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Characterization of the prostanoid receptor(s) on human blood monocytes at which prostaglandin E_2 inhibits lipopolysaccharide-induced tumour necrosis factor-α generation

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1 The prostanoid receptor(s) that mediates inhibition of bacterial lipopolysaccharide (LPS)-induced tumour necrosis factor-α (TNFα) generation from human peripheral blood monocytes was classified by use of naturally occurring and synthetic prostanoid agonists and antagonists.

2 In human monocytes that were adherent to plastic, neither prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) nor the stable prostacyclin and thromboxane mimetics, cicaprost and U-46619, respectively, promoted the elaboration of TNFα-like immunoreactivity, as assessed with a specific ELISA, indicating the absence of excitatory prostanoid receptors on these cells.

3 Exposure of human monocytes to LPS (3 ng ml^{-1}, $\sim EC_{84}$) resulted in a time-dependent elaboration of TNFα which was suppressed in cells pretreated with prostaglandin E_1 (PGE_1), PGE_2 and cicaprost. This effect was concentration-dependent with mean pIC_{50} values of 7.14, 7.34 and 8.00 for PGE_1, PGE_2 and cicaprost, respectively. PGD_2, $PGF_{2\alpha}$ and U-46619 failed to inhibit the generation of TNFα at concentrations up to 10 μM.

4 With respect to PGE_2, the EP-receptor agonists, 16,16-dimethyl PGE_2 (non-selective), misoprostol (EP_2/EP_3-selective), 11-deoxy PGE_1 (EP_2-selective) and butaprost (EP_2-selective) were essentially full agonists as inhibitors of LPS-induced TNFα generation with mean pIC_{50} values of 6.21, 6.02, 5.67 and 5.59, respectively. In contrast to the results obtained with butaprost and 11-deoxy PGE_1, another EP_2-selective agonist, AH 13205, inhibited TNFα generation by only 21% at the highest concentration (10 μM) examined. EP-receptor agonists which have selectivity for the EP_1- (17-phenyl-ω-trinor PGE_2) and EP_3-receptor (MB 28,767, sulprostone) were inactive or only weakly active as inhibitors of TNFα generation.

5 Pretreatment of human monocytes with the TP/EP_4-receptor antagonist, AH 23848B, at 10, 30 and 100 μM suppressed LPS-induced TNFα generation by 10%, 28% and 77%, respectively, but failed to shift significantly the location of the PGE_2 concentration-response curves.

6 Given that AH 13205 was a poor inhibitor of TNFα generation, studies were performed to determine if it was a partial agonist and whether it could antagonize the inhibitory effect of PGE_2. Pretreatment of human monocytes with 10 and 30 μM AH 13205 inhibited the generation of TNFα by 31% and 53%, respectively, but failed to shift significantly the location of the PGE_2 concentration-response curves at either concentration examined.

7 Since PGD_2 and 17-phenyl-ω-trinor PGE_2 (EP_1-agonist) did not suppress TNFα generation, the EP_1/EP_2/DP-receptor antagonist, AH 6809, was employed to assess if EP_2-receptors mediated the inhibitory effect of PGE_2. Pretreatment of human monocytes with 10 μM AH 6809 did not affect LPS-induced TNFα generation but produced a parallel 3.5 fold rightwards shift of the PGE_2 concentration-response curve.

8 Collectively, these data suggest that human peripheral blood monocytes express at least two distinct populations of inhibitory prostanoid receptors that mediate inhibition of LPS-induced TNFα generation. One of these probably represents IP receptors based upon the selectivity of cicaprost for this subtype. The other population has the pharmacology of EP-receptors, but the rank order of potency for a range of synthetic EP-receptor agonists was inconsistent with an interaction with any of the currently defined subtypes. Given the pharmacological behaviour of butaprost, AH 6809 and AH 23848B in these cells, we propose that multiple (EP_2- and/or EP_4- and/or IP) or novel EP-receptors mediate the inhibitory effect of PGE_2 on TNFα generation.

Keywords: Human monocytes; EP_2-receptors; EP_4-receptors; TNFα generation; cicaprost; IP-receptors; butaprost

Introduction

Elegant studies performed over the last 15 years (for example see Armstrong *et al*., 1983; Jones *et al*., 1985; Dong *et al*., 1986; and reviews by Kennedy *et al*., 1982; Coleman *et al*., 1984; 1994b) have provided pharmacological evidence for five main classes of receptor for the naturally occurring prostanoid agonists. These receptors have been given the prefix DP-, EP-, FP-, IP- and TP- and belong to the G-protein-coupled receptor superfamily with seven transmembrane-spanning domains. Due to the lack of selective antagonists, this taxonomy was formulated predominantly from rank orders of agonist potency obtained in various pharmacological preparations, where each prostanoid is at least one order of magnitude more potent than the others at a specific prostanoid receptor. Molecular biological techniques have recently confirmed this pharmacological classification, with the cloning and expression of cDNAs for representatives of the five prostanoid receptors in a number of species including man (Hirata *et al*., 1991; Abramovitz *et al*., 1994; Boie *et al*., 1994; 1995; Jinhong *et al*., 1994; Kunapuli *et al*., 1994; Regan *et al*., 1994a,b).

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We have recently shown (Seldon *et al*., 1995) that prostaglandin E_2 (PGE_2) inhibits the induction of the tumour necrosis factor-α (TNFα) gene in human peripheral blood monocytes in response to bacterial lipopolysaccharide (LPS), suggesting that repression of this gene may be mediated by prostanoid receptors of the EP-subtype. Currently, pharmacological evidence and primary sequence information of partial and full length cDNA clones indicates the presence of at least four EP-receptor variants (see Coleman *et al*., 1994b). EP_1-receptors generally mediate contraction of smooth muscle and are coupled to the hydrolysis of inositol phospholipids with subsequent Ca^{2+} - mobilization via the G_q/G_{11} class of pertussis toxin-insensitive G-proteins (Funk *et al*., 1993; Watabe *et al*., 1993; Coleman *et al*., 1994b). In contrast, prostanoid receptors of the EP_2-subtype are positively coupled to adenylyl cyclase via Gs and mediate relaxation of a variety of smooth muscle preparations (Nials *et al*., 1993; Coleman *et al*., 1994b). The EP_3-receptors comprise a family of similar but structurally distinct proteins of which at least four variants can theoretically be derived from the same gene (Regan *et al*., 1994a; Kotani *et al*., 1995). This heterogeneity presumably explains the fact that EP_3 receptors subserve a variety of functions including inhibition of autonomic neurotransmission (Mantelli *et al*., 1991; Molderings *et al*., 1994), smooth muscle contraction (Qian *et al*., 1994), and inhibition of lipolysis (Strong *et al*., 1992) and water reabsorption (Sonnenberg & Smith, 1988). Indeed, transfection experiments of the cDNAs encoding these EP_3-variants into Chinese hamster ovary (CHO) cells revealed that they can couple to at least three second messenger systems including the stimulation of phospholipase C (via G_q/G_{11}), and the activation (G_s) and inhibition (G_i/G_o) of adenylyl cyclase (Namba *et al*., 1993; An *et al*., 1994; Kotani *et al*., 1995). Recently, the existence of a novel EP-receptor subtype (denoted EP_4) was originally proposed from studies performed in piglet saphenous vein where the pharmacological behaviour of prostanoid agonists and antagonists did not resemble an interaction with the other defined EP-receptor subtypes (Coleman *et al*., 1994a). Similar data were obtained subsequently for a number of other tissues (see Coleman *et al*., 1994b) including rabbit jugular vein (Milne *et al*., 1995), rabbit saphenous vein (Lydford *et al*., 1996) and rat trachea (Lydford & McKechnie, 1994) suggesting that the EP_4-receptor might be distributed ubiquitously. Unequivocal evidence for a novel human and murine EP-receptor subtype has come from cloning and expression studies, which have identified a receptor that has little homology with existing EP-subtypes while sharing many of the characteristics of the pharmacologically defined EP_4-receptor (Honda *et al*., 1993; Bastien *et al*., 1994; Nishigaki *et al*., 1995). The most salient characteristics of this novel receptor are the findings that PGE_2 can exhibit subnanomolar affinity, the EP_2-selective agonists, butaprost and AH 13205, have relatively low affinity, and the TP-receptor antagonist, AH 23848B, acts as a competitive antagonist with a pA_2 of between 5 and 6 (Honda *et al*., 1993; Bastien *et al*., 1994; Coleman *et al*., 1994a; Nishigaki *et al*., 1995; Lydford *et al*., 1996).

