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Activation of EP_4 and EP_2 receptors inhibits TNFα release from human alveolar macrophages.

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Abstract

Prostaglandin E_2 (PGE_2) has been shown to inhibit mediator release from human alveolar macrophages (AMs), but the prostanoid receptor(s) mediating this response have not been documented. To investigate this we conducted a range of pharmacological and expression based studies in monocyte derived macrophages (MDMs) and AMs.

MDMs were obtained by *in vitro* differentiation of monocytes from the peripheral blood of healthy human volunteers. Human AMs were obtained by perfusion of lung tissue from carcinoma resection patients.

In MDMs, PGE_2 potently inhibited lipopolysaccharide-induced TNFα release ($p[A]_{50}$ = 8.51 ± 0.11, maximum inhibition = 95.9% ± 4.8%). In human AMs, PGE_2 also inhibited TNFα release but the concentration-effect curve observed was very flat and inhibition was incomplete. The shape of the PGE_2 curve in AMs suggested that its effects were mediated by activation of a heterogeneous receptor population. Expression studies combined with the use of various EP receptor agonists and a selective EP_4-receptor antagonist (Ono-AE2-227) confirmed that the inhibitory effects of PGE_2 in both AMs and MDMs were mediated by activation of EP_4 and EP_2 receptors.

These data indicate that both EP_4 and EP_2 selective agonists may have anti-inflammatory properties in lung diseases where macrophages play a role.

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Introduction

Alveolar macrophages are a key component of innate immune defence in the lungs. They remove inhaled material, including particulates and microorganisms, by phagocytosis and by releasing a wide range of mediators which recruit other cells, notably neutrophils, into the lungs. This pro-inflammatory response is important in clearing foreign agents but it has been hypothesised that in certain conditions it is excessive, leading to destruction of host tissues and the development of chronic inflammatory disease [1]. This has promoted considerable interest in discovering pharmacological agents that dampen down release of pro-inflammatory mediators from macrophages.

One such agent is prostaglandin E_2 (PGE_2) which elicits a range of biological effects via its interaction with G-protein coupled receptors, designated EP_1, EP_2, EP_3 and EP_4 [2]. EP receptors all have high affinity for PGE_2, but differ in their affinities for various synthetic agonists and antagonists [3;4]. PGE_2 has been shown to affect many macrophage functions, including tumouricidal activity [5;6], phagocytosis [7;8] and mediator release [9-13]. The effects of PGE_2 on mediator release are generally considered to be anti-inflammatory as it has been demonstrated to inhibit the release of a number of cytokines and chemokines, whilst inducing expression of the anti-inflammatory cytokine interleukin-10 (IL-10) [14;15]. These observations suggest that it may be possible to develop EP receptor agonists as therapeutic drugs to treat inflammatory diseases. However, few studies have assessed the effects of such agents on human AMs. PGE_2 mediated inhibitions of IL-1 [16], IL-8 [17], TNFα [18] and fibronectin [10] have been reported in these cells but the receptor type(s) mediating these responses were not investigated. The aim of this present study was therefore to address this issue and by so doing provide information on the potential efficacy of EP agonists as anti-inflammatory drugs for pulmonary diseases such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF) or acute respiratory distress syndrome (ARDS).

Materials and methods

Reagents

LPS (*E. coli* 026:B6), 3-isobutyl-1-methylxanthine (IBMX), indomethacin and Foetal Calf Serum (FCS) were from Sigma-Aldrich, Poole, Dorset UK. PGE_2, PGE_1-OH, sulprostone, butaprost (methyl ester) were from Cayman Chemical, Ann Arbor, Michigan USA. The selective EP_4-receptor antagonist, Ono-AE2-227 [19] was synthesised by the Medicinal Chemistry Department, AstraZeneca R&D Charnwood. RPMI, DMEM, Iscoves modified Dulbecco's medium containing GlutaMAX™ (IMDM), Geneticin, L-glutamine and Penicillin/Streptomycin were from Invitrogen Ltd, Paisley UK.

Generation of HEK cells stably transfected with the human EP_2 or EP_4 receptor

The human EP_2 and EP_4 cDNA sequences were cloned into the mammalian expression vector pIRESneo2 (Clontech Laboratories Inc. Mountain View, CA

USA). Plasmid DNA was transfected into HEK cells using FuGENE 6 transfection reagent (Roche Molecular Systems, Inc., Alameda, CA USA), and expressing cells selected using 1mg/ml Geneticin solution. Specific cell surface expression of EP_2 or EP_4 was confirmed by antibody staining (data not shown).

Preparation of macrophages

Human MDMs were prepared from peripheral blood monocytes isolated from healthy human volunteers. Venous blood was layered onto lymphoprep (Axis-Shield UK, Kimbolten, Cambs UK), and centrifuged at 800*g* for 20 minutes with no brake. The mononuclear cell layer was removed and platelet contamination removed by several low speed (80*g*) centrifugation/wash steps using 5% v/v autologous serum in 0.9% w/v NaCl. Monocytes were prepared by negative selection using human monocyte isolation kit II, (Miltenyi Biotec, Surrey UK) according to the manufacturers instructions. Cells were cultured in IMDM /10% v/v FCS/penicillin (100U/ml)/streptomycin (100 mg/ml) with 50 ng/ml human M-CSF (R&D Systems. Abingdon, Oxon UK). Medium was changed every 5 days. Cells were used 10-14 days after isolation.

Human AMs were obtained from resection tissue from lung cancer patients. Macrophages were flushed from the tissue with PBS. Macrophages were purified by one hour adhesion to tissue culture plastic in serum free RPMI. Contaminating cells were removed by stringent washing in RPMI. Cells were incubated for 1-3 days in RPMI/10% FCS before use in cytokine release experiments.

