnight incubation at 4°C with a goat anti-human antibody directed against PKI α . Unbound antibody was washed away and a biotinylated swine anti-rabbit IgG secondary antibody was added for 60 min at room temperature. After a further wash in the same buffer, fluorescein-conjugated streptavidin (diluted 1:100) was added to the cells for a further 60 min. The cells were washed again and counterstained with the nuclear marker DAPI (1 μ g/ml, 5 min). The subcellular localization of PKI α was then visualized by laser scanning confocal microscopy.

Measurement of GM-CSF and G-CSF. HASM cells (naive and virus-infected) were pretreated for 30 min with indomethacin (10 μ M), and, where indicated, receptor antagonist(s) before being exposed for a further 5 or 30 min to prostanoid or 8-Br-cAMP, respectively. IL-1 β was then added (10 pg/ml for G-CSF, \sim EC₄₀; 100 pg/ml for GM-CSF, \sim EC₅₀), and the cells were incubated at 37°C in a thermostatically controlled incubator under a 5% CO₂ atmosphere. At 24 h, the amount of GM-CSF and G-CSF released into the culture supernatant was quantified by sandwich ELISA (Human DuoSet Development System; R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions. The detection limit of these assays are 7.8 and 15.6 pg/ml for GM-CSF and G-CSF, respectively.

Western Blot Analysis. HASM cells were treated with 3% FCS for 24 h. The medium was removed and the cells were washed with HBSS, lysed, and proteins extracted in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 0.05% (w/v) sodium deoxycholate, 0.025% (w/v) SDS, and 0.1% (v/v) Triton X-100 supplemented with phenylmethylsulfonyl fluoride (0.1 mg/ml), leupeptin (10 μ g/ml), and aprotinin (25 μ g/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) bromophenol blue] and boiled for 5 min. Denatured proteins (25 μ g) 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 TBT-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, PKI α , 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-1 β was from R & D Systems; DMEM and HBSS were from Flow Laboratories (Rickmansworth, Hert-

fordshire, 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-oxoxanthine-2-carboxylic acid), SQ 29,548 (1S-[1 α ,2 α (Z),3 α ,4 α]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid), BW A868C [3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino) hydantoin], PGD₂, PGE₂, U-46619 (9,11-dideoxy-11 α ,9 α -methanoepoxy-prostaglandin F_{2 α}), and all isoprostanes were obtained from Cayman Chemical (Ann Arbor, MI). L-161,982 ([4'-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-dihydro-[1,2,4] triazol-4-ylmethyl]-biphenyl-2-sulfonic acid (3-methyl-thiophene-2-carbonyl)-amide] was donated by Merck Frosst (Montreal, Canada). Goat anti-human PKI α (code sc1944) and goat anti-human β -actin (code sc1615) were from Autogen Bioclear (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 \pm 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 pEC₅₀ or pIC₅₀ values were subsequently interpolated from curves of best fit.

Estimates of antagonist affinity were calculated using the equation $pK_B = \log (CR - 1) - \log [B]$ as described by Schild (1949), where CR is the concentration ratio calculated from the EC₅₀ of agonist in the presence of the antagonist divided by the EC₅₀ of the agonist alone, K_B is the equilibrium dissociation constant, and [B] is the concentration of antagonist. In the experiments described herein, the term apparent pA₂ is substituted for pK_B as antagonists were used at one concentration only, which precludes assumptions being made about the nature of the antagonism.

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

Effect of 8-Isoprostanes on IL-1 β -Induced GM-CSF and G-CSF Release. None of the E- or F α -ring 8-isoprostanes examined in the present study elaborated GM-CSF or G-CSF from HASM cells. However, pretreatment of cells with 8-iso-PGE₁ and 8-iso-PGE₂ suppressed the ability of IL-1 β to generate GM-CSF with >80% inhibition achieved at the highest concentration (10 μ M) tested (IL-1 β , 1.26 \pm 0.22 ng/ml; IL-1 β + 8-iso-PGE₁, 0.29 \pm 0.04 ng/ml; IL-1 β , 1.13 \pm 0.26 ng/ml; IL-1 β + 8-iso-PGE₂, 0.15 \pm 0.08 ng/ml). This effect was concentration-dependent with $-\log$ IC₅₀ (molar) values of 7.10 \pm 0.13 (n = 29) and 7.85 \pm 0.05 (n = 19) for 8-iso-PGE₁ and 8-iso-PGE₂, respectively (Fig. 1a). These values were significantly different from one another ($P < 0.05$) with 8-iso-PGE₂ being 5.6 times more potent than 8-iso-PGE₁.

In contrast, 8-iso-PGE₁ and 8-iso-PGE₂ enhanced in a concentration-dependent manner the release of G-CSF from IL-1 β -stimulated HASM cells by between 225 to 650% at the highest concentration (10 μ M) of drug tested (IL-1 β , 1.26 \pm 0.42 pg/ml; IL-1 β + 8-iso-PGE₁, 4.78 \pm 1.46 ng/ml; IL-1 β , 2.07 \pm 0.70 ng/ml; IL-1 β + 8-iso-PGE₂, 5.92 \pm 1.41 ng/ml). This response was concentration-dependent with $-\log$ EC₅₀ (molar) values (8-iso-PGE₁ = 6.45 \pm 0.13, n = 25; 8-iso-PGE₂ = 6.71 \pm 0.54, n = 12) that were 4- to 14-fold lower than for the inhibition of GM-CSF release (Fig. 1b).

The F α -ring 8-isoprostanes 8-iso-PGF_{1 α} , 8-iso-PGF_{2 α} , and

8-iso-PGF_{3α} were without significant effect on the release of CSFs from IL-1β-stimulated HASM cells at concentrations up to 10 μM (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 EP₁-, EP₂-, and DP-receptor subtypes (Coleman et al., 1987; Keery and Lumley, 1988; Woodward et al., 1995). Pretreatment of cells with AH 6809 (10 μM) produced a parallel ~10-fold shift to the right of the mean 8-iso-PGE₁ and 8-iso-PGE₂ concentration-response curves that described the inhibition of GM-CSF release from which apparent pA₂ values of 5.73 ± 0.3 and 5.67 ± 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 EP₄-Receptor Antagonist on the Modulation of CSF Release by E-Ring 8-Isoprostanes. Pretreatment of HASM cells with the selective EP₄-receptor antagonist L-161,982, at a concentration (2 μM) that is ~500 times greater than its K_B (calculated by Schild analysis; Schild, 1949) for the human EP₄-receptor subtype expressed in human embryonic kidney 293 cells (Machwate et al., 2001), did not antagonize the effect of 8-iso-PGE₁ or 8-iso-PGE₂ on GM-CSF or G-CSF release (Fig. 3; Table 1). However, L-161,982 was pharmacologically active in this tissue and significantly (*P* < 0.05) inhibited the ability of PGE₁-OH (3 μM), a selective EP₄-receptor agonist, to augment G-CSF release (IL-1β, 1.39 ± 0.44 ng/ml; IL-1β + PGE₁-OH, 2.12 ± 0.66 ng/ml; IL-1β + L-161,982, 1.50 ± 0.42 ng/ml; IL-1β + PGE₁-OH + L-161,982, 1.36 ± 0.29 ng/ml, *n* = 3).