The objectives of the present study were to characterize the EP-receptor subtype which mediates the inhibition of LPS-induced TNFα generation in human peripheral blood monocytes and to assess if other prostanoid receptors are expressed by these cells which subserve the same functional response.

A preliminary account of some of these data was presented to the British Pharmacological Society (Meja *et al*., 1996).

Methods

Isolation and purification of human mononuclear cells

Blood was collected from normal healthy individuals by antecubital venepuncture into acid citrate dextrose (in mM: disodium citrate 160, glucose 110 - pH 7.4) and mixed with 6% w/v Hespan (hydroxymethyl starch) to sediment erythrocytes. After standing at room temperature for 90 min, the leukocyte-rich plasma was removed and centrifuged at 312 *g* for 7 min. The resulting cell pellet was gently resuspended in approximately 7 ml of buffer A (in mM: KH_2PO_4 5, K_2HPO_4 5, NaCl 110, - pH 7.4) made 50% v/v with Percoll and layered over a discontinuous Percoll density gradient (63% and 73% (v/v)) in buffer A. Mononuclear cells were subsequently separated from polymorphonuclear cells by centrifugation at 1200 *g* for 30 min at 18°C. With this procedure, mononuclear cells were recovered from the 50%/63% v/v Percoll interface.

Mononuclear cells were washed twice in Ca^{2+}/Mg^{2+}-free HBSS to remove Percoll and finally suspended in Ca^{2+}/Mg^{2+}-free HBSS at a concentration of 10^6 cells ml^{-1}. Cells (5×10^5) were added to 24 well culture plates (Greiner Labortecnik Ltd, Dursley, Gloucestershire) containing 500 μl Dutch modified RPMI 1640 (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and allowed to adhere to the plastic for 90 min at 37°C in a humidified incubator under an atmosphere of 5% CO_2. The purity of the adherent cell population was routinely >97%. Plates were agitated, non-adherent cells decanted and the resulting monocytes were cultured for 18 h in 1 ml Dutch-modified RMPI 1640 in the absence and presence of LPS (3 ng ml^{-1}; EC_{84}; Seldon *et al*., 1995). The ability of a range of prostanoids to modify the release of TNFα by LPS into the culture supernatant was subsequently measured by ELISA (see below).

Measurement of TNFα

TNFα was measured by an amplified sandwich ELISA. Ninety six well round-bottom plates (Greiner Labortecnik Ltd, Dursley, Gloucestershire) were coated with 100 μl of a mouse anti-human TNFα monoclonal antibody diluted 1 : 400 in buffer B (in mM: Na_2CO_3 15, $NaHCO_3$ 36, NaN_3 15 - pH 9.6) and left for 2 h at 37°C. Plates were subsequently washed in buffer C (in mM: NaCl 145, KCl 4, NaH_2PO_4 10, Na_2HPO_4 3 and 0.05% v/v Tween-20, pH 7.4) and immediately treated with BSA (5% w/v) for 30 min at 37°C. After a further wash with buffer C, 100 μl TNFα standards, quality controls and unknown samples (diluted 1 in 4 in buffer C) were added to the plates and left for 18 h at 4°C. Plates were washed in buffer C, incubated for 2 h with 100 μl of a rabbit anti-human polyclonal TNFα antibody (diluted 1 : 500 in Buffer C supplemented with 10% v/v FCS), washed again and then incubated for a further 2 h at room temperature with 100 μl of an alkaline phosphatase-labelled sheep anti-rabbit polyclonal IgG antibody (diluted 1 : 2000 in buffer C supplemented with 10% FCS). Plates were washed once more and developed with a *p*-nitrophenyl phosphate assay kit (code 50-80-00, KPL/Dynatech Laboratories Ltd, Billingshurst, Sussex) according to the manufacturer's instructions. TNFα was measured colorimetrically at 405 nm and quantified by interpolation from a standard curve constructed to known concentrations of human recombinant TNFα. The detection limit of this assay is 8 pg ml^{-1}.

Assessment of monocyte viability

Cell respiration, an indicator of viability, was assessed by measuring the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described by Hirst *et al*. (1992).

Drugs and analytical reagents

FCS, RPMI 1640 and HBSS were from Flow Laboratories (Rickmansworth, Hertfordshire), Percoll was obtained from Pharmacia/LKB (Milton Keynes, Buckinghamshire), LPS (from *Salmonella enteritidis*), flurbiprofen, MTT, PGD_2, PGE_2, $PGF_{2\alpha}$ and U-46619 (9,11-dideoxy-9α, 11α -methanoepoxy $PGF_{2\alpha}$) were from the Sigma Chemical Company (Poole, Dorset) and 16,16-dimethyl-PGE_2, 11-deoxy-PGE_1, 17-phenyl-ω-trinor-PGE_2 and misoprostol were from Cascade Biochemicals Ltd (Reading Berkshire). All other drugs including syn-

thetic prostanoid were generously donated by the following: M & B 28767 (15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13-E-enoic acid; Rhône-Poulenc Rorer, Dagenham, Essex); sulprostone, cicaprost and rolipram (Schering AG, Berlin, Germany); butaprost (Bayer Ltd, Stoke Poges, Slough) and AH 23848B ([1α(Z),2β, 5α]-$\pm$)-7-[5-[[(1,1′-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl) cyclopentyl]-4-heptanoic acid), AH 13205 (*trans*-2-[4-(1-hydroxyhexyl)pentyl)phenyl]-5-oxocyclopentane heptanoic acid) and AH 6809 (6-isopropoxy-9-oxoxanthine-2-carboxylic acid; Glaxo-Wellcome Ltd, Stevenage). TNFα was obtained from British Biotechnology (code BDP 28) and the National Institute for Biological Standards and Controls (code 87/650) for standards and quality controls respectively. Mouse anti-human TNFα, rabbit anti-human TNFα and alkaline phosphatase-labelled sheep anti-rabbit IgG antibodies were purchased from Serotec Ltd (Kidlington, Oxford), Genzyme Corporation (West Malling, Kent) and Stratech Scientific Ltd (Luton, Bedfordshire), respectively. All other reagents were from BDH (Poole, Dorset).

Dissolution and storage of drugs

Stock solutions were made at a concentration of 10 mM in NaOH (M & B 28767, flurbiprofen), ethanol (PGD_2, PGE_2, $PGF_{2\alpha}$, U-46619, butaprost, sulprostone, AH 23848B) or DMSO (16,16-dimethyl-PGE_2, 11-deoxy-PGE_1, 17-phenyl-ω-trinor-PGE_2, misoprostol, AH 13205). Drugs were subsequently diluted to the desired working concentration in enriched Dutch-modified RPMI 1640. Cicaprost was provided at a 50 μg ml^{-1} solution and was similarly diluted in Dutch-modified RPMI 1640. LPS was dissolved in distilled water and stored at -70°C. Human recombinant TNFα for both quality controls and standards was obtained as a lyophilized powder and re-constituted at 1 μg ml^{-1} in distilled water and stored at -70°C. When required TNFα was diluted in enriched Dutch-modified RPMI 1640.

Data and statistical analyses

Data points and values in the text and figure legends, represent the mean$\pm$s.e.mean of n independent determinations. Concentration-response curves were analysed by least-squares non-linear iterative regression with the 'PRISM' curve fitting programme (GraphPad software, San Diego, CA) and EC_{50} and IC_{50} values were subsequently interpolated from curves of best-fit. Equi-effective molar concentration ratios (e.c.r.) were calculated by use of the formula (IC_{50} for PGE analogue)/(IC_{50} for PGE_2). When statistical evaluation was required, data were analysed by Student's t test for paired data or by one-way ANOVA/Newman-Keuls multiple comparison test. The null hypothesis was rejected when $P<0.05$.