Real time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) expression studies

RNA was isolated from cell homogenates using Trizol (Invitrogen) and prepared using RNeasy miniprep column (Qiagen, Crawley, West Sussex UK) with DNAse. RNA was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturers recommended protocol. Control (-RT) reactions were done in the absence of reverse transcriptase.
The primers used comprised the following nucleotides based on published sequences: EP_1-Forward, ATGGTGGGCCAGCTTGTC; EP_1-Reverse, GCCACCAACACCAGCATTG; EP_2-Forward, GCCTGCAACTTCAGTGTCATTC; EP_2-Reverse, GTCCGCAGCGGCTTCT ; EP_3-Forward, GACGGCCATTCAGCTTATGG; EP_3-Reverse, CTGATTGAAGATCATTTTCAACATCA; EP_4-Forward, ACATGTACGCGGGCTTCAG; EP_4-Reverse, GCCGCACACAAGCACGTT. All primers were supplied by Eurogentec Ltd, Southampton, Hampshire UK. The 18S probe had the following modification: 5'-Yakima Yellow, 3'- Black Hole Quencher 1. Real-time PCR was conducted on an Mx3000P real-time PCR machine (Stratagene Europe, Amsterdam The Netherlands). Samples were run in triplicate for each gene. PCR conditions: 95°C, 5 minutes; (95°C, 20 seconds, 60°C, 90 seconds)x50; 95°C, 1 minute, followed by dissociation curve from 55°C to 95°C. Data was collected at the end of each cycle and throughout each dissociation curve. 8-point, 2-fold standard curves were run for each gene using a Human Reference cDNA (Stratagene) as template. Expression values calculated from a standard curve and normalised to 18S levels.

Flow Cytometry

MDMs and AMs were harvested by scraping, washed in PBS and EP receptor proteins labelled using BD Pharmingen Cytofix/cytoperm^{TM} kit according to the manufacturers instructions. Rabbit IgG control was from Sigma-Aldrich, EP_2 and EP_4 antibodies were from Cayman Chemical and secondary goat anti rabbit IgG-Alexafluor488 antibody was from Invitrogen Ltd. Primary antibodies were used at 2μg/ml final concentration, secondary antibody at 4μg/ml. 10 μl/ml FcR Block (Miltenyi Biotec, Surrey UK) was added at each stage. Fluorescence was measured using a Beckman Coulter FC500 flowcytometer.

cAMP assay

EP_4 or EP_2 receptor transfected HEK cells were harvested using Accutase^{TM} (Innovative Cell Technologies Inc. San Diego CA), and plated at 50,000 cells/well in DMEM/10% FCS in poly-D-lysine coated 96 well plates (BD Labware, Bedford MA). The following day, cells were washed twice and incubated in serum-free DMEM containing IBMX (1mM). 20 minutes later, Ono-AE2-227 was added for 15 minutes, and then PGE_2 added for a further 20 minutes. cAMP in cell lysates was measured using RPN225 cAMP EIA Biotrak system (GE Healthcare Amersham Biosciences, Chalfont St Giles, Bucks UK) according to protocol 3 of the manufacturers instructions.

Cytokine release assay

MDMs and AMs were harvested, washed and plated at 50,000 cells/well in a 96 well plate in IMDM containing 0.5% FCS for 1-2 hours. EP agonists were then added as indicated, followed 15 minutes later by LPS (100ng/ml final concentration) and cells incubated for 24 hours. In experiments using Ono-AE2-227, this agent was added 20 minutes before PGE_2. Where used, indomethacin was added 2 hours before addition of PGE_2. Supernatants were harvested and cytokines measured using optEIA ELISA kits (BD Biosciences, Erembodegen, Belgium) according to manufacturers instructions.

Data Analysis

Logistic curve fitting

Where agonist E/[A] curve data were clearly monophasic they were fitted to the following form of the Hill equation:

$$E = \frac{\alpha[A]^{nH}}{[A]^{nH} + [A]_{50}^{nH}} \qquad (1)$$

in which α, $[A]_{50}$ and n_H are the asymptote (maximum effect), location (potency) and slope parameters, respectively. $[A]_{50}$ values were assumed to be log-normally distributed and quoted as $p[A]_{50}$ ($-\log[A]_{50}$) values.

In most instances data from individual experiments was fitted to equation 1. However the AM data were more variable than the MDM data and in this case the mean data were fitted to equation 1.
All cytokine data were expressed as % inhibitions of the LPS induced response. cAMP data was expressed as % maximum response to PGE_2.

Antagonist affinity estimation.

$[A]_{50}$ data obtained in antagonist experiments were fitted to the following linear form of the Schild equation [20]:

$$\log_{10}[A]_{50} = \log_{10}[A]_{50}^{C} + \log_{10}\left(1 + [B]^{n} / K_{B}\right) \qquad (2)$$

where $[A]_{50}^{C}$ is the estimated control $[A]_{50}$ value, [B] is the concentration of the antagonist, K_B is the antagonist equilibrium constant and n is equivalent to the Schild plot slope parameter (unity for simple competition). If n was not significantly different from unity, it was constrained to unity for pK_B ($-\log K_B$) estimation.

Computer simulation of two-receptor systems:

In order to interpret the data from the experiments using Ono-AE2-227 it was necessary to consider the action of a selective antagonist on a one agonist: two-receptor system. The model of Furchgott [21] was used to simulate the expectations of such an interaction. This model is an extension of the traditional model for a one-receptor system [22;23]) in which pharmacological effect (E) is assumed to be a function of stimulus (S). In the two-receptor case the combined stimulus is imparted by the interaction of the agonist at each receptor, thus :

$$S = S_1 + S_2 = e_1p_1 + e_2p_2 \qquad (3)$$

where e_1 and e_2 are the efficacies of the agonist at each receptor and p_1 and p_2 are the respective fractional occupancies.
Furchgott assumed that the relation between S and E can be described by:

$$\frac{E}{E_m} = \frac{S^n}{1 + S^n} = \frac{(e_1p_1 + e_2p_2)^n}{1 + (e_1p_1 + e_2p_2)^n} \qquad (4)$$

When a competitive antagonist able to interact with each receptor is present the occupancies p_1 and p_2 are defined by:

$$p_1 = \frac{[A]}{K_{A1}(1 + [B]/K_{B1}) + [A]} \qquad (5)$$

$$p_2 = \frac{[A]}{K_{A2}(1 + [B]/K_{B2}) + [A]} \qquad (6)$$

where K_{A1}, K_{A2}, K_{B1} and K_{B2} are the dissociation constants of the agonist and antagonist respectively for the two receptors.
Equations (4), (5) and (6) were used to generate theoretical agonist concentration-effect curves in the presence and absence of the competitive antagonist in the MDM and AM systems. These simulations were then superimposed on the experimental data to see how well the latter fitted the model.