Effect of Prostanoid Receptor Agonists on CSF Release. PGE₂ (100 nM and 10 μM) significantly suppressed the ability of IL-1β to promote GM-CSF release from HASM cells under experimental conditions where PGD₂ and the thromboxane mimetic U-46619 were inactive (Fig. 4a). In contrast, all three prostanoids at 10 μM significantly augmented IL-1β-induced G-CSF output, although PGE₂ was the most effective (Fig. 4b).

Effect of Selective TP- and DP₁-Receptor Antagonists on the Modulation of CSF Release by E-Ring Isoprostanes. Pretreatment (30 min) of HASM cells with SQ 29,548 (1 μM) or BW A868C (1 μM), at concentrations that are ~500 and 2000 times greater than their affinity at TP- and DP₁-receptors, respectively (Swayne et al., 1988; Giles et al., 1989), failed to antagonize the effect of E-ring 8-isoprostanes [at 100 nM (~EC₄₀) and 10 μM (~EC₉₅)] on CSF secretion (Fig. 5; Table 1). The enhancement by PGD₂ (10 μM) of G-CSF release similarly was insensitive to BW A868C (Table 1).

Effect of Concurrent Antagonism of EP₂- and EP₄-Receptors on PGE₂-Induced G-CSF Release. Consistent with the data presented in Figs. 2 and 3, pretreatment (30 min) of HASM cells with AH 6809 (10 μM) or L-161,982 (2 μM) failed to antagonize the enhancement by E-ring 8-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 (~5-fold) of the mean 8-iso-PGE₁ and 8-iso-PGE₂ 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-PGE₁ and 8-iso-PGE₂ 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 mM) inhibited and augmented the elaboration by IL-1β of GM-CSF and G-CSF release, respectively (Fig. 7, a and b).

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-PGE₁, 8-iso-PGE₂, and 8-Br-cAMP on CSF release from HASM cells, a virus vector, Ad5.CMV.PKIα, encoding an endogenous, potent, and highly selective inhibitor of PKA (PKIα) was used (Glass et al., 1986; Olsen and Uhler, 1991). In uninfected cells, PKIα was not

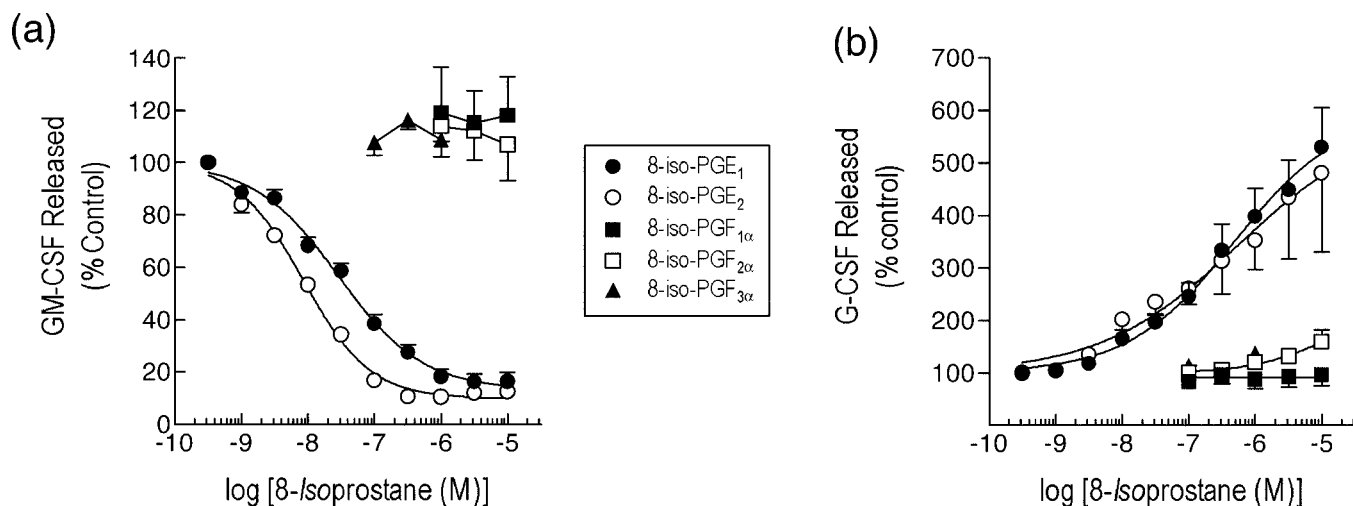


Fig. 1. Effect of E- and F_α-ring 8-isoprostanes on IL-1β-induced GM-CSF and G-CSF release. Adherent HASM cells were pretreated for 5 min with 8-isoprostane before being exposed to IL-1β. Cells were maintained at 37°C in a thermostatically controlled incubator under a 5% CO₂ atmosphere, and the amount 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 ± S.E.M. of 19 to 29 determinations using tissue from different donors. Indomethacin (10 μM) was present in the culture medium throughout the experiment.

detected by Western blotting in any experiment. However, 48 h after infection with Ad5.CMV.PKI α (MOI = 100), a single peptide was labeled by the anti-PKI α antibody that migrated as a 12-kDa band on SDS polyacrylamide gels (Fig. 8a). Confocal microscopy confirmed that at this time the number of PKI α ⁺ cells expressed as a percentage of cells staining for the nuclear marker DAPI was greater than 90% (Fig. 8b).