Results

We have previously established that exposure of human peripheral blood monocytes to LPS evokes a time- and concentration-dependent elaboration of TNFα with a $t_{1/2}$ and EC_{50} of 2.8 h and 238 pg ml^{-1} respectively (Seldon *et al*., 1995). In the experiments described herein, LPS was used at 3 ng ml^{-1} (EC_{84}) and TNFα was measured in the culture supernatant 18 h after the addition of the stimulus. Under the conditions used in this study, the generation of TNFα in response to LPS is absolutely dependent upon new protein synthesis and is unaffected by flurbiprofen, at a concentration (10 μM) that inhibits cyclo-oxygenases 1 and 2 (see Seldon *et al*., 1995 for further details).

Effect of naturally occurring prostaglandins, cicaprost and U-46619

In the absence of LPS, the naturally occurring prostanoids PGD_2, PGE_2 and $PGF_{2\alpha}$, and the thromboxane and prostacyclin mimetics, U-46619 and cicaprost, respectively, did not promote the generation of TNFα, which indicates an absence of excitatory prostanoid receptors on human monocytes that positively regulate TNFα gene transcription. In contrast, PGE_1, PGE_2 and cicaprost suppressed the elaboration of TNFα in LPS-stimulated cells in a concentration-dependent manner (Figure 1) with respective pIC_{50} values of 7.14 ± 0.05 ($n=4$), 7.34 ± 0.06 ($n=11$) and 8.00 ± 0.22 ($n=4$). In each case, TNFα generation was inhibited by 85 to 90% at the highest concentration (10 μM) of agonist tested (Figure 1), although the concentration-response curves that described this effect were shallow and had slopes significantly less than unity (Figure 1).

The addition of PGE_2 to monocyte cultures 60 min after LPS failed to inhibit TNFα generation (data not shown).

PGD_2, $PGF_{2\alpha}$ and U-46619 were essentially inactive at suppressing TNFα release at concentrations up to 10 μM under identical experimental conditions (Figure 1).

Effect of EP-selective prostanoid agonists

To identify the receptor(s) that mediates the inhibitory action of PGE_2 on TNFα generation, eight PGE analogues which have varying degrees of selectivity for the EP_1-, EP_2- and EP_3-receptor subtypes were evaluated for inhibitory activity. Sulprostone, MB 28,767, 17-phenyl-ω-trinor PGE_2 and AH 13205 were very weak agonists with pIC_{50}s >5 (Figure 2; Table 1). In contrast, the position of the log concentration response curves of the other analogues studied was parallel to that obtained for PGE_2 (implying that similar asymptotes would be achieved) although they were 13 to 56 fold less potent (Figure 2, Table 1). The rank order of potency for the EP-receptor agonists tested was: $PGE_1 \approx PGE_2 >$ 16,16-dimethyl $PGE_2 \geqslant$ misoprostol $>$ 11-deoxy PGE_1 = butaprost $>>$ 17-phenyl-ω-trinor $PGE_2 >$ AH 13205 = MB 28,767 $>$ sulprostone. The pIC_{50} values and equi-effective molar concentration-ratios are presented in Table 1. Consistent with the data obtained for PGE_1 and PGE_2, the concentration-response curves that described the suppression of TNFα generation by the active EP-receptor agonist tested were shallow with slopes significantly less than 1 (Figure 2).

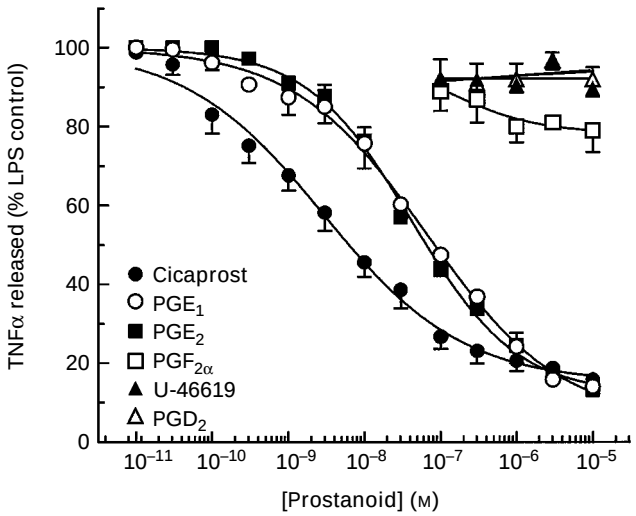

Figure 1 Effect of prostanoids on LPS-induced TNFα biosynthesis from human peripheral blood monocytes. Adherent monocytes were pretreated for 5 min with varying concentrations of PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, cicaprost and U-46619 before being exposed to LPS (3 ng ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of TNFα released into the culture supernatant was quantified at 18 h by a sandwich ELISA. Each data point represents the mean, and vertical lines show s.e.mean of three to five determinations made in different cell preparations. See Methods for further details.

Effect of AH 23848B on the inhibition of TNFα generation evoked by PGE_2

Pretreatment of human monocytes with 10, 30 and 100 μM of the putative EP_4-receptor antagonist, AH 23848B (Coleman *et al*., 1994a, b), inhibited the generation of TNFα by 10±4% ($n=4$), 28±5% ($n=4$) and 76.7±14.6% ($n=3$), respectively (Figure 3). However, AH 23848B at 10 and 30 μM did not cause any significant shift in the location of the PGE_2 concentration-response curves (Figure 3). The pEC_{50} values of PGE_2 in the absence and presence of 10 and 30 μM AH 23848B were 7.49±0.09 ($n=3$), 7.44±0.09 ($n=3$) and 7.79±0.11 ($n=3$) respectively ($P>0.05$; one-way ANOVA). Cell viability was not affected by any concentration of AH 23848B studied.

Effect of AH 13205 on the inhibition of TNFα generation evoked by PGE_2

The EP_2-selective agonist, AH 13205 (Nials *et al*., 1993), was a poor inhibitor of TNFα generation from monocytes (Table 1), which could indicate that it exhibits partial agonist activity in this system. Studies were thus performed to assess if AH 13205 could antagonize the inhibitory effect of the full agonist, PGE_2.

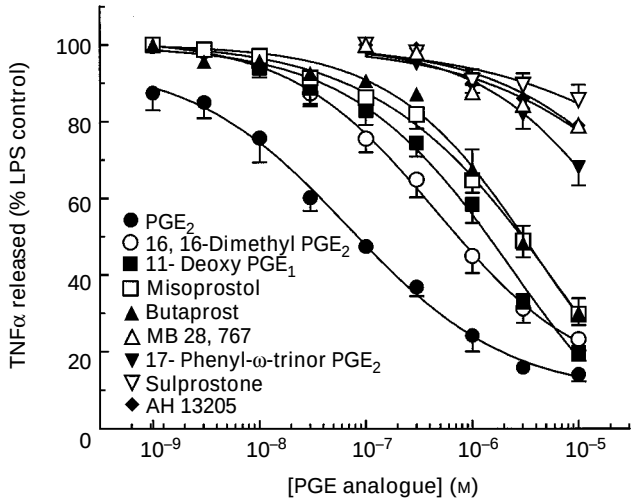

Figure 2 Effect of EP-selective prostanoid agonists on LPS-induced TNFα biosynthesis from human peripheral blood monocytes. Adherent monocytes were pretreated for 5 min with varying concentrations of PGE_2 and eight synthetic PGE analogues before being exposed to LPS (3 ng ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of TNFα released into the culture supernatant was quantified at 18 h by a sandwich ELISA. Each data point represents the mean and vertical lines show s.e.mean of three to five determinations made in different cell preparations. See Methods for further details.

Pretreatment of human monocytes with 10 and 30 μM AH 13205 inhibited the generation of TNFα by 31.2±4% ($n=4$) and 53±5% ($n=4$), respectively (Figure 4). However, despite suppressing cytokine production, AH 13205 failed to shift the location of the PGE_2 concentration-response curves significantly at either concentration examined (Figure 4). The pEC_{50} values of PGE_2 in the absence and presence of 10 and 30 μM AH 13205 were 7.82±0.34 ($n=3$), 7.32±0.16 ($n=3$) and 7.05±0.32 ($n=3$), respectively ($P>0.05$; one-way ANOVA). Cell viability was not affected by either concentration of AH 13205 studied.