All of the data fitting procedures and simulations were carried out using Microsoft Excel. Results are expressed and plotted as mean values $\pm$ s.e. Statistical differences were assessed by the use of Student's *t* test or ANOVA as appropriate and considered significant at the level $p < 0.05$.

Results

Inhibition of TNFα release from human macrophages by EP receptor agonists

Preliminary experiments revealed that PGE_2 potently inhibited LPS-induced release of a variety of mediators, including TNFα, GM-CSF and MIP-1β from MDMs (Figure 1). Of these the most robust signal was TNFα so this readout was used in all subsequent studies in both MDMs and AMs. PGE_2 caused a concentration-dependent inhibition of LPS-induced TNFα release from both MDMs (Figure 2a) and AMs (Figure 2b). In MDMs the E/[A] curve appeared monophasic and logistic curve-fitting (equation 1) yielded a $p[A]_{50}$ of 8.51 ± 0.11; n = 7 and a maximum inhibition (α) of 95.9% ± 4.8%. In AMs, the PGE_2 E/[A] curve appeared biphasic precluding logistic curve fitting and suggesting that more than one receptor type may be involved in the response. To further investigate this we tested the effects of a range of EP receptor agonists. PGE_1-OH (EP_4 & EP_2) [24;25] and butaprost (an EP_2 receptor selective agonist) [4;25], were active in both systems. In MDMs they produced similar maximum inhibitions of TNFα as PGE_2 (95.7% ± 1.2%; n = 7 and 94.4% ± 4.78%; n = 4, respectively) but were 10-fold (PGE_1-OH; $p[A]_{50}$ = 7.51 ± 0.07; n = 7) and 245-fold (butaprost; $p[A]_{50}$ = 6.12 ± 0.13; n = 4), less potent than PGE_2. By measuring cAMP elevation in HEK cells transfected with human EP_4 we confirmed that PGE_1-OH is a relatively potent EP_4 agonist ($p[A]_{50}$ = 8.95 ± 0.15; n = 4) and that butaprost is inactive at EP_4 up to concentrations of 10 μM (data not shown). In AMs the PGE_1-OH and butaprost E/[A] curves were not fully defined at the highest concentrations tested, again precluding logistic curve fitting, but these two agonists were clearly less potent than PGE_2. Sulprostone, (an EP_1/EP_3 selective agonist) [4], did not cause significant inhibition in either system at concentrations as high as 10 μM. The activity of butaprost suggested the presence of EP_2 receptors in both systems but the relatively high potency of PGE_2 and PGE_1-OH suggested an additional receptor was involved. Given the inactivity of sulprostone, the most likely candidate was the EP_4 receptor.

Expression of EP receptors in human macrophages

Real-time quantitative RT-PCR was used to determine the pattern of mRNA expression for the four known EP receptors in MDMs and AMs. This analysis demonstrated significant expression of EP_2 and EP_4 receptors in both systems but little or no expression of EP_1 and EP_3 receptors (Figure 3a). We also assessed EP_2 and EP_4 protein expression in MDMs and AMs by flow cytometry (Figures 3b and 3c). These studies confirmed that both macrophage systems expressed significant levels of EP_2 (percentage of cells with positive expression; MDMs 96.3 ± 2.2; n = 4; AMs 87.7 ± 5.8; n = 3) and EP_4 receptors (percentage of cells with positive expression; MDMs 78.5 ± 7.4; n = 4; AMs 33.0 ± 12.4; n = 3).

Characterisation of Ono-AE2-227 in HEK cells stably transfected with human EP_4 or EP_2 receptors.

The expression pattern and agonist potency order obtained indicated EP_4 and EP_2 receptor involvement in inhibition of TNFα release from MDMs and AMs. To

provide further evidence for this, a reported selective EP_4 receptor antagonist, Ono-AE2-227 was synthesised and characterised in HEK cells stably expressing human EP_4 or EP_2 receptors. In the EP_4 system, this compound inhibited PGE_2-mediated elevations of cAMP and the resulting curve displacements did not deviate significantly from parallelism (ANOVA) (Figure 4a). This apparent competitive behaviour was confirmed by analysis of the computed $[A]_{50}$ values using equation (2) which yielded a slope value of 1.11 ± 0.07; $n = 5$. This value was not significantly different from unity and the resulting pK_B estimate was 9.17 ± 0.11; $n = 5$. The inset of figure 4a shows an example of a Clark plot [26] generated from one of the five experiments.
In contrast, in the EP_2 system this compound did not block the effects of PGE_2 at concentrations up to 1μM (Figure 4b). These data confirmed that Ono-AE2-227 is a potent EP_4 receptor antagonist with high selectivity over EP_2 receptors.

Effect of Ono-AE2-227 on PGE_2-induced inhibitions of TNFα release from MDMs and AMs.

In MDMs, Ono-AE2-227 produced parallel rightward shifts of the PGE_2 E/[A] curve indicating involvement of EP_4 receptors in this response, but the interaction was inconsistent with simple competitive behaviour (Figure 5a; compare with Figure 4a). This was confirmed by fitting the $[A]_{50}$ data to equation (2) which yielded a slope value (0.53 ± 0.03; $n = 4$) significantly less than unity (data not shown). We used a theoretical model that describes the behaviour of an agonist in a system in which its response is mediated through two distinct receptor types (see Methods). Figure 5b shows the result of this analysis in which we super-imposed the model-generated curves on the experimental data (see figure legend for model parameters used). The good accordance of the lines with the data points support our hypothesis; Ono-AE2-227 initially shifts the PGE_2 control curve by blocking EP_4 receptors but as its concentration is increased the PGE_2 curves hit a "resistant phase" that is most likely the result of EP_2 receptor activation.