HASM cells exposed to IL-1 β elaborated GM-CSF and G-CSF in an amount that was not significantly altered after infection with Ad5.CMV.PKI α or Ad5.CMV.Null (MOI = 100, 48 h) (Fig. 7, a and b). 8-iso-PGE₁ (10 μ M) and 8-iso-PGE₂ (10 μ M) inhibited and enhanced, respectively, IL-1 β -stimulated GM-CSF and G-CSF release by a mechanism that was prevented in cells expressing the PKI α 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.PKI α , 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

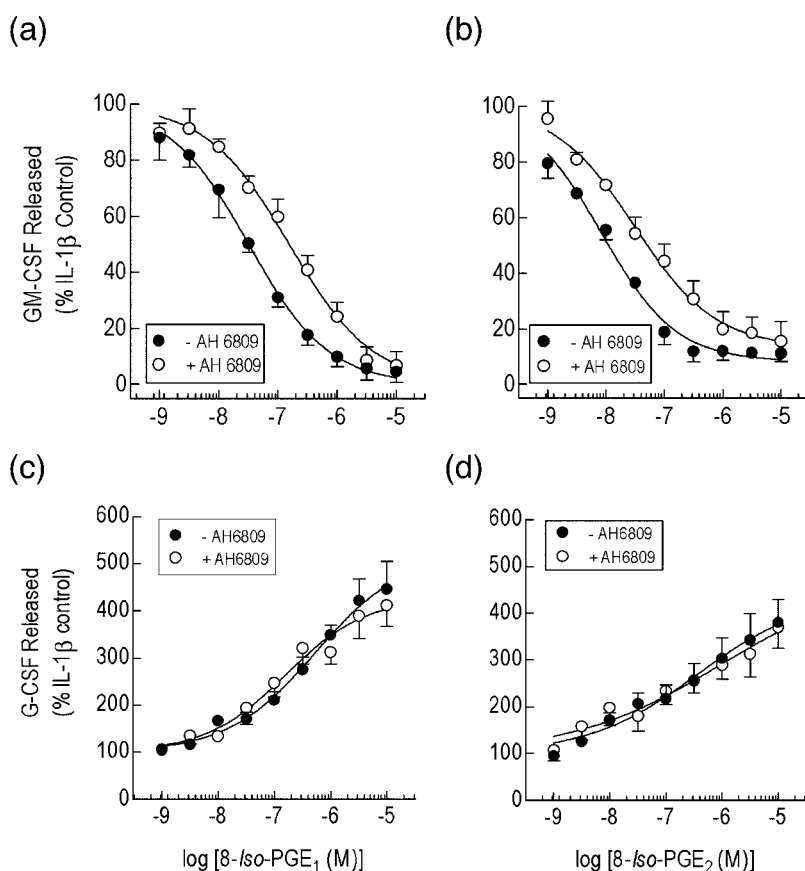


Fig. 2. Effect of AH 6809 on the inhibition and augmentation, respectively, of G-CSF and GM-CSF release from HASM cells by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with AH 6809 (10 μ M) and then for a further 5 min with 8-iso-PGE₁ or 8-iso-PGE₂. IL-1 β was then added to the 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 \pm S.E.M. of nine and six determinations for 8-iso-PGE₁ or 8-iso-PGE₂, respectively, using tissue from different donors. Indomethacin (10 μ M) was present throughout the experiment.

TABLE 1

Effect of BW A868C on the augmentation of IL-1 β -induced G-CSF release by E-ring 8-isoprostanes

Values in parentheses indicate number of determinations. Adherent HASM cells were pretreated (30 min) with BW A868C (1 μ M) and then for a further 5 min with 8-iso-PGE₁, 8-iso-PGE₂, or vehicle. IL-1 β (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.

Treatment	G-CSF Released	
	- BW A868C	+ BW A868C
	ng/ml	
IL-1 β	0.97 \pm 0.08 (3)	1.05 \pm 0.15 (3)
IL-1 β + PGD ₂ (10 μ M)	2.71 \pm 0.22* (3)	3.30 \pm 0.58* (3)
IL-1 β	0.88 \pm 0.17 (4)	0.78 \pm 0.13 (4)
IL-1 β + 8-iso-PGE ₁ (100 nM)	1.53 \pm 0.20* (4)	1.59 \pm 0.09* (4)
IL-1 β + 8-iso-PGE ₁ (10 μ M)	2.69 \pm 0.31* (4)	2.79 \pm 0.27* (4)
IL-1 β	0.88 \pm 0.17 (4)	0.78 \pm 0.13 (4)
IL-1 β + 8-iso-PGE ₂ (100 nM)	1.52 \pm 0.19* (4)	1.36 \pm 0.23* (4)
IL-1 β + 8-iso-PGE ₂ (10 μ M)	3.34 \pm 0.48* (4)	3.13 \pm 0.50* (4)

* $P < 0.05$, significant augmentation of IL-1 β -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 F α -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 F α -ring 8-isoprostanes were examined. Without exception, 8-iso-PGF $_{1\alpha}$, 8-iso-PGF $_{2\alpha}$, and 8-iso-PGF $_{3\alpha}$ were without effect on the release of CSFs. In contrast, the secretion from IL-1 β -stimulated HASM cells of GM-CSF and G-CSF was inhibited and enhanced, respectively, by both 8-iso-PGE $_1$ and 8-iso-PGE $_2$. These results were identical to those evoked by PGE $_2$, 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-PGE $_1$ was 10-fold less potent than 8-iso-PGE $_2$ 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 EP $_2$ -receptor subtype mediated the inhibitory effect of E-ring 8-isoprostanes on GM-CSF release, AH 6809, an EP $_1$ /EP $_2$ /DP-receptor antagonist, was used. Clarke et al. (2004a) have established previously that neither PGD $_2$ nor 17-phenyl- ω -trinor PGE $_2$ (a selective EP $_1$ -receptor agonist) has any effect on IL-1 β -induced GM-CSF release from HASM cells. Therefore, AH 6809 was used in the present study as a selective EP $_2$ -

receptor antagonist and produced a rightwards shift of the mean 8-iso-PGE $_1$ and 8-iso-PGE $_2$ concentration-response curves from which apparent pA $_2$ values of 5.73 and 5.67 were derived, respectively. These values agree with the affinity of AH 6809 in other EP $_2$ -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 EP $_2$ -receptor subtype. Two further pieces of evidence support this conclusion. First, 8-Br-cAMP mimicked the effect of 8-iso-PGE $_1$ and 8-iso-PGE $_2$, which is consistent with the positive coupling of the EP $_2$ -receptors to adenylyl cyclase. Second, L-161,982, a highly selective antagonist of the EP $_4$ -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 spasmogenic activity of 8-iso-PGE $_2$ 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-PGE $_1$ or 8-iso-PGE $_2$ 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.