Effect of AH 6809 on the inhibition of TNFα generation evoked by PGE_2

AH 6809 is an EP_1- and DP-receptor antagonist (Coleman *et al*., 1987; Keery & Lumley, 1988) that was recently found to

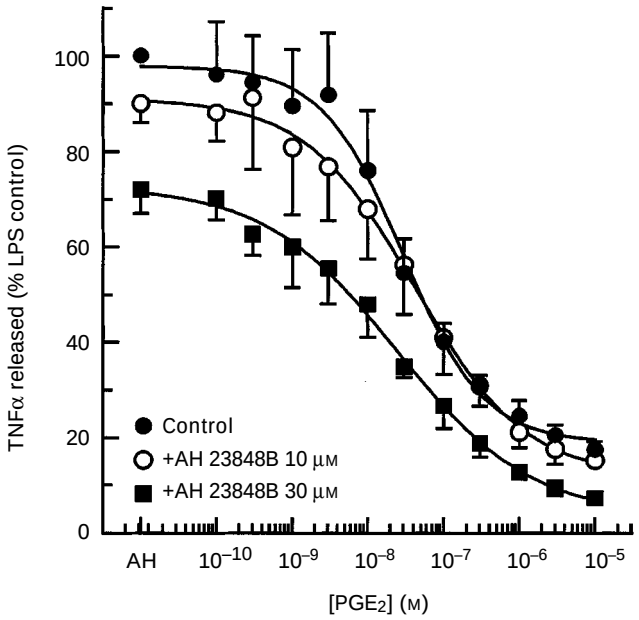

Figure 3 Effect of AH 23848B, an antagonist at TP- and EP_4-receptors, on the inhibition of LPS-induced TNFα generation from human peripheral blood monocytes evoked by PGE_2. Adherent monocytes were pretreated for 25 min with AH 23848B or its vehicle followed for a further 5 min with varying concentrations of PGE_2. Monocytes were then exposed to LPS (3 ng ml^{-1}) and maintained at 37°C for 18 h in a thermostatically-controlled incubator under a 5% CO_2 atmosphere. The amount of TNFα released into the culture supernatant was subsequently quantified by a sandwich ELISA. Each data point represents the mean, and vertical lines show s.e.mean, of four determinations made in different cell preparations. 'AH' refers to the effect of AH 23848B on TNFα generation in the absence of PGE_2. See Methods for further details.

Table 1 Potency order of EP-selective prostanoid agonists at suppressing TNFα generation from human peripheral blood monocytes

EP-receptor agonist	n	*Receptor selectivity**	*Inhibition of TNFα* (pIC_{50})	*Relative potency* ($PGE_2=1$)
PGE_2	11	$EP_1=EP_2=EP_3=EP_4$	7.34±0.06 (86.8±1.7%)	1
PGE_1	4	$EP_1=EP_2=EP_3=EP_4$	7.14±0.05 (85.9±1.9%)	1.58
16, 16-Dimethyl PGE_2	5	$EP_1=EP_3>EP_2$	6.21±0.13 (76.2±3.0%)	13.5
Misoprostol	5	$EP_2=EP_3>EP_1$	6.02±0.11 (80.3±2.1%)	20.9
11-Deoxy PGE_1	5	$EP_2>EP_3>EP_1$	5.67±0.11 (70.2±2.9%)	46.8
Butaprost	5	$EP_2>>EP_1>EP_3$	5.59±0.12 (69.9±3.8%)	56.2
17-Phenyl-ω-trinor PGE_2	5	$EP_1>EP_3>EP_2$	>5 (32.1±4.7%)	>219
AH 13205	3	$EP_2>EP_1>EP_3$	>5 (21.4±1.7%)	>219
MB 28,767	3	$EP_3>EP_2>EP_1$	>5 (18.9±0.5%)	>219
Sulprostone	3	$EP_3>EP_1>>EP_2$	>5 (14.1±4.1%)	>219

Values in parentheses show the percentage inhibition of TNFα release evoked by 10 μM EP-receptor agonist. Relative potency values>1 indicate that the agonist in question is less potent than PGE_2. *Selectivity based on activity in isolated tissues.

block PGE_2-induced cyclic AMP accumulation in COS-7 cells that had been transfected with a plasmid expressing the human recombinant EP_2-receptor (Woodward *et al*., 1995). Since PGD_2 and 17-phenyl-ω-trinor PGE_2 (EP_1-agonist) failed to suppress TNFα generation (Figure 1; Table 1), AH 6809 was used to determine if EP_2-receptors mediated the inhibitory effect of PGE_2.

Pretreatment of human monocytes with 10 μM AH 6809, a concentration that produced a 30 fold shift in the PGE_2 response curve in COS-7 cells (Woodward *et al*., 1995), did not affect LPS-induced TNFα generation *per se* but produced a small (~3.5 fold), but nevertheless significant, rightwards shift of the PGE_2 concentration-response curve from which a pA_2 of 5.4 was derived (Figure 5). The pEC_{50} values of PGE_2 in the absence and presence of 10 μM AH 6809 were 7.81 ± 0.04 ($n=4$) and 7.26 ± 0.07 ($n=4$), respectively ($P<0.05$, Student's t test). Cell viability was not affected by AH 6809 (10 μM).

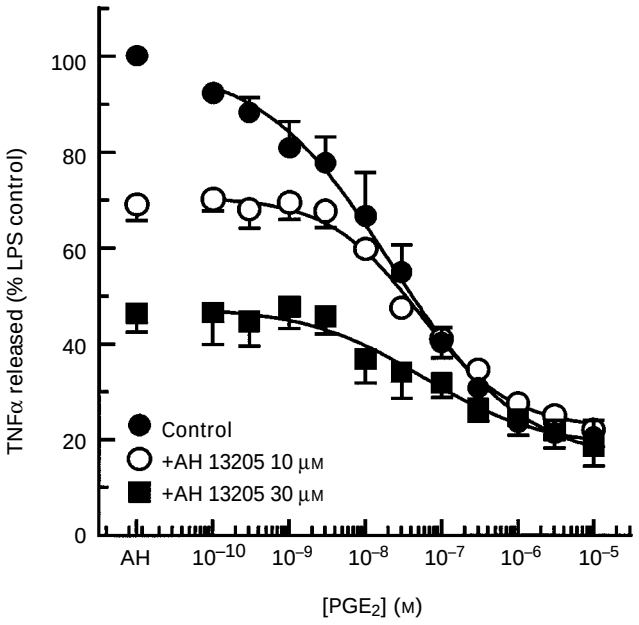

Figure 4 Effect of the EP_2-selective agonist, AH 13205, on the inhibition of LPS-induced TNFα generation from human peripheral blood monocytes evoked by PGE_2. Adherent monocytes were pretreated for 25 min with AH 13205 or its vehicle followed for a further 5 min with varying concentrations of PGE_2. Monocytes were then exposed to LPS (3 ng ml^{-1}) and maintained at 37°C for 18 h in a thermostatically-controlled incubator under a 5% CO_2 atmosphere. The amount of TNFα released into the culture supernatant was subsequently quantified by a sandwich ELISA. Each data point represents the mean, and vertical lines show s.e.mean, of four determinations made in different cell preparations. 'AH' refers to the effect of AH 13205 on TNFα generation in the absence of PGE_2. See Methods for further details.

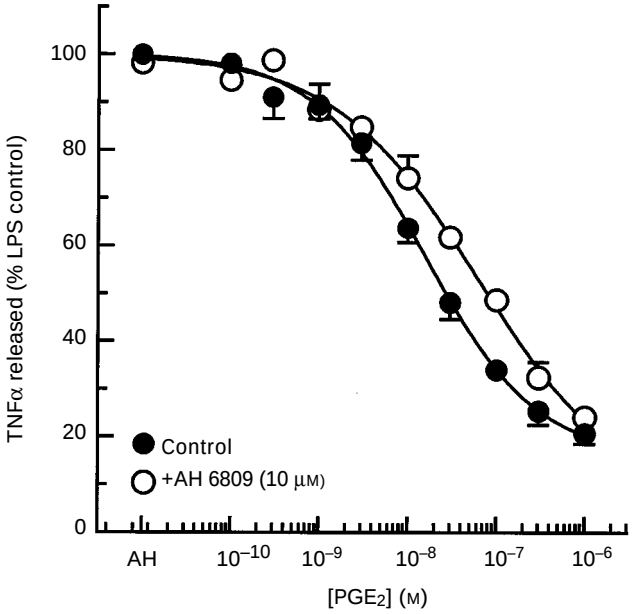

Figure 5 Effect of AH 6809, an antagonist at EP_1, EP_2- and DP-receptors, on the inhibition of LPS-induced TNFα generation from human peripheral blood monocytes evoked by PGE_2. Adherent monocytes were pretreated for 25 min with 10 μM AH 6809 or its vehicle followed for a further 5 min with varying concentrations of PGE_2. Monocytes were then exposed to LPS (3 ng ml^{-1}) and maintained at 37°C for 18 h in a thermostatically-controlled incubator under a 5% CO_2 atmosphere. The amount of TNFα released into the culture supernatant was subsequently quantified by a sandwich ELISA. Each data point represents the mean, and vertical lines show s.e.mean, of four determinations made in different cell preparations. 'AH' refers to the effect of AH 6809 on TNFα generation in the absence of PGE_2. See Methods for further details.