In AMs, Ono-AE2-227 amplified the response to LPS in a concentration-dependent manner. Thus, when expressed as a percentage of the response to LPS alone, Ono-AE2-227 elicited "negative inhibitions" of the LPS-induced TNFα release in the presence of low concentrations of PGE_2 (Figure 6a). Clearly these basal effects of Ono-AE2-227 were complicating the analysis of the data. The most likely explanation for this potentiation was that endogenous PGE_2 released from the AMs had an inhibitory effect on cytokine release and that Ono-AE2-227 blocked this response. Based on this assumption, we repeated the experiment in AMs which had been treated with the cyclo-oxygenase inhibitor, indomethacin. This protocol eliminated the basal effects of Ono-AE2-227 and the resulting data is shown in Figure 6b. Under these conditions, inhibition was seen with the EP_4 antagonist but the interaction was again clearly not consistent with simple competition; Clark analysis yielded a slope value of 0.66. Again we modelled the data using the one-agonist, two-receptor model and found good accordance with the experimental data (Figure 6c, see legend for model parameters used).

Discussion

To our knowledge this is the first study to fully characterise the receptors involved in PGE_2 mediated inhibition of cytokine release in both human MDMs and human AMs. The agonist potency order obtained in both these systems was similar (Figure 2) suggesting that MDMs are a good surrogate system for AMs. The relatively high potency of PGE_2 and PGE_1-OH and the activity of butaprost (EP_2 selective) suggested that both EP_4 and EP_2 receptors were present in these cells. This assertion was supported by the biphasic nature of the PGE_2 E/[A] curve in AMs. Such data is consistent with the activation of a mixed receptor population in which the agent in question exhibits partial agonism, i.e. PGE_2 sub-maximally activates EP_4 and EP_2 receptors in AMs leading to an apparent additive effect. The higher potency phase results from EP_4 receptor activation and the lower potency phase from EP_2 receptor activation. In contrast, the PGE_2 E/[A] curve appears monophasic in MDMs because in this system it behaves as a full agonist, i.e. maximum activation of EP_4 receptors at low concentrations means that subsequent activation of EP_2 receptors at higher concentrations cannot produce an additive effect (see Tables in figure 5 and 6 figure legends for the efficacy (e) values used to simulate the data in MDMs and AMs respectively). Sulprostone was inactive in both MDMs and AMs, indicating the absence of EP_1 and EP_3 receptors.

Our initial pharmacological findings were supported by expression studies in these cells that demonstrated significant expression of EP_4 and EP_2 receptor mRNA and protein, but barely detectable levels of EP_1 and EP_3 receptor mRNA (Figure 3). These data are similar to those reported in other human macrophage systems [27] although Takayama *et al*. found mRNA for only EP_4 in human MDMs [15]. This may be due to the increased sensitivity of the quantitative real time RT-PCR used in this study over the static endpoint RT-PCR method used in the Takayama study.

The EP_4-receptor selective antagonist, Ono-AE2-227 produced rightward shifts of the PGE_2 E/[A] curve in MDMs that were parallel but clearly did not accord with simple competition at a homogeneous population of receptors (Figure 5a). A theoretical model describing the action of an agonist with two distinct receptor populations was able to accommodate our data set well (Figure 5b). The finding that low concentrations of Ono-AE2-227 shifted the PGE_2 E/[A] curve indicate that EP_4 receptors are the dominant receptor type mediating the agonist responses. The PGE_2 E/[A] curve obtained in the presence of 1 μM Ono-AE2-227 is likely to represent EP_2 activity in MDMs, since greater than 99.9% of the EP_4 receptors are occupied by this concentration of antagonist (see Figure 4a).

In the AM system, Ono-AE2-227 clearly potentiated the LPS response in a concentration dependent manner (Figure 6a). This suggested that these cells were releasing endogenous PGE_2, a phenomenon that has been reported by other investigators [16;28]. We did not observe such an effect in our MDM system suggesting that the activation state of these cells may differ. Addition of indomethacin eliminated this basal effect of Ono-AE2-227 in AMs, confirming that it was the result of endogenous prostanoid release. In the presence of indomethacin, Ono-AE2-227 blocked the responses to PGE_2 but the curve shifts did not conform to the behaviour expected of a one-receptor system. The data could again be

accommodated by the theoretical one agonist:two receptor model, requiring only a small alteration of the model parameters corresponding to a 2 and 4-fold lower protein expression levels of EP_4 and EP_2 receptors respectively (compare model parameters in legends to figures 5 and 6). The lower maximum inhibitions observed in the AMs are consistent with the lower expression of EP_4 and EP_2 receptors suggested by the modelling and indicated by our protein expression studies (figures 3b and 3c). This may be a consequence of partial receptor down-regulation [29] induced by the endogenous release of PGE_2 from these cells.

Our findings are consistent with previously reported data from human monocytes and rodent macrophages, where EP_2 and EP_4 receptors have been demonstrated to mediate the anti-inflammatory effects of PGE_2 [11;12;30]. By extending these studies to human AMs, we have provided evidence to suggest that EP_4, EP_2 or mixed EP_4/EP_2 receptor agonists could be useful anti-inflammatory drugs in diseases such as COPD and ARDS.

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Figure Legends.

Figure 1. Effects of PGE_2 on TNFα, GM-CSF and MIP-1β release from MDMs.

E/[A] curves for PGE_2-induced inhibitions of LPS stimulated TNFα (a), GM-CSF (b) and MIP-1β (c) from MDMs. The data are the mean of 3-7 experiments with vertical lines indicating the s.e. Lines drawn through the MDM data are the result of curve fitting using equation (1).