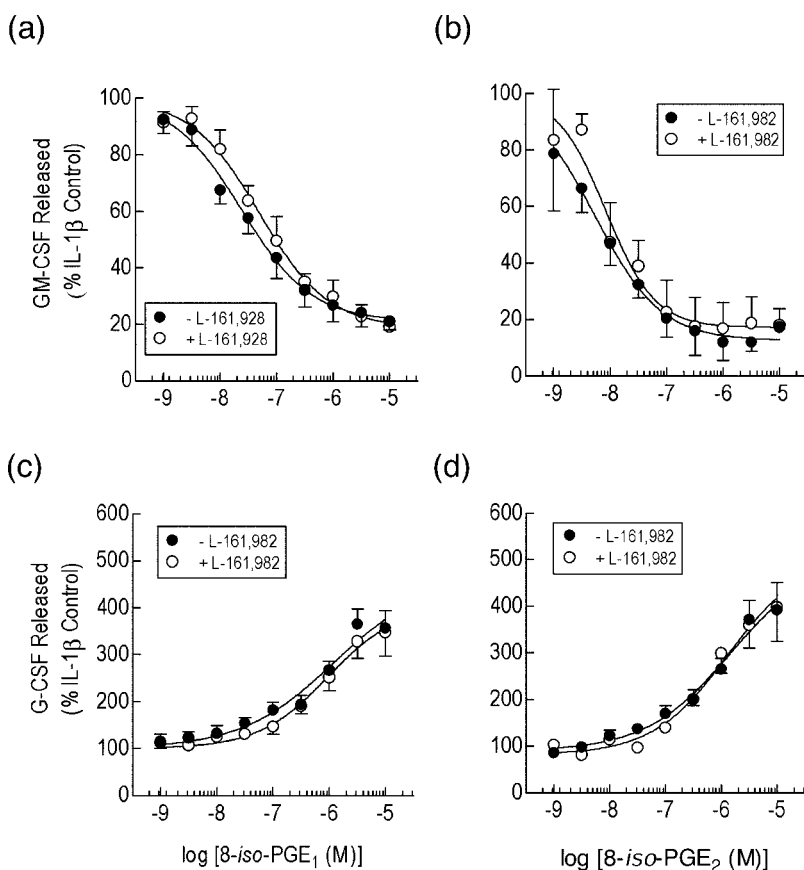


Fig. 3. Effect of an EP $_4$ -receptor antagonist, L-161,982, on the inhibition and augmentation, respectively, of G-CSF and GM-CSF release from HASM cells by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with L-161,982 (2 μ M) and then for a further 5 min with 8-iso-PGE $_1$ or 8-iso-PGE $_2$. IL-1 β 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 \pm S.E.M. of eight determinations using tissue from different donors. Indomethacin (10 μ M) was present throughout the experiment.

G-CSF Release

AH 6809 was also used to assess the role of EP₂-receptors in the enhancement by E-ring 8-isoprostanes of G-CSF secretion

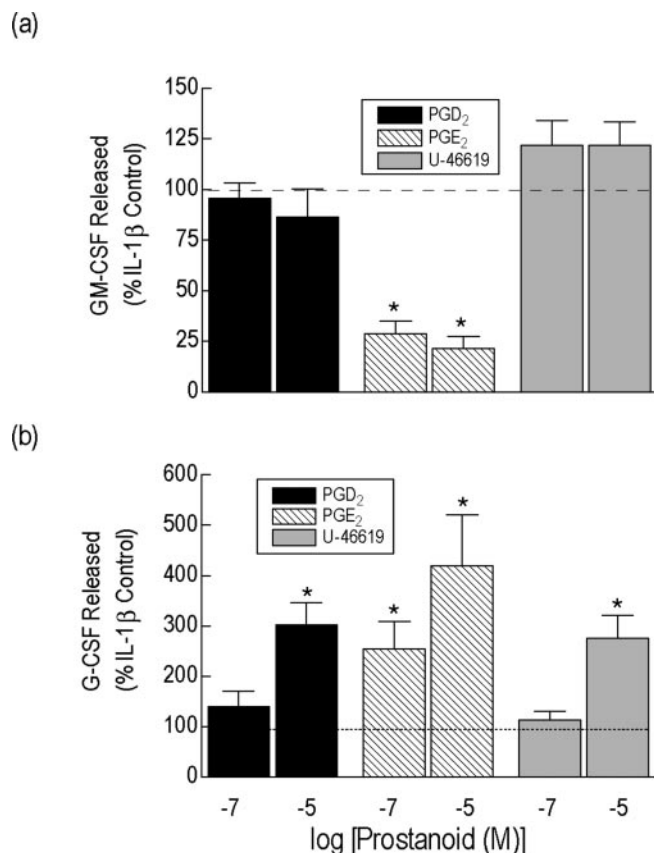


Fig. 4. Effect of PGD₂, PGE₂, and the TP-receptor agonist U-46619 on CSF release from IL-1β-stimulated HASM cells. Adherent HASM cells were pretreated for 5 min with prostanoid before being exposed to IL-1β. Cells were maintained at 37°C in a thermostatically controlled incubator under a 5% CO₂ atmosphere, and the amount of GM-CSF (a) and G-CSF (b) released into the culture supernatant was quantified at 24 h by a sandwich ELISAs. 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.

TABLE 2

Effect of AH 6809 and L-161,982 alone and in combination on the EC₅₀ values for 8-iso-PGE₁- and 8-iso-PGE₂-induced G-CSF release

Values in parentheses indicate number of determinations. Adherent HASM cells were pretreated (30 min) with AH 6809 (10 μM) or L-161,982 (2 μM) alone and in combination and then for a further 5 min with 8-iso-PGE₁ and 8-iso-PGE₂. 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.