Table 2 Comparison of the potencies of PGE_2 and synthetic EP-receptor agonists at suppressing TNFα generation from human peripheral blood monocytes with other tissues expressing inhibitory/relaxant EP receptors

	Equi-effective molar concentration ratio ($PGE_2 = 1$)							
EP-selective agonist	*Human monocyte* (↓TNFα)	*Human monocyte** (↑cAMP)	*Jurkat T-cells*** (↑cAMP)	*Rabbit ear artery¶* (Relaxation)	*Piglet saphenous vein‡* (Relaxation)	*Rabbit jugular vein**** (Relaxation)	*Rat trachea***** (Relaxation)	*Rabbit saphenous vein¶* (Relaxation)
PGE_2 (pEC_{50}/pIC_{50})	7.34	6.41†	7.10	7.04	8.69	9.34	7.81	9.39
PGE_1	1.58	ND	ND	ND	ND	ND	ND	1.1
16, 16-Dimethyl PGE_2	13.5	3.4	150	9.1	1.6	2.1	2.8	6.8
Misoprostol	20.9	>26	ND	1.3	ND	8.3	16.1	96
11-Deoxy PGE_1	46.8	3.1	3	11.9	1.6	2.1	2.4	4.9
Butaprost	56.2	>26	>1500	16¶¶	40.9	685	ND	382
17-Phenyl-ω-trinor PGE_2	>219	ND	ND	ND	ND	ND	ND	ND
AH 13205	>219	>26	>1500	37	3102	2320‡	1,118	1,496
MB 28,767	>219	ND	ND	ND	ND	ND	ND	ND
Sulprostone	>219	ND	>1500	>770¶¶	6,480††	>3,000	Inactive	15,572

*Data from Milne *et al*. (1994); **Data from De Vries *et al*. (1995); ***Data from Lawrence & Jones (1992); ****Data from Lydford & McKechnie (1994); ¶Data from Lydford *et al*. (1996); ¶¶Data from Humbles *et al*. (1991); †pEC_{30}; ††Data from Coleman *et al*. (1994a,b); ‡Data from Milne *et al*. (1995). ND: not determined.

Discussion

The objective of this study was to classify the inhibitory prostanoid receptor(s) on human monocytes at which PGE_2 suppresses LPS-induced TNFα generation. The sub-micromolar potency of PGE_1 and PGE_2 in these cells indicated that this response was mediated by an EP-receptor. Indeed, with the exception of the prostacyclin mimetic, cicaprost (see below), all of the other prostanoids studied (PGD_2, $PGF_{2\alpha}$, U-46619) were essentially inactive.

Four distinct EP-receptor subtypes have been described and studies with naturally occurring and synthetic prostanoid agonists and antagonists were performed to classify the EP-receptor subtype(s) on monocytes. The finding that sulprostone, 17-phenyl-ω-trinor PGE_2 and MB 28,767 were greater than three orders of magnitude less active than PGE_2 in this system provides persuasive evidence that monocytes do not express EP_1- or EP_3-receptors that negatively regulate TNFα biosynthesis (see Lawrence *et al*., 1991). Further support for this conclusion was the finding that at a concentrtion of 10 μM, the DP/EP_1-receptor antagonist, AH 6809 (Coleman *et al*., 1987; Keery & Lumley, 1988), produced only a modest 3.5 fold shift to the right of the PGE_2 concentration-response curves which equates to a pA_2 of 5.4 (see below for a discussion of the effect of AH 6809 at EP_2-receptors). This antagonism was significantly less than would be predicted for an interaction with either DP- or EP_1-receptors, where pA_2s of 6.0 to 6.6 and 6.4 to 7 are predicted respectively (see Coleman *et al*., 1994b).

We and others have previously demonstrated that PGE_2 elevates the cyclic AMP content and activates cyclic AMP-dependent protein kinase in human monocytes (Eriksen *et al*., 1985; Milne *et al*., 1994; Seldon *et al*., 1995) and monocytic cell lines (Loh *et al*., 1993; Blaschke *et al*., 1996). Given that TNFα gene expression is negatively regulated by cyclic AMP-elevating agents (Taffet *et al*., 1990; Severn *et al*., 1992; Prabhakar *et al*., 1994; Seldon *et al*., 1995), it was considered plausible that EP_2- and/or EP_4-receptors (which are positively coupled to adenylyl cyclase) might mediate the inhibition of TNFα biosynthesis (see Coleman *et al*., 1994b). Although agonism at certain spliced variants of the EP_3-receptor also leads to an increase in cyclic AMP (Namba *et al*., 1993), the expression of such receptors by human monocytes was incompatible with poor activity of sulprostone and M&B 28,767 (see above).

If EP_2-receptors are involved, then a prediction based on data obtained with established EP_2-containing tissues such as the rabbit ear artery is that butaprost and AH 13205 (Gardiner, 1986; Nials *et al*., 1993), should be 10 to 100 times less potent than PGE_2 (Lydford *et al*., 1996; see Table 2). However, while butaprost fell within the predicted range (e.c.r. = 56), it was relatively weak compared to smooth muscle preparations expressing the EP_2-receptor subtype, and AH 13205 was considerably less active (e.c.r. > 219) than would be anticipated. Furthermore, misoprostol was greater than 20 times less effective than PGE_2 on human monocytes, unlike the rabbit ear artery and the cat trachea (both EP_2) where it is equi-effective (Coleman *et al*., 1988; Lydford *et al*., 1996). While these results cast doubt over the involvement of EP_2-receptors, the ability of butaprost to suppress TNFα generation and the knowledge that it is a weak agonist at EP_2-receptors expressed on other leukocytes (Wise & Jones, 1994; Teixeira *et al*., 1997), prompted us to perform more detailed studies.

It was reasoned that the low potency of AH 13205 might reflect partial agonist activity at EP_2-receptors. A prediction of this proposal is that AH 13205 would antagonize the effect of a full agonist such as PGE_2. However, even at high concentrations (10 and 30 μM), AH 13205 exhibited marked agonism at preventing the generation of TNFα. Since both agonism and antagonism result from an interaction with the same receptor, these results are clearly inconsistent with the behaviour of a partial agonist which evokes agonism at concentrations equal to those that produce blockade (Kenakin, 1987).

Woodward *et al*. (1995) have recently demonstrated that the DP- and EP_1-receptor blocking drug, AH 6809, is also an antagonist at the human recombinant EP_2-receptor subtype expressed in COS-7 cells. Since human monocytes did not express EP_1- or DP-receptors, AH 6809 was used to assess if the EP_2-subtype was involved in the suppression of TNFα generation. As mentioned above, pretreatment of monocytes with 10 μM AH 6809 produced a parallel shift to the right of the PGE_2 concentration-response curve. However, the concentration-ratio (~3.5) obtained yielded a pA_2 of 5.4 which is less than predicted for blockade of the recombinant EP_2-receptor subtype expressed in CHO cells ($pA_2 = 6.5$; Woodward *et al*., 1995) and in human myometrium ($pA_2 = 5.85$; Brown *et al*., 1997). While this would suggest that EP_2-receptors are unlikely to mediate the inhibitory effect of PGE_2, AH 6809 exhibits several additional properties that question this interpretation. For example, Coleman *et al*. (1985) have shown that AH 6809 binds avidly to plasma proteins which renders it inactive. Since the culture medium in this study was supplemented with 10% FCS, it is likely that AH 6809 would be susceptible to the same fate and would lead to an underestimation of its affinity at EP_2-receptors. In addition, AH 6809 is a phosphodiesterase inhibitor (Keery & Lumley, 1988) and could produce less than expected antagonism through its ability to act synergistically with PGE_2 in elevating cyclic AMP. Thus, although the overall activity profile of PGE analogues in monocytes is inconsistent with an interaction with EP_2-receptors (see Table 2), the activity of butaprost in this system, and the ability of AH 6809 to antagonize the effect of PGE_2, suggests that this receptor subtype might contribute (in part) to the suppression of TNFα generation.