Figure 1

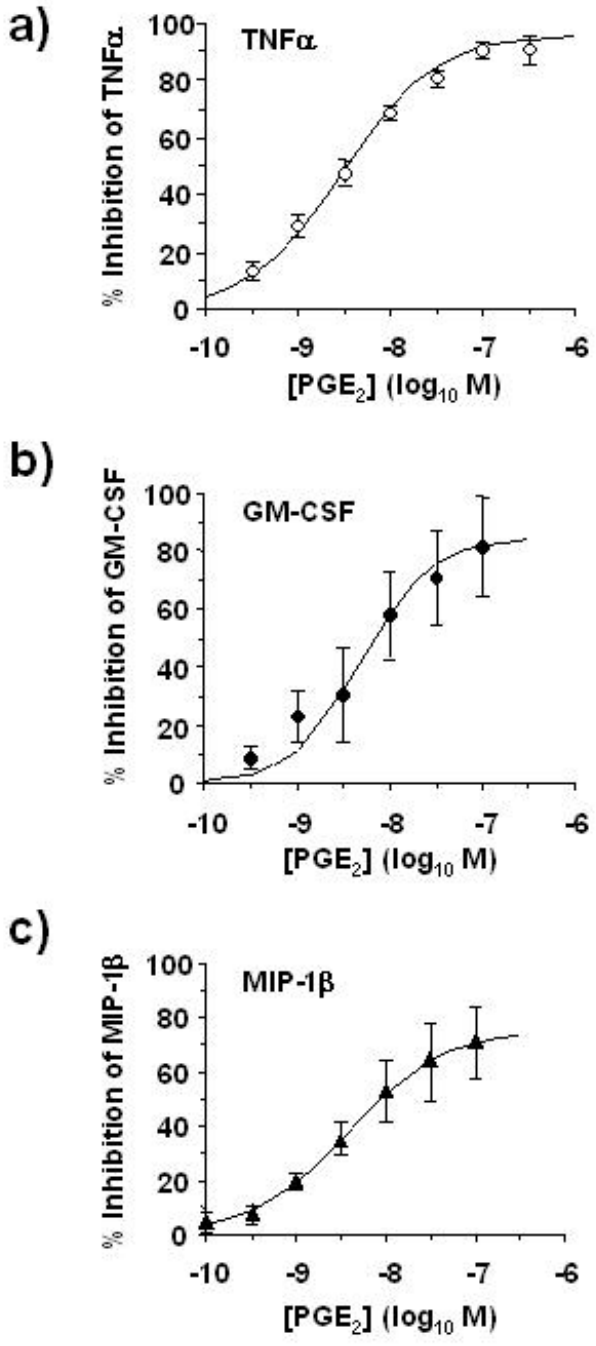


Figure 2. Effects of EP receptor agonists in human MDMs and AMs.

E/[A] curves for agonist induced inhibitions of LPS stimulated TNFα release from MDMs (a) and AMs (b). Agonists used were PGE_2 (○), PGE_1-OH (●), Butaprost (△) and Sulprostone (▲). The data are the mean of 4-7 experiments with vertical lines indicating the s.e. Lines drawn through the MDM data are the result of curve fitting using equation (1). The AM data could not be fitted.

Figure 2

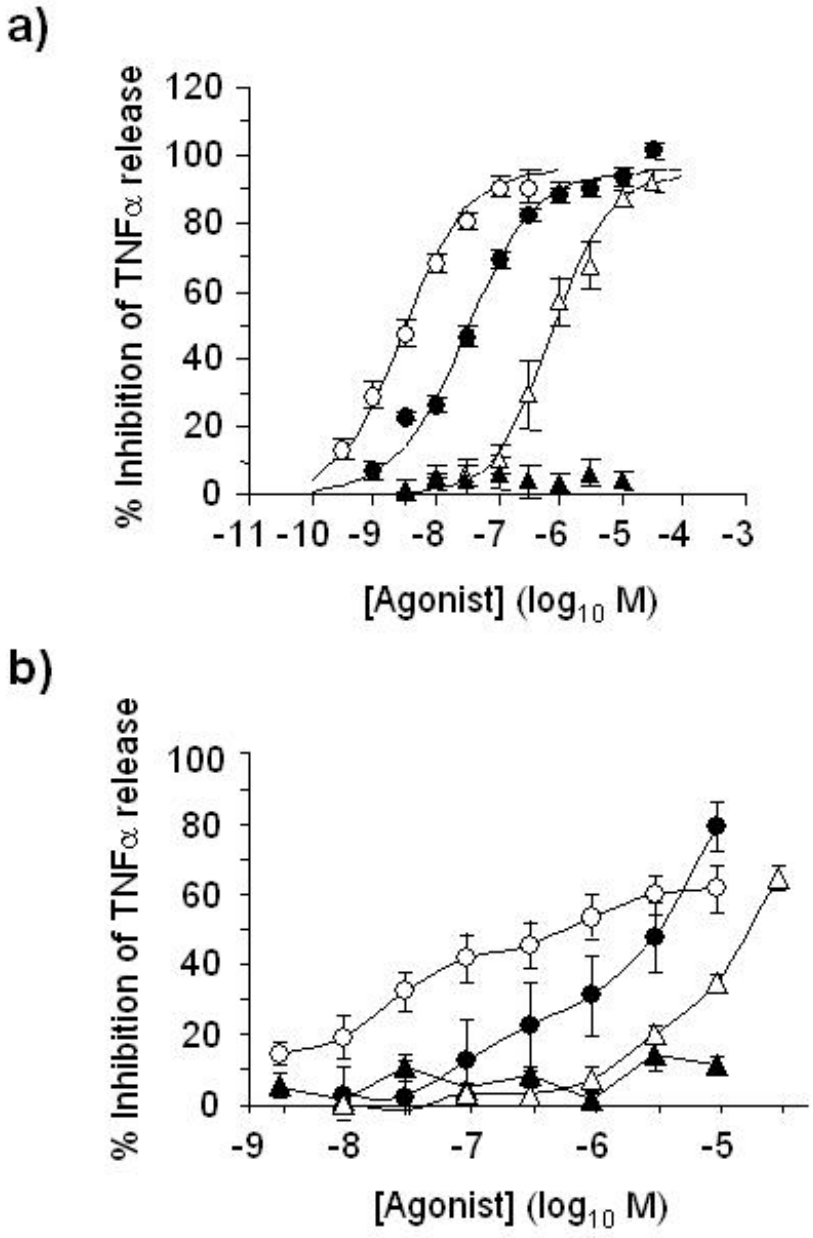


Figure 3. Expression of EP receptors in human macrophages.

Panel (a) shows mRNA levels in MDMs (□) and AMs (■) detected using quantitative RT-PCR. Data is the mean of 3 MDM donors and 3 AM donors. EP_2 and EP_4 were highly expressed in both cell types; EP_1 and EP_3 receptor expression was very low.
Panels (b and c) show protein levels of EP_2 and EP_4 receptors respectively in MDMs and AMs assessed using flow cytometry. The white peak represents the signal from the IgG control, the black peak is the signal seen with anti-receptor antibody.