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

* *P* < 0.05, significant difference in -log EC₅₀ values with respect to vehicle or single antagonist-treated cells.

tion from HASM cells. However, on this functional output, AH 6809 was inactive. Because 8-Br-cAMP mimicked the behavior of 8-iso-PGE₁ and 8-iso-PGE₂, it was reasoned that these isoprostanes may also be agonists at additional prostanoid receptors that can couple positively to adenylyl cyclase. Of the four known EP-receptor subtypes, agonism of the EP₄-, and certain splice variants of the EP₃-, 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 EP₃-subtype is unlikely to mediate the actions of 8-iso-PGE₁ and 8-iso-PGE₂ reported herein. Likewise, despite coupling to adenylyl cyclase in many circumstances, the EP₄-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-PGE₁ or 8-iso-PGE₂ 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 EP₂- and EP₄-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_A- and ET_B-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-PGE₁ and 8-iso-PGE₂ 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 EP₄-receptor agonist, PGE₁-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 EP₂- and EP₄-blocked tissues displaced to the right the mean 8-iso-PGE₁ and 8-iso-PGE₂ concentration-response curves approximately 5-fold. Thus, selective antagonism of EP₂- or EP₄-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-PGE₁ and 8-iso-PGE₂. On HASM cells, we have found that PGE₂, butaprost (EP₂-receptor agonist), and PGE₁-OH also augment G-CSF release together with a number of other ligands (e.g., 16,16-dimethyl-PGE₂, ONO-AE1-259, and misoprostol) that have selectivity for the EP₂- and/or EP₄-receptor subtype (Clarke et al., 2005). Furthermore, although butaprost- and PGE₁-OH-induced G-CSF secretion was antagonized by AH 6809 and L-161,982, respectively, with the expected affinity, the same response evoked by PGE₂ 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 EP₂- and EP₄-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-PGE₁ and 8-iso-PGE₂. 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

visualized. Three additional explanations could therefore account for these results.

Intramolecular Cross-Talk between EP₂- and EP₄-Receptors. Agonism of EP₂-receptors may reduce the affinity of L-161,982 for the EP₄-receptor subtype. This phenomenon has been demonstrated for endothelin receptor subtypes in Girardi cardiac myocytes where endothelin-1, acting through the ET_A-receptor, reduces the affinity of BQ788, a selective ET_B-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 EP₂- (pA₂ = 6) and EP₄-receptor subtypes (pA₂ = 8.4) (Woodward et al., 1995; Machwate et al., 2001).

Agonism of DP₁- 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 the our knowledge there are no reports that 8-iso-PGE₁ or 8-iso-PGE₂ 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 pA₂ of ~6, which is consistent with an EP₂-receptor-mediated process. Furthermore, the K_i of PGE₂, which is isomeric with 8-iso-PGE₂, for the human cloned IP-receptor is approximately 2000 and 12,500 times lower than its affinity at the EP₂- and EP₄-receptor subtypes, respectively (Breyer et al., 2001), and it is known that chiral inversion at the C8 position can further reduce potency of PGE₂ 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 DP₁-receptors in mediating the excitatory actions of E-ring 8-isoprostanes was also excluded on the basis that 8-iso-PGE₁, 8-iso-PGE₂ as well as PGD₂ enhanced IL-1β-induced G-CSF release by a mechanism that was insensitive to the highly selective DP₁-receptor antagonist BW A868C.

Agonism of Novel "Isoprostane" Receptors. 8-iso-PGE₁ and 8-iso-PGE₂ 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 PGE₁-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 EP₂- and EP₄-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 isoquinolinesulfonamides 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 PKIα, an endog-

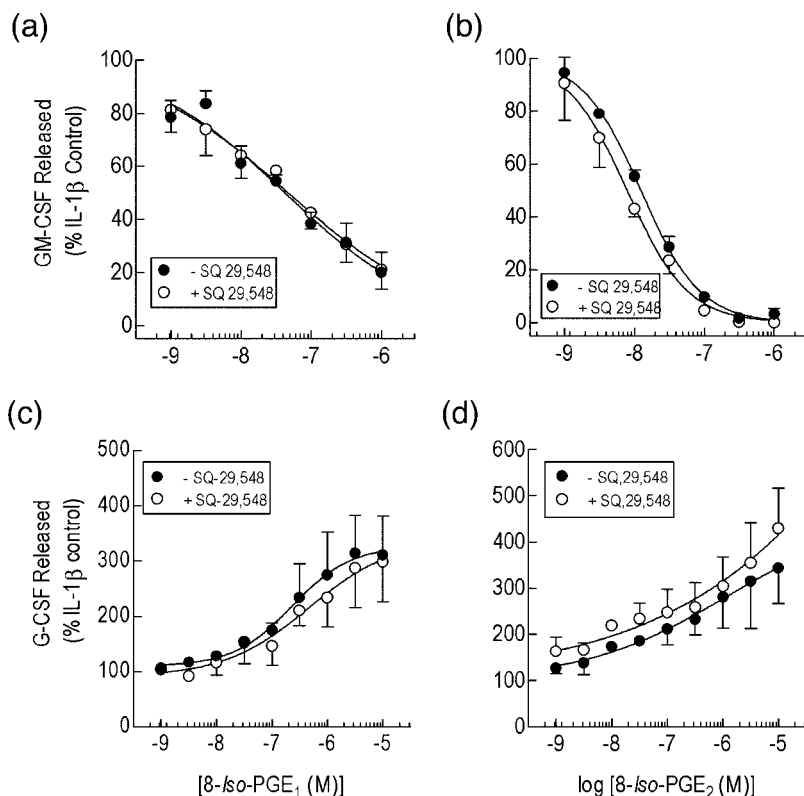


Fig. 5. Effect of a TP-receptor antagonist, SQ 29,548, on the inhibition and augmentation, respectively, of G-CSF and GM-CSF release from HASM cells by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with SQ 29,548 (1 μM) and then for a further 5 min with 8-iso-PGE₁ or 8-iso-PGE₂. 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 three determinations using tissue from different donors. Indomethacin (10 μM) was present throughout the experiment.