Recently, a fourth human EP-receptor was cloned by Bastien *et al*. (1994) which has a number of pharmacological characteristics that distinguish if from the EP_2-subtype. According to Coleman *et al*. (1994b), the demonstration of subnanomolar potency and susceptibility to competitive antagonism by AH 23848B indicates activity at EP_4-receptors. Indeed, these criteria have categorized the piglet and rabbit saphenous veins as EP_4-containing preparations (Coleman *et al*., 1994a, b; Lydford *et al*., 1996). In this study, PGE_2 had relatively low potency ($pEC_{50} = 7.34$) and was insensitive to AH 23848B, characteristics that, when considered alone, do not implicate EP_4-receptors (see Table 2). However, AH 23848B *per se* demonstrated appreciable agonist activity in human monocytes with an intrinsic activity (relative to PGE_2) of 0.88 at 100 μM. Qualitatively identical results have been obtained with EP_4- (but not EP_2-) containing tissues such as CHO cells and rabbit saphenous vein (Lydford *et al*., 1996) and has lead to the proposal that AH 23848B might act as a partial agonist at the EP_4-receptor in some preparations (Lydford *et al*., 1996). This is an important consideration since the high intrinsic activity of AH 23848B against monocyte TNFα production would then explain why it failed to antagonize the inhibitory action of PGE_2 (see Kenakin, 1987). Additionally, affinity of AH 23848B for EP_4-receptors could also reconcile our data with those of Milne *et al*. (1995), who demonstrated that AH 23848B competitively antagonized ($pA_2 = 5.6$) PGE_2-induced cyclic AMP accumulation in human monocytes (Milne *et al*., 1995) presumably at a concentration that exerted no intrinsic agonist activity.

It is clear from the above discussion that a major difficulty in identifying unequivocally EP_4-receptors is a lack of selective tools. Moreover, a survey of the literature suggests that current criteria used to classify this subtype might be inappropriate. This is suggested from data obtained in a number of diverse tissues. For example, PGE_2 is very potent at eliciting relaxation ($pD_2 = 9.34$) of the rabbit jugular vein but is insensitive to AH 23848B (Milne *et al*., 1995). Conversely, PGE_2 relaxes the rat isolated trachea with sub-micromolar potency ($pD_2 = 7.81$), yet this response is competitively antagonized by AH 23848B with an affinity consistent with an interaction at EP_4-receptors (Lydford *et al*., 1996). Similarly, the potency of PGE_2 ($pIC_{30} = 6.41$) in elevating the cyclic AMP content in human monocytes is 50 to 1000 fold weaker than expected for an interaction at EP_2- or EP_4-receptors, despite the fact that AH

23848B behaves as an antagonist in this system (Milne *et al*., 1995). In another study, PGE_2-induced cyclic AMP accumulation in Jurkat T-cells was antagonized by AH 23848B but in a non-competitive manner, and the overall rank order of potency of a range of synthetic agonists did not mirror the EP-receptor pharmacology shown for established EP_2- or EP_4-containing preparations (De Vries *et al*., 1995). Thus, while these inconsistencies might indicate the expression of novel EP-receptors (e.g. EP_5), the possibility remains that species variation, differences in EP_4-receptor density between tissues, or multiple, pharmacologically distinct EP_4-receptor gene products are responsible for these anomalies.

The results obtained with cicaprost (Sturzenbechner *et al*., 1986) suggest that IP-receptors are also expressed by human monocytes and mediate inhibition of TNFα generation. Although no antagonists are currently available with which to confirm unequivocally this assertion, the knowledge that cicaprost is a potent and highly selective IP-receptor agonist (Dong *et al*., 1986) and was 8 times more potent that PGE_2 at inhibiting the elaboration of TNFα provides persuasive evidence. These results are consistent with previous findings which have documented the ability of cicaprost to inhibit TNFα generation from the monocytic tumour cell line, THP-1 (Crutchley *et al*., 1992), and from peripheral blood mononuclear cells (Eisenhut *et al*., 1993; Sinha *et al*., 1995; Greten *et al*., 1996). In addition, Greten *et al*. (1996) have recently shown that the phosphodiesterase 4 inhibitor, rolipram, potentiates the inhibitory effect of cicaprost on TNFα generation which is in agreement with the traditional notion that IP-receptors are positively coupled to adenylyl cyclase.

It is noteworthy, that the classification of receptors by use of functional responses (e.g. gene induction) that are manifest over periods of hours is subject to additional concerns. In particular, are the drugs used for the classification stable for the duration of the experiment? In this study, LPS-stimulated monocytes were cultured in medium supplemented with 10% FCS, and TNFα was measured 18 h later. It is clear that our inability to ascribe the rank order of agonist potency to an existing EP-receptor subtype could be attributable to degradation of the prostanoids or their binding (inactivation) by FCS with time. While we did not assess these possibilities empirically, we are confident that the prostanoids studied are stable over the time-frame where TNFα gene repression occurs. This is based on the finding that the elaboration of TNFα was not prevented if PGE_2 was added to the cultures 60 min after LPS. Thus, a relatively brief exposure (<60 min) of monocytes to PGE_2 is sufficient to represss TNFα gene induction.

Collectively, the results of this study can broadly be interpreted in at least one of two ways. Perhaps the most simplistic explanation is that the suppression of monocyte TNFα production by PGE_2 is mediated by multiple prostanoid receptor subtypes. This is an attractive proposal since it explains the data obtained with butaprost, AH 6809 and AH 23848B, and the atypical rank order of agonist potency in these cells (see Table 2). Based upon the data presented herein, at least two scenarios can be considered. It is known that PGE_1 and its analogues (butaprost, misoprostol) are moderately potent agonists at the IP-receptor (Wise & Jones, 1996), which raises the possibility that IP- as well as EP-receptors mediate the inhibitory effects of PGE_2 in this system. A similar case can be made for the regulation of TNFα production by EP_2- and EP_4-receptors. Indeed, many leukocytes express multiple EP-subtypes (Zeng *et al*., 1995; Fedyk *et al*., 1995; Blaschke *et al*., 1996) and EP_2- EP_3- and EP_4-receptors have been unequivocally identified at the mRNA or protein level on the human monocytic cell line, U-937 (Zeng *et al*., 1995; Blaschke *et al*., 1996). Furthermore, recent radioligand binding studies conducted with U-937 cells have demonstrated that [^{3}H]-PGE_2 labelled two independent populations of high affinity (K_ds = 3.1 and 137 nM) binding sites (Loh *et al*., 1993). The finding that the concentration-response curves which described the inhibition of TNFα generation by PGE_2 and the other active EP-receptor agonists were routinely shallow is also consistent with the notion of multiple subtypes. The other explanation of these data is that PGE_2 suppresses TNFα generation by interacting with an EP-receptor that is pharmacologically distinct from those subtypes currently defined.

In conclusion, the results of this study suggest that human peripheral blood monocytes express both IP- and EP-receptors that negatively regulate TNFα generation from LPS-stimulated human monocytes. Due to a lack of selective antagonists, the receptor subtype(s) that mediates the effect of PGE_2 was not resolved, although the participation of multiple (EP_2- and/or EP_4- and/or IP) or novel EP-receptors should be investigated further when more selective ligands become available.

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References

ABRAMOVITZ, M., BOIE, Y., NGUYEN, T., RUSHMORE, T.H., BAYNE, M.A., METTERS, K.M., SLIPETZ, D.M. & GRYGORCZYK, R. (1994). Cloning and expression of a cDNA for the human prostanoid FP receptor. *J. Biol. Chem*., **269,** 2632–2636.

AN, S., YANG, J., SO, S.W., ZENG, L. & GOETZL, E.J. (1994). Isoforms of the EP_3 subtype of human prostaglandin E_2 receptor transduce both intracellular calcium and cyclic AMP signals. *Biochemistry*, **33,** 14496–14502.

ARMSTRONG, R.A., JONES, N.L. & WILSON, N.L. (1983). Ligand binding to thromboxane receptors on human platelets: correlation with biological activity. *Br. J. Pharmacol*., **79,** 953–964.