Representative raw data is shown but expression was assessed in 4 MDM and 3 AM donors.

Figure 3

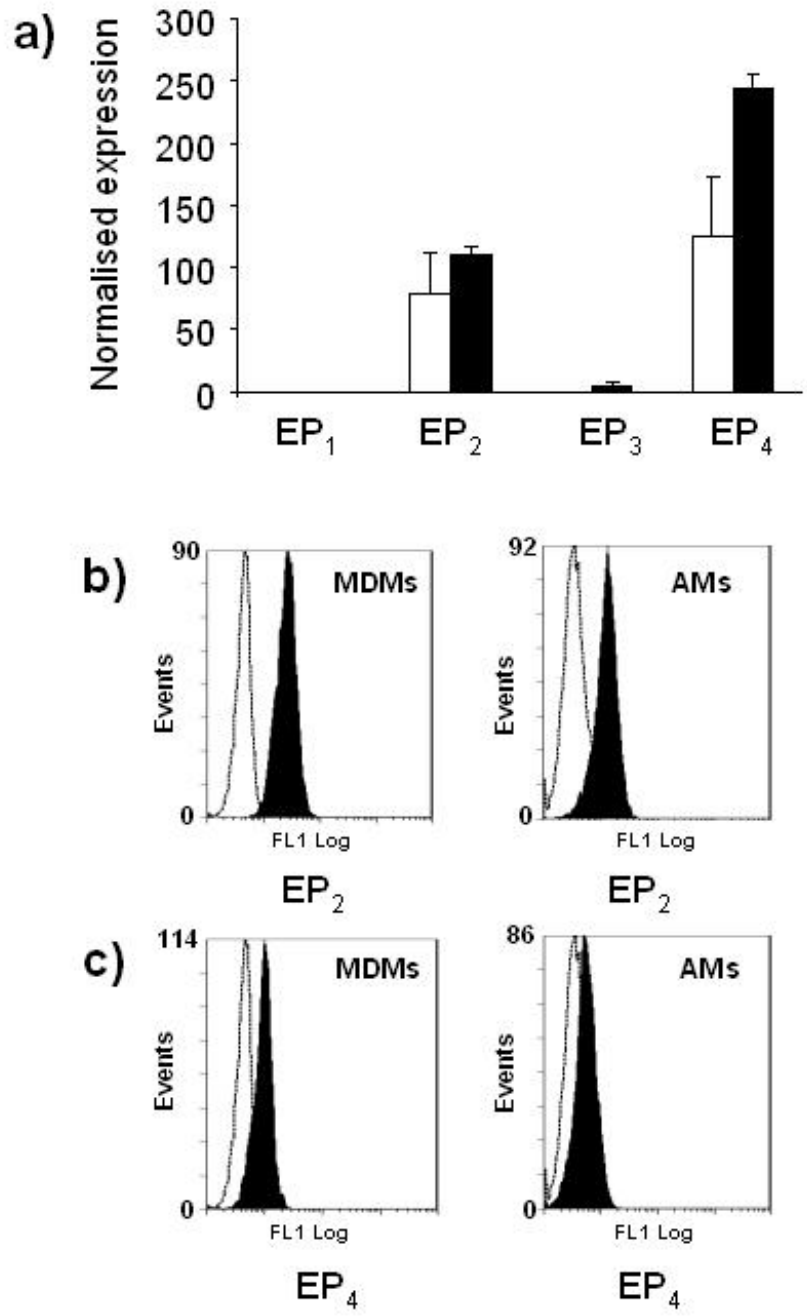


Figure 4. Effect of Ono-AE2-227 on PGE_2-induced increase in cAMP levels in HEK cells stably expressing human EP_4 or EP_2 receptors.

E/[A] curves to PGE_2 in the presence of 10-1000nM Ono-AE2-227 in EP_4 (a) and EP_2 (b) expressing cells. Data series are PGE_2 alone (○), or in the presence of 10 nM (●), 100 nM (△) or 1 μM (▲) Ono AE2-227. In both cases the data are the mean of 5

experiments with vertical lines indicating the s.e. Lines drawn through the data are the result of curve fitting using equation (1). The inset to (a) illustrates the corresponding $[A]_{50}$ data for one of the five experiments, in Clark plot form. The adherence of the data with the unit slope drawn through them indicates consistency with simple competition. The pK_B value estimated from this experiment was 9.15.