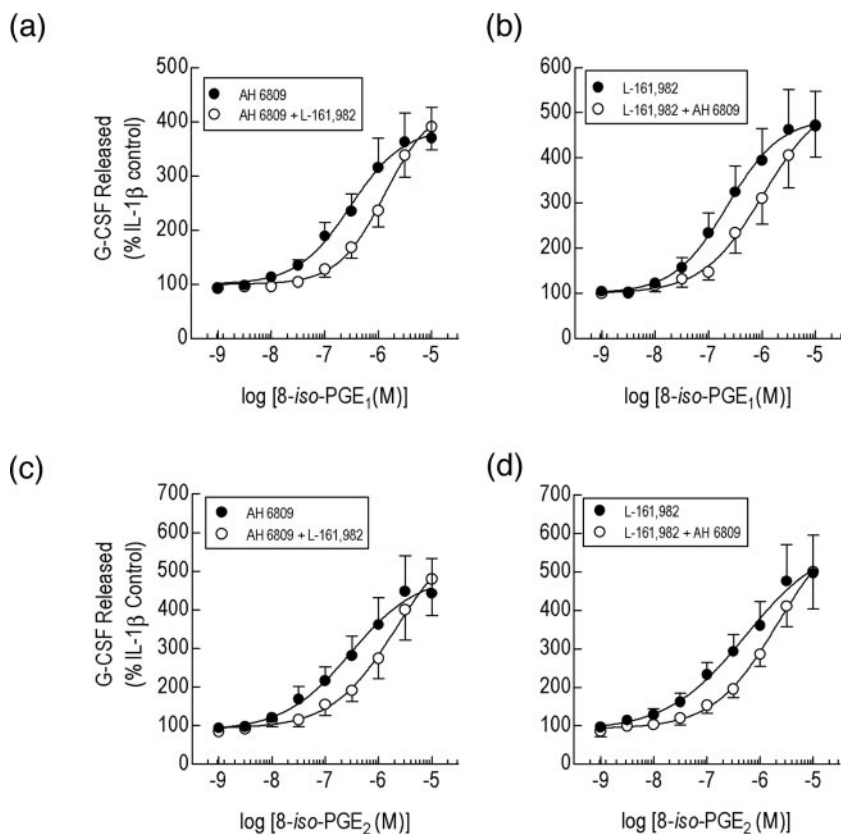


Fig. 6. Effect of dual blockade of EP₂- and EP₄-receptors on the augmentation of IL-1 β -induced G-CSF evoked by E-ring 8-isoprostanes. Adherent HASM cells were pretreated (30 min) with AH 6809 (10 μ M) or L-161,982 (2 μ M) alone and in combination and then for a further 5 min with 8-iso-PGE₁ (a and b) and 8-iso-PGE₂ (c and d). IL-1 β (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 \pm S.E.M. of eight and seven determinations for using tissue from with 8-iso-PGE₁ and 8-iso-PGE₂, respectively. Indomethacin (10 μ M) was present throughout the experiment.

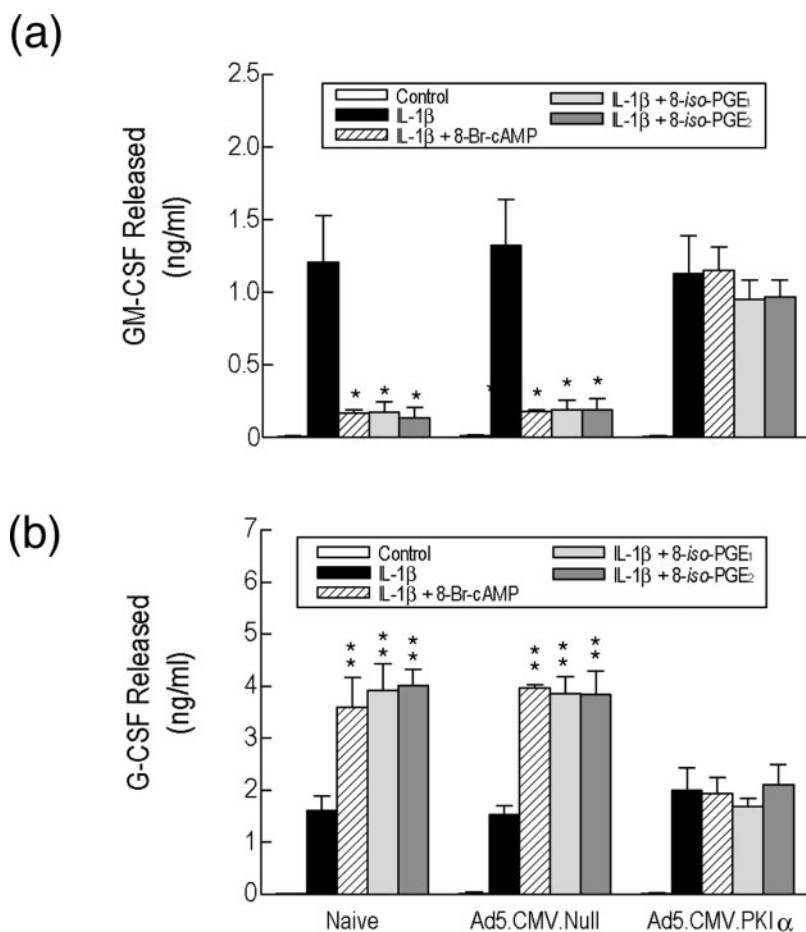


Fig. 7. Effect of PKI α on the regulation by 8-Br-cAMP and E-ring 8-isoprostanes of GM-CSF and G-CSF secretion from HASM cells. Adherent cells were infected with Ad5.CMV.Null or Ad5.CMV.PKI α (MOI = 100, 48 h). 8-Br-cAMP (1 mM), 8-iso-PGE₁ (10 μ M), or 8-iso-PGE₂ (10 μ M) was then added for 30 min. At this point, cells were exposed to IL-1 β for 24 h to promote GM-CSF (a) and G-CSF (b) release, which was measured by ELISA. Each bar represents the mean \pm S.E.M. of four independent determinations using tissue from different donors. Indomethacin (10 μ M) was present throughout the experiment. *, $P < 0.05$, significant inhibition of IL-1 β -stimulated GM-CSF release. **, $P < 0.05$, significant augmentation in G-CSF release over the effect of IL-1 β 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, PKI α 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)

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 PKI α . Under these conditions, CREB phosphorylation and the modulation CSF release evoked by 8-iso-PGE $_1$ and 8-iso-PGE $_2$ were abolished in HASM cells expressing PKI α , indicating that the CSF2 and CSF3 genes are both regulated, directly or indirectly, by PKA. It is noteworthy that the EP $_4$ -receptor can also couple via Gs to the activation of the phosphatidylinositol 3-kinase/protein kinase B α 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 PKI α (Glass et al., 1986; Scarpetta and Uhler, 1993; Collins and Uhler, 1997).