BASTIEN, L., SAWYER, N., GRYGORCZYK, R., METTERS, K.M. & ADAM, M. (1994). Cloning, functional expression, and characterization of the human prostaglandin E_2 receptor EP_2 subtype. *J. Biol. Chem*., **269,** 11873–11877.

BLASCHKE, V., JUNGERMANN, K. & PÜSCHEL, G.P. (1996). Exclusive expression of the Gs-linked prostaglandin E_2 receptor subtype 4 mRNA in human mononuclear Jurkat and KM-3 cells and coexpression of subtype 4 and 2 mRNA in U-937 cells. *FEBS Letts*., **394,** 39–43.

BOIE, Y., RUSHMORE, T.H., DARMON-GOODWIN, A., GRYGORCZYK, R., SLIPETZ, D.M., METTERS, K.M. & ABRAMOVITZ, M. (1994). Cloning and expression of a cDNA for the human prostanoid IP receptor. *J. Biol. Chem*., **269,** 12173–12178.

BOIE, Y., SAWYER, N., SLIPETZ, D.M., METTERS, K.M. & ABRAMOVITZ, M. (1995). Molecular cloning and characterization of the human prostanoid DP-receptor. *J. Biol. Chem*., **270,** 18910–18916.

BROWN, C.J., BOERSMA, J.I. & CRANKSHAW, D.J. (1997). Effect of AH6809 on prostanoid-induced relaxation of human myometrium *in vitro*. *Br. J. Pharmacol*., **120,** 338P.

COLEMAN, R.A., DENYER, L.H. & SHELDRICK, R.L.G. (1985). The influence of protein binding on the potency of the prostanoid EP_1-receptor blocking drug, AH 6809. *Br. J. Pharmacol*., **86,** 803P.

COLEMAN, R.A., GRIX, S.P., HEAD, S.A., LOUTTIT, J.B., MALLETT, A. & SHELDRICK, R.L.G. (1994a). A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins*, **47,** 151–168.

COLEMAN, R.A., HUMPHREY, P.P.A., KENNEDY, I. & LUMLEY, P. (1984). Prostanoid receptors - the development of a working classification. *Trends. Pharmacol. Sci*., **5,** 303 – 306.

COLEMAN, R.A., HUMPHREY, P.P.A., SHELDRICK, R.L.G. & WHITE, B.P. (1988). Gastric antisecretory prostanoids: actions at different prostanoid receptors. *Br. J. Pharmacol*., **95,** 724P.

COLEMAN, R.A., KENNEDY, I. & SHELDRICK, R.L.G. (1987). New evidence with selective agonists and antagonists for the subclassification of PGE_2-sensitive (EP) receptors. *Adv. Prostaglandin Thromboxane Leukotriene Res*., **17,** 467 – 470.

COLEMAN, R.A., SMITH, W.L. & NURUMIYA, S. (1994b). International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev*., **46,** 205 – 229.

CRUTCHLEY, D.J., CONANAN, L.B. & QUE, B.G. (1994). Effect of prostacyclin analogs on the synthesis of tissue factor, tumor necrosis factor-α and interleukin-1β in human monocytic THP-1 cells. *J. Pharmacol. Exp. Ther*., **271,** 446 – 451.

DE VRIES, G.W., GUARINO, P., MCLAGHLIN, A., CHEN, J., ANDREWS, S. & WOODWARD, D.F. (1995). An EP receptor with a novel pharmacological profile in the T-cell line Jurkat. *Br. J. Pharmacol*., **115,** 1231 – 1234.

DONG, Y.J., JONES, R.L. & WILSON, N.H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Br. J. Pharmacol*., **87,** 97 – 107.

EISENHUT, T., SINHA, B., GRÖTTRUP-WOLFERS, E., SEMMLER, J., SIESS, W. & ENDRES, S. (1993). Prostacyclin analogs suppress the synthesis of tumor necrosis factor-α in LPS-stimulated human peripheral blood mononuclear cells. *Immunopharmacology*, **26,** 259 – 264.

ERIKSEN, E.F., RICHELSEN, B., BECK-NIELSEN, H., MELSEN, F., NIELSEN, H.K. & MOSEKILDE, L. (1985). Prostaglandin E_2 receptors on human peripheral blood monocytes. *Scand. J. Immunol*., **21,** 167 – 172.

FEDYK, E.R., RIPPER, J.M., BROWN, D.M. & PHIPPS, R.P. (1996). A molecular analysis of PGE receptor (EP) expression on normal and transformed B-lymphocytes: coexpression of EP_1, EP_2, $EP_{3\beta}$ and EP_4. *Mol. Immunol*., **33,** 33 – 45.

FUNK, C.D., FURCI, L., FITZGERALD, G.A., GRYGORCZYK, R., ROCHETTE, C., BAYNE, M.A., ABRAMOVITZ, M., ADAM, M. & METTERS, K.M. (1993). Cloning and expression of a cDNA for the human prostaglandin EP_1 receptor. *J. Biol. Chem*., **268,** 26767 – 26772.

GARDINER, P. (1986). Characterisation of prostanoid relaxant/inhibitory receptors (ψ) using a highly selective agonist, TR4979. *Br. J. Pharmacol*., **87,** 45 – 56.

GRETEN, T.F., SINHA, B., HASLBERGER, C., EIGLER, A. & ENDRES, S. (1996). Cicaprost and the type IV phosphodiesterase inhibitor, rolipram, synergize in suppression of tumor necrosis factor-α synthesis. *Eur. J. Pharmacol*., **299,** 229 – 233.

HIRATA, M., HAYASHI, A., USHIKUBI, F., YOKATA, Y., KAGEYAMA, R., NAKANISHI, S. & NARUMIYA, S. (1991). Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature*, **349,** 617 – 629.

HIRST, S.J., BARNES, P.J. & TWORT, C.H.C. (1992). Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am. J. Respir. Cell. Mol. Biol*., **7,** 574 – 581.

HONDA, A., SUGIMOTO, Y., NAMBA, T., WATABE, A., IRIE, A., NEGISHI, M., NARUMIYA, S. & ICHIKAWA, A. (1993). Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_2 subtype. *J. Biol. Chem*., **268,** 7759 – 7762.

HUMBLES, A.A., NIALS, A.T. & COLEMAN, R.A. (1991). Pharmacological characterisation of the prostanoid receptors in the rabbit isolated ear artery. *Br. J. Pharmacol*., **104,** 418P.

JINHONG, Y., MENGHANG, X., EDWARD, J.G. & SONGZHU, A. (1994). Cloning and expression of the EP_3-subtype of human receptors for prostaglandin E_2. *Biochem. Biophys. Res. Commun*., **198,** 999 – 1006.

JONES, R.L., WILSON, N.H. & ARMSTRONG, R.A. (1985). Characterisation of thromboxane receptors on human platelets. *Adv. Exp. Med. Biol*., **192,** 67 – 81.

KEERY, R.J. & LUMLEY, P. (1988). AH 6809, a prostaglandin DP-receptor blocking drug on human platelets. *Br. J. Pharmacol*., **94,** 745 – 754.

KENAKIN, T.P. (1987). *Pharmacologic Analysis of Drug-Receptor Interaction*. New York: Raven Press.

KENNEDY, I., COLEMAN, R.A., HUMPHREY, P.P.A., LEVY, G.P. & LUMLEY, P. (1982). Studies on the characterisation of prostanoid receptors: a proposed classification. *Prostaglandins*, **24,** 667 – 689.

KOTANI, M., TANAKA, I., OGAWA, Y., USUI, T., MORI, K., ICHIKAWA, A., NARIMIYA, S., YOSHIMI, T. & NAKAO. (1995). Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP_3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol. Pharmacol*., **48,** 869 – 879.

KUNAPULI, S.P., GUANG, F.M., BASTEPE, M., LIU-CHEN, L.Y., LI, S., CHEUNG, P.P., DERIEL, J.K. & ASHBY, B. (1994). Cloning and expression of a prostaglandin E-receptor EP_3-subtype from human erythroleukemia cells. *Biochem. J*., **298,** 263 – 267.

LAWRENCE, R.A., JONES, R.L. & WILSON, N.H. (1991). Characterization of the receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig ileum. *Br. J. Pharmacol*., **105,** 271 – 278.