Figure 4

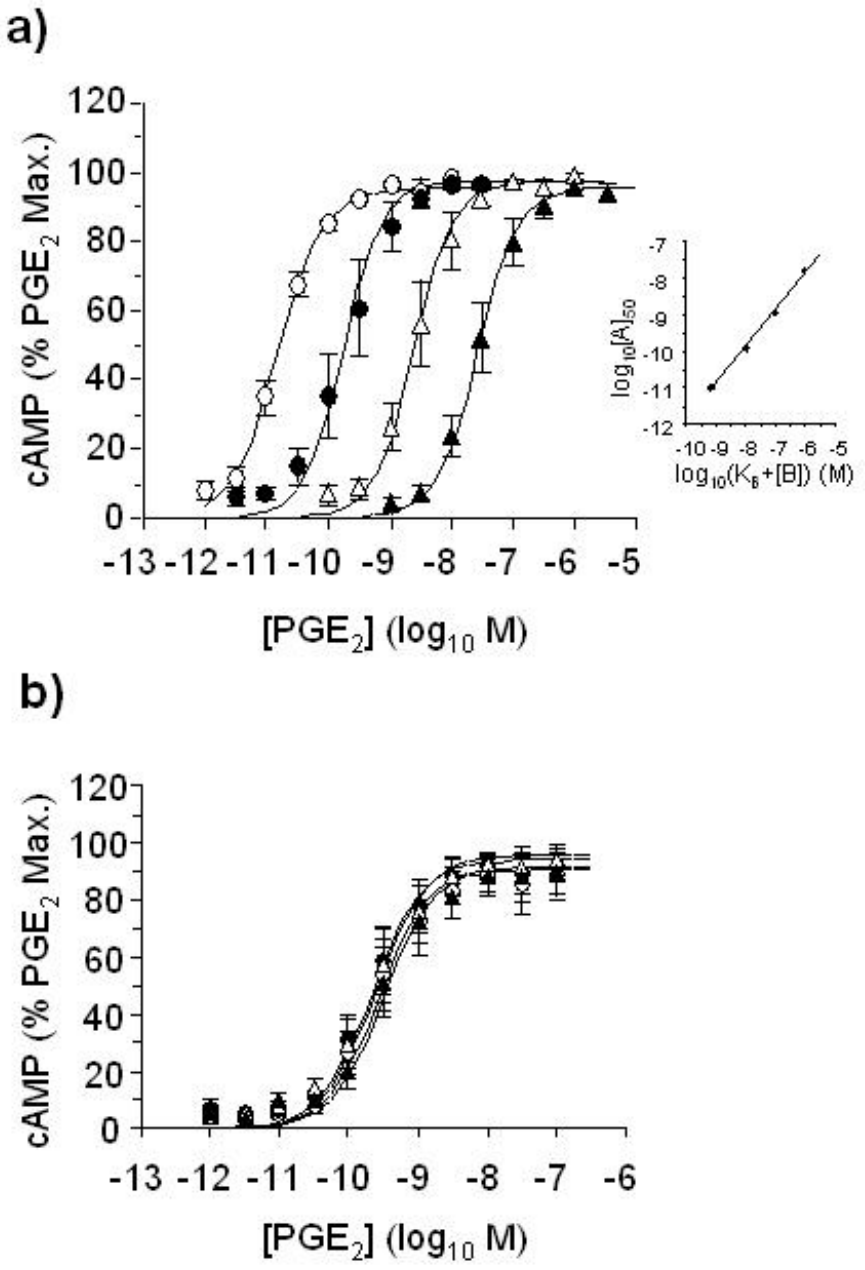


Figure 5. Effect of Ono-AE2-227 on PGE_2-induced inhibition of LPS stimulated TNFα release from MDMs.

E/[A] curves to PGE_2 in the presence of 10-1000nM Ono-AE2-227. Data series are PGE_2 alone (○), or in the presence of 10 nM (●), 100 nM (△) or 1 μM (▲) Ono AE2-227. The data are the mean of 4 experiments with vertical lines indicating the s.e. In (a) the lines drawn through the data are the results of curve fitting using equation (1). In (b) the data was fitted to the theoretical model described in the Methods Section. The parameters used are given below:

	Control	10nM	100 nM	1000nM
E_m	100%	100%	100%	100%
n	0.9	0.9	0.9	0.9
ε_1	1.0	1.0	1.0	1.0
ε_2	1.0	1.0	1.0	1.0
R_1	10.0	10.0	10.0	10.0
R_2	10.0	10.0	10.0	10.0
K_{A1}	30nM	210nM	1.8μM	21μM
K_{A2}	1μM	1μM	1μM	1μM

EP_4 is receptor one and EP_2 receptor two in the model. $e_1 = \varepsilon_1 R_1$ and $e_2 = \varepsilon_2 R_2$, where ε = intrinsic efficacy and R = receptor concentration. To simulate the addition of Ono-AE2-227, K_{A1} is multiplied by the factor $(1 + [B]/K_{B1})$, where [B] is 10, 100 or 1000 nM and K_{B1} is 0.67nM (as estimated from the experiments in EP_4 transfected HEK cells). This factor was also corrected for the protein binding of Ono-AE2-227 which we measured as approximately 99.8% in human plasma; the effective concentrations of [B] in the presence of 0.5% v/v FCS were therefore 4nM, 40nM and 480nM. K_{A2} is constant as Ono-AE2-227 does not bind to EP_2 receptors, i.e the factor $(1 + [B]/K_{B2}) = 1$ at all concentrations used.

Figure 5

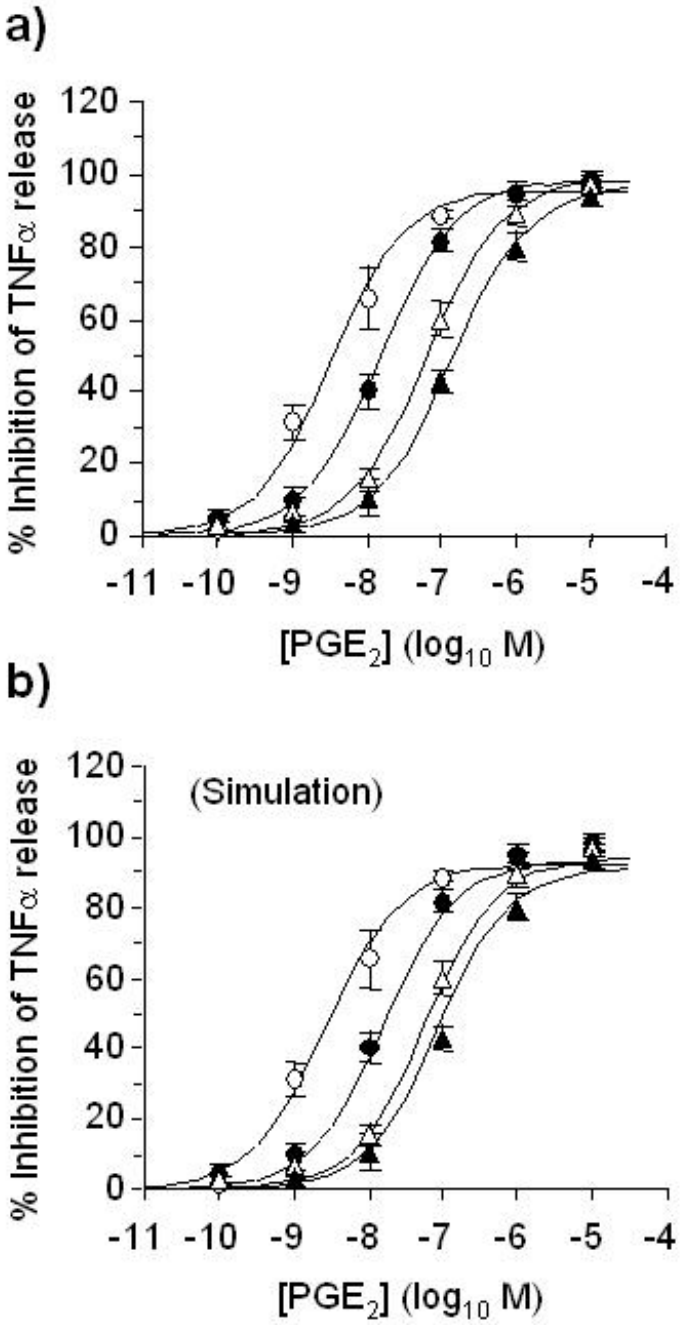


Figure 6. Effect of Ono-AE2-227 on PGE_2-induced inhibition of LPS stimulated TNFα release from AMs.