Divergent Signaling

IL-1 β 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-1 β 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-1 β -induced GM-CSF secretion by 8-iso-PGE $_1$ and 8-iso-PGE $_2$ was mediated exclusively by prostanoid receptors of the EP $_2$ -subtype, studies with selective antagonists revealed that blockade of both the EP $_2$ - and EP $_4$ -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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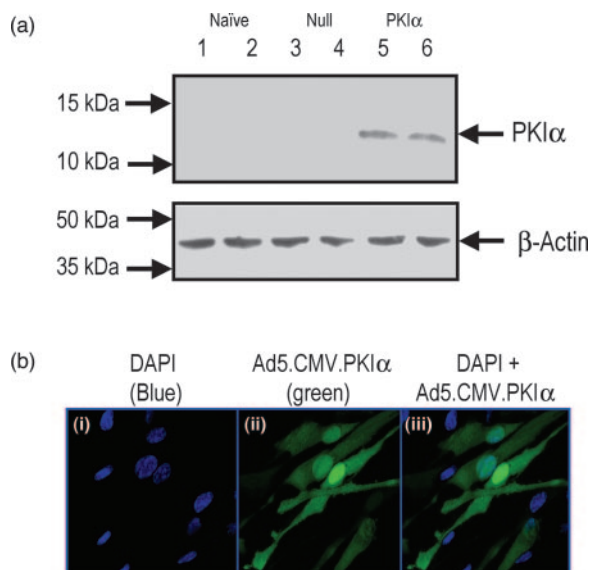


Fig. 8. Expression of PKI α in HASM cells infected with Ad5.CMV.PKI α . Adherent cells were cultured until 50% confluent and then infected with Ad5.CMV.PKI α (MOI = 100) or left untreated (naïve) for 48 h at 37°C. Cells were growth arrested in serum-free medium and processed for PKI α expression by Western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

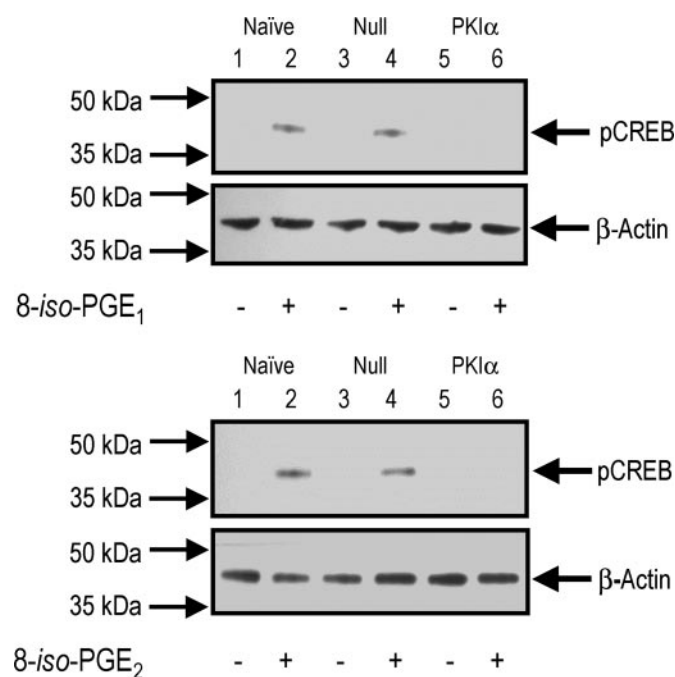


Fig. 9. Effect of PKI α on 8-Br-cAMP- and E-ring 8-isoprostane-induced CREB phosphorylation in HASM cells. Adherent cells were infected with Ad5.CMV.Null or Ad5.CMV.PKI α (MOI = 100, 48 h). 8-Br-cAMP (1 mM), 8-iso-PGE $_1$ (10 μ M), or 8-iso-PGE $_2$ (10 μ M) was added for 30 min, and the cells were processed for CREB phosphorylation and β -actin content (to confirm equal loading) by Western blotting. Data are representative of three independent determinations. Indomethacin (10 μ M) was present throughout the experiment. Lanes 1 and 2, naïve; lanes 3 and 4, Ad5.CMV.Null; and lanes 5 and 6, Ad5.CMV.PKI α .