LAWRENCE, R.A. & JONES, R.L. (1992). Investigation of the prostaglandin E (EP-) receptor subtype mediating relaxation of the rabbit jugular vein. *Br. J. Pharmacol*., **105,** 817 – 824.

LOH, C.L., ROTONDO, D. & DUTTA-ROY, A.K. (1993). Characterization of prostaglandin E_2 receptors expressed on human monocytic leukaemic cell line, U937. *Biochim. Biophys. Acta,* **1177,** 43 – 48.

LYDFORD, S.J. & MCKECHNIE, K. (1994). Characterization of the prostaglandin E_2-sensitive (EP)-receptor in the rat isolated trachea. *Br. J. Pharmacol*., **112,** 133 – 136.

LYDFORD, S.J., MCKECHNIE, K.C.W. & DOUGALL, I.G. (1996). Pharmacological studies on prostanoid receptors in the rabbit isolated saphenous vein: a comparison with the rabbit ear artery. *Br. J. Pharmacol*., **117,** 13 – 20.

MANTELLI, L., AMERINI, S., RUBINO, A. & LEDDA, F. (1991). Prejunctional prostanoid receptors on cardiac adrenergic terminals belong to the EP_3 subtype. *Br. J. Pharmacol*., **102,** 573 – 576.

MEJA, K., SELDON, P.M., BARNES, P.J. & GIEMBYCZ, M.A. (1996). Characterisation of the prostanoid receptors that mediates inhibition of lipopolysaccharide-induced tumour necrosis factor-α generation from human monocytes. *Br. J. Pharmacol*., **119,** 158P.

MILNE, S.A., ARMSTRONG, R.A. & WOODWARD, D.F. (1995). Comparison of the EP receptor subtypes mediating relaxation of the rabbit jugular and pig saphenous veins. *Prostaglandins*, **49,** 225 – 237.

MILNE, S.A., LEE, J., ARMSTRONG, R.A. & WOODWARD, D.F. (1994). Human monocytes and cultured CHO cells both express EP_4 receptors positively coupled to adenylyl cyclase. *Br. J. Pharmacol*., **113,** 8P.

MOLDERINGS, G.J., COLLING, E., LIKUNGA, J., JAKSCHIK, J. & GOTHERT, M. (1994). Modulation of noradrenaline release from the sympathetic nerves of the human saphenous vein and pulmonary artery by pre-synaptic EP_3- and DP-receptors. *Br. J. Pharmacol*., **111,** 733 – 738.

NAMBA, T., SUGIMOTO, Y., NEGISHI, M., IRIE, A., USHIKUBI, F., KAKIZUKA, A., ITO, S., ICHIKAWA, A. & NARUMIYA, S. (1993). Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP_3 determines G-protein specificity. *Nature*, **365,** 166 – 170.

NIALS, A.T., VARDEY, C.J., DENYER, L.H., THOMAS, M., SPARROW, S.J., SHEPHERD, G.D. & COLEMAN, R.A. (1993). AH 13205, a selective prostanoid EP_2-agonist. *Cardiovasc. Drug. Rev*., **11,** 165 – 179.

NISHIGAKI, N., NEGISHI, M., HONDA, A., SUGIMOTO, Y., NAMBA, T., NARUMIYA, S. & ICHIKAWA, A. (1995). Identification of prostaglandin E receptor 'EP_2' cloned from mastocytoma cells as EP_4 subtype. *FEBS Letts*., **364,** 339 – 341.

PRABHAKAR, U., LIPSHUTZ, D., O'LEARY-BARTUS, J., SLIVJAK, M.J., SMITH, E.F., LEE, J.C. & ESSER, K.M. (1994). Characterization of cyclic AMP-dependent inhibition of LPS-induced TNFα production by rolipram, a specific phosphodiesterase inhibitor. *Int. J. Immunopharmacol*., **16,** 805 – 816.

QIAN, Y.-M., JONES, R.L., CHAN, K.-M., STOCK, A.I. & HO, J.K.S. (1994). Potent contractile actions of prostanoid EP_3-receptor agonists on human isolated pulmonary artery. *Br. J. Pharmacol*., **113,** 369 – 374.

REGAN, J.W., BAILEY, T.J., DONELLO, J.E., PIERCE, K.L., PEPPERL, D.J., ZHANG, D., KEDZIE, K.M., FAIRBAIRN, C.E., BOGARDUS, A.M., WOODWARD, D.F. & GIL, D.W. (1994a). Molecular cloning and expression of human EP_3 receptors: evidence for three variants with different carboxy termini. *Br. J. Pharmacol*., **112,** 377 – 385.

REGAN, J.W., BAILEY, T.J., PEPPERL, D.J., PIERCE, K.L., BOGARDUS, A.M., DONELLO, J.E., FAIRBAIRN, C.E., KEZDIE, K.M., WOODWARD, D.F. & GIL, D.W. (1994b). Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP_2-subtype. *Mol. Pharmacol*., **46,** 213–220.

SELDON, P.M., BARNES, P.J., MEJA, K. & GIEMBYCZ, M.A. (1995). Suppression of lipopolysaccharide-induced tumor necrosis factor-α generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase. *Mol. Pharmacol*., **48,** 747–757.

SEVERN, A., RAPSON, N.T., HUNTER, C.A. & LIEW, F.Y. (1992). Regulation of tumor necrosis factor production by adrenaline and β-adrenergic agonists. *J. Immunol*., **148,** 3441–3445.

SINHA, B., SEMMLER, J., EISENHUT, T., EIGLER, A. & ENDRES, S. (1995). Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by combination of phosphodiesterase inhibitors and prostanoids. *Eur. J. Immunol*., **25,** 147–153.

SONNENBURG, W.K. & SMITH, W.L. (1988). Regulation of cyclic AMP metabolism in rabbit cortical collecting tubule cells by prostaglandins. *J. Biol. Chem*., **263,** 6155–6160.

STRONG, P., COLEMAN, R.A. & HUMPHREY, P.P.A. (1992). Prostanoid-induced inhibition of lipolysis in rat isolated adipocytes: probable involvement of EP_3 receptors. *Prostaglandins*, **43,** 559–566.

STURZENBECHNER, S., HABEREY, M., MULLER, B., SCHILLINGER, E., SCHRODER, G., SKUBALLA, W., STOCK, G., VORBRUGGEN, H. & WITT, W. (1986). Pharmacological profile of a new carbacyclin derivative with high metabolic stability and oral activity in the rat. *Prostaglandins*, **31,** 95–109.

TAFFET, S.M., SINGHEL, K.J., OVERHOLTZER, J.F. & SHURTLEFF, S.A. (1990). Regulation of tumor necrosis factor expression in a macrophage-like cell line by lipopolysaccharide and cyclic AMP. *Cell. Immunol*., **155,** 1230–1236.

TEIXEIRA, M.M., AL-RASHED, S., ROSSI, A.G. & HELLEWELL, P.G. (1997). Characterization of the prostanoid receptors mediating inhibition of PAF-induced aggregation of guinea-pig eosinophils. *Br. J. Pharmacol*., **121,** 77–82.

WATABE, A., SUGIMOTO, Y., HONDA, A., IRIE, A., NAMBA, T., NEGISHI, M., ITO, S., NARUMIYA, S. & ICHIKAWA, A. (1993). Cloning and expression of cDNA for a mouse EP_1 subtype of prostaglandin E receptor. *J. Biol. Chem*., **268,** 21075–21078.

WISE, H. & JONES, R.L. (1994). Characterization of prostanoid receptors on rat neutrophils. *Br. J. Pharmacol*., **113,** 581–587.

WISE, H. & JONES, R.L. (1996). Focus on prostacyclin and its novel mimetics. *Trends Pharmacol. Sci*., **17,** 17–21.

WOODWARD, D.F., PEPPERL, D.J., BURKEY, T.H. & REGAN, J.W. (1995). 6-Isopropoxy-9-oxoxanthine-2-carboxylic acid (AH6809), a human EP_2-receptor antagonist. *Biochem. Pharmacol*., **50,** 1731–1733.

ZENG, L., AN, S. & GOETZL, E.J. (1995). Independent down-regulation of EP_2 and EP_3 subtypes of the prostaglandin E_2 receptors on U937 human monocytic cells. *Immunology*, **86,** 620–628.

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