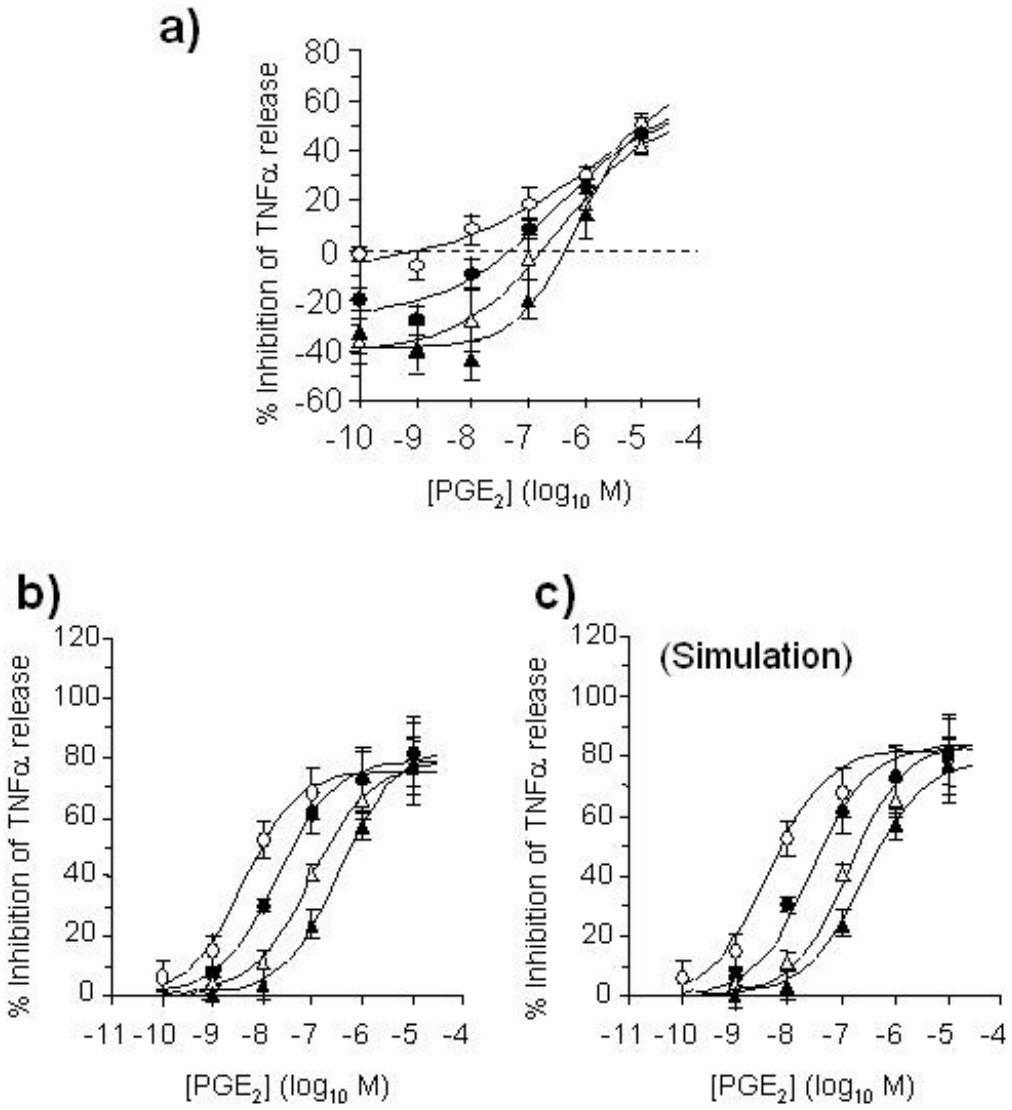
E/[A] curves to PGE_2 in the presence of 10-1000nM Ono-AE2-227. The data are the mean of 4 experiments with vertical lines indicating the s.e. Data series are PGE_2 alone (○), or in the presence of 10 nM (●), 100 nM (△) or 1 μM (▲) Ono AE2-227. In (b), the experiment was carried out in the presence of 10 μM indomethacin.

In (a and b) the lines drawn through the data are the results of curve fitting the mean data using equation (1). In (c) the data used in (b) was fitted to the theoretical model described in the Methods Section. The parameters used are given below:

	Control	10nM	100 nM	1000nM
E_m	100%	100%	100%	100%
n	0.8	0.8	0.8	0.8
ε_1	1.0	1.0	1.0	1.0
ε_2	1.0	1.0	1.0	1.0
R_1	5.0	5.0	5.0	5.0
R_2	2.5	2.5	2.5	2.5
K_{A1}	30nM	210nM	1.8μM	21μM
K_{A2}	1μM	1μM	1μM	1μM

See figure 5 legend for further explanation.

Figure 6


a)
% Inhibition of TNFα release
80
60
40
20
0
-20
-40
-60
-10 -9 -8 -7 -6 -5 -4
[PGE2] (log10 M)
b)
% Inhibition of TNFα release
120
100
80
60
40
20
0
-11 -10 -9 -8 -7 -6 -5 -4
[PGE2] (log10 M)
c)
(Simulation)
% Inhibition of TNFα release
120
100
80
60
40
20
0
-11 -10 -9 -8 -7 -6 -5 -4
[PGE2] (log10 M)