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References

- Ammit AJ, Hoffman RK, Amrani Y, Lazaar AL, Hay DW, Torphy TJ, Penn RB, and Panettieri RA (2000) Tumor necrosis factor- α -induced secretion of RANTES and interleukin-6 from human airway smooth-muscle cells. Modulation by cyclic adenosine monophosphate. *Am J Respir Cell Mol Biol* **23**:794–802.
- Breyer RM, Bagdassarian CK, Myers SA, and Breyer MD (2001) Prostanoid receptors: subtypes and signaling. *Annu Rev Pharmacol Toxicol* **41**:661–690.
- Clarke DL, Belvisi MG, Catley MC, Yacoub MH, Newton R, and Giembycz MA (2004a) Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which PGE₂ inhibits the release of GM-CSF. *Br J Pharmacol* **141**:1141–1150.
- Clarke DL, Belvisi MG, Smith SJ, Hardacker E, Yacoub MH, Meja KK, Newton R, Slater DM, and Giembycz MA (2005) Prostanoid receptor expression by human airways smooth muscle cells and regulation of the secretion of granulocyte colony-stimulating factor. *Am J Physiol*, in press.
- Clarke DL, Giembycz MA, Patel HJ, and Belvisi MG (2004b) E-Ring 8-isoprostanes inhibit ACh release from parasympathetic nerves innervating guinea-pig trachea through agonism of prostanoid receptors of the EP₃-subtype. *Br J Pharmacol* **141**:600–609.
- Clarke DL, Patel HJ, Mitchell JA, Yacoub MH, Giembycz MA, and Belvisi MG (2001) Regulation of the release of colony-stimulating factors from human airway smooth muscle cells by prostaglandin E₂. *Am J Respir Crit Care Med* **163**:A515.
- Coleman RA, Kennedy I, and Sheldrick RL (1987) New evidence with selective agonists and antagonists for the subclassification of PGE₂-sensitive EP-receptors. *Adv Prostaglandin Thromboxane Leukotriene Res* **17A**:467–470.
- Coleman RA, Smith WL, and Narumiya S (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution and structure of the receptors and their subtypes. *Pharmacol Rev* **46**:205–229.
- Collins SP and Uhler MD (1997) Characterization of PKI γ , a novel isoform of the protein kinase inhibitor of cAMP-dependent protein kinase. *J Biol Chem* **272**:18169–18178.
- Cowley EA (2003) Isoprostane-mediated secretion from human airway epithelial cells. *Mol Pharmacol* **64**:298–307.
- Davies SP, Reddy H, Caivano M, and Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**:95–105.
- Engh RA, Girod A, Kinzel V, Huber R, and Bossemeyer D (1996) Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isouquinolinesulfonyle protein kinase inhibitors H7, H8 and H89. Structural implications for selectivity. *J Biol Chem* **271**:26157–26164.
- Fujino H and Regan JW (2003) Prostanoid receptors and phosphatidylinositol 3-kinase: a pathway to cancer? *Trends Pharmacol Sci* **24**:335–340.
- Fukuroda T, Ozaki S, Ihara M, Ishikawa K, Yano M, Miyauchi T, Ishikawa S, Onizuka M, Goto K, and Nishikibe M (1996) Necessity of dual blockade of endothelin ET_A and ET_B receptor subtypes for antagonism of endothelin-1-induced contraction in human bronchi. *Br J Pharmacol* **117**:995–999.
- Fukuroda T, Ozaki S, Ihara M, Ishikawa K, Yano M, and Nishikibe M (1994) Synergistic inhibition by BQ-123 and BQ-788 of endothelin-1-induced contractions of the rabbit pulmonary artery. *Br J Pharmacol* **113**:336–338.
- Giles H, Leff P, Bollofo ML, Kelly MG, and Robertson AD (1989) The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br J Pharmacol* **96**:291–300.
- Glass DB, Cheng HC, Kemp BE, and Walsh DA (1986) Differential and common recognition of the catalytic sites of the cGMP-dependent and cAMP-dependent protein kinases by inhibitory peptides derived from the heat-stable inhibitor protein. *J Biol Chem* **261**:12166–12171.
- Hillock CJ and Crankshaw DJ (1999) Inhibitory prostanoid EP receptors in human non-pregnant myometrium. *Eur J Pharmacol* **378**:99–108.
- Janssen LJ (2001) Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *Am J Physiol* **280**:L1067–L1082.
- Janssen LJ, Premji M, Netherton S, Catalli A, Cox G, Keshavjee S, and Crankshaw DJ (2000) Excitatory and inhibitory actions of isoprostanes in human and canine airway smooth muscle. *J Pharmacol Exp Ther* **295**:506–511.
- Kawikova I, Barnes PJ, Takahashi T, Tadjkarimi S, Yacoub MH, and Belvisi MG (1996) 8-Epi-PGF_{2 α} , a novel noncyclooxygenase-derived prostaglandin, constricts airways *in vitro*. *Am J Respir Crit Care Med* **153**:590–596.
- Keery RJ and Lumley P (1988) AH6809, a prostaglandin DP-receptor blocking drug on human platelets. *Br J Pharmacol* **94**:745–754.
- Kharitonov SA and Barnes PJ (2002) Biomarkers of some pulmonary diseases in exhaled breath. *Biomarkers* **7**:1–32.
- Liu T, Stern A, Roberts LJ, and Morrow JD (1999) The isoprostanes: novel prostaglandin-like products of the free radical-catalyzed peroxidation of arachidonic acid. *J Biomed Sci* **6**:226–235.
- Machwate M, Harada S, Leu CT, Seedor G, Labelle M, Gallant M, Hutchins S, Lachance N, Sawyer N, Slipetz D, et al. (2001) Prostaglandin receptor EP₄ mediates the bone anabolic effects of PGE₂. *Mol Pharmacol* **60**:36–41.
- Meja KK, Catley MC, Cambridge LM, Barnes PJ, Lum H, Newton R, and Giembycz MA (2004) Adenovirus-mediated delivery and expression of a cAMP-dependent protein kinase inhibitor gene to BEAS-2B epithelial cells abolishes the anti-inflammatory effects of rolipram, salbutamol and prostaglandin E₂: a comparison with H-89. *J Pharmacol Exp Ther* **309**:833–844.
- Metcalf D (1985) The granulocyte-macrophage colony-stimulating factors. *Science (Wash DC)* **229**:16–22.
- Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, and Roberts LJ (1990) A series of prostaglandin F₂-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci USA* **87**:9383–9387.
- Morrow JD and Roberts LJ (2002) The isoprostanes: their role as an index of oxidant stress status in human pulmonary disease. *Am J Respir Crit Care Med* **166**:S25–S30.
- Olsen SR and Uhler MD (1991) Isolation and characterization of cDNA clones for an inhibitor protein of cAMP-dependent protein kinase. *J Biol Chem* **266**:11158–11162.
- Ozaki S, Ohwaki K, Ihara M, Ishikawa K, and Yano M (1997) Co-expression studies with endothelin receptor subtypes indicate the existence of intracellular cross-talk between ET_A and ET_B receptors. *J Biochem* **121**:440–447.
- Scarpetta MA and Uhler MD (1993) Evidence for two additional isoforms of the endogenous protein kinase inhibitor of cAMP-dependent protein kinase in mouse. *J Biol Chem* **268**:10927–10931.
- Schild HO (1949) pA_x and competitive drug antagonism. *Br J Pharmacol Chemother* **4**:277–280.
- Spicuzza L, Barnes PJ, Di Maria GU, and Belvisi MG (2001) Effect of 8-isoprostaglandin F_{2 α} on acetylcholine release from parasympathetic nerves in guinea pig airways. *Eur J Pharmacol* **416**:231–234.
- Swayne GT, Maguire J, Dolan J, Raval P, Dane G, Greener M, and Owen DA (1988) Evidence for homogeneity of thromboxane A₂ receptor using structurally different antagonists. *Eur J Pharmacol* **152**:311–319.
- Taber DF, Morrow JD, and Roberts LJ (1997) A nomenclature system for the isoprostanes. *Prostaglandins* **53**:63–67.
- Takio K, Wade RD, Smith SB, Krebs EG, Walsh KA, and Titani K (1984) Guanosine cyclic 3',5'-phosphate dependent protein kinase, a chimeric protein homologous with two separate protein families. *Biochemistry* **23**:4207–4218.
- Ungurin MD, Carriere MC, Denis D, Lamontagne S, Sawyer N, Stocco R, Tremblay N, Metters KM, and Abramovitz M (2001) Key structural features of prostaglandin E₂ and prostanoid analogs involved in binding and activation of the human EP₁ prostanoid receptor. *Mol Pharmacol* **59**:1446–1456.
- Woodward DF, Pepper DJ, Burkey TH, and Regan JW (1995) 6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809), a human EP₂ receptor antagonist. *Biochem Pharmacol* **50**:1731–1733.
- Zahler S and Becker BF (1999) Indirect enhancement of neutrophil activity and adhesion to cultured human umbilical vein endothelial cells by isoprostanes (iPF_{2 α} -III and iPE₂-III). *Prostaglandins Other Lipid Mediat* **57**:319–331.

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