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PPAR-α and -γ but not -δ agonists inhibit airway inflammation in a murine model of asthma: *in vitro* evidence for an NF-κB-independent effect

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1 Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that have been proposed to regulate inflammation by antagonising the nuclear factor-κB (NF-κB) signalling pathway. We investigated the role of PPARs using synthetic agonists in murine models of airway inflammation, and addressed the possible effect on NF-κB signalling *in vitro* using a human epithelial cell line, A549.

2 Sensitised BALB/c mice exposed to an aerosol solution of ovalbumin had an increased number of airway eosinophils, neutrophils and lymphocytes. When given intranasally an hour before the aerosol challenge, a PPAR-α (GW 9578) and PPAR-γ (GI 262570) selective agonist as well as a dual PPAR-α/γ (GW 2331) agonist selectively inhibited allergen-induced bronchoalveolar lavage eosinophil and lymphocyte but not neutrophil influx. In contrast, a PPAR-δ agonist (GW 501516) was inactive.

3 When given intranasally an hour before challenge, PPAR-α and PPAR-γ selective agonists as well as a dual PPAR-α/γ agonist did not inhibit lipopolysaccharide-induced bronchoalveolar lavage neutrophil influx or tumour necrosis factor-α (TNF-α) and KC production.

4 In A549 cells, selective agonists for PPAR-α, -γ and -δ did not inhibit intracellular adhesion molecule-1 expression following stimulation with proinflammatory cytokines. In addition, IL-8 release and the activation of an NF-κB-responsive reporter gene construct were inhibited only at micromolar concentrations, suggesting that these effects were not PPAR-mediated.

5 Our *in vivo* data show that agonists of PPAR-α and -γ, but not -δ, inhibit allergen-induced bronchoalveolar lavage eosinophil and lymphocyte influx. *In vitro* data suggest that this effect might not be mediated by antagonism of the NF-κB pathway.
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Keywords: Peroxisome proliferator-activated receptor; lung; inflammation; NF-κB

Abbreviations: FCS, foetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM-1, intracellular adhesion molecule-1; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptors; Th, T helper cells; TNF-α, tumour necrosis factor-α

Introduction

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily. The three known PPAR subtypes, α, γ and δ, show different tissue distributions and are associated with selective ligands. PPAR-α is predominantly expressed in tissues that demonstrate high catabolism for fatty acids such as liver, heart, kidney and muscles. PPAR-γ is most highly expressed in white adipose tissue where it plays a major regulatory role in adipocyte differentiation and lipid metabolism. PPAR-δ is ubiquitously expressed and so far no specific role has been described for this isoform (Berger & Moller, 2002). Although most studies in the PPAR field have focused on the understanding of how PPAR-α and -γ regulate lipid metabolism, recent evidence suggests that these receptors may play a role in the regulation of inflammation. It has been demonstrated that PPAR-α and -γ are both expressed in murine and human monocytes/macrophages (Chinetti *et al*., 1998). PPAR-γ has also been shown to be expressed in murine (Clark *et al*., 2000) and human (Yang *et al*., 2000) T lymphocytes. *In vivo*, inflammation induced by LTB_4, a PPAR-α ligand, was shown to be prolonged in PPAR-α-deficient mice (Devchand *et al*., 1996), suggesting an anti-inflammatory role for this receptor. In contrast, in mice injected with lipopolysaccharide (LPS), activation of PPAR-α induced a significant increase in plasma tumour necrosis factor-α (TNF-α) levels. This result was confirmed to be mediated by PPAR-α using deficient mice (Hill *et al*., 1999). Contradictory data have also been generated concerning the role of PPAR-γ in inflammatory models *in vivo*. Activation of PPAR-γ has been shown to inhibit inflammation in a model of inflammatory bowel disease (Su *et al*., 1999) and a mouse model of artherosclerosis (Li *et al*., 2000). In contrast, plasma TNF-α levels following challenge with LPS (Thieringer *et al*., 2000) or the development of arthritis (Wiesenberg *et al*.,

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1998) were not affected by PPAR-γ ligand treatment. Similarly, *in vitro* experiments have also produced contradictory results for both PPAR-α and -γ (Clark, 2002). This discrepancy, at least for PPAR-γ, is most likely because of the use of nonselective ligands (Spiegelman, 1998) or the use of very high concentrations of more selective ligands (Chawla *et al*., 2001).

Although, as described above, the role of PPARs in various inflammatory disease has been studied, it is only recently that a possible role for PPARs in airway inflammatory disease such as asthma has been postulated. Asthma is a chronic inflammatory disease of the airways believed to be driven by type 2 T helper cells (Th). A prominent feature of this disease is an intense eosinophilic infiltration that is believed to be the main protagonist in inflicting injury to the bronchial mucosa. This inflammation contributes to bronchial obstruction, hyperreactivity and to the symptoms of asthma (Maddox & Schwartz, 2002). When compared with healthy subjects, immunoreactivity for PPAR-γ has been shown to be augmented in the bronchial submucosa, the airway epithelium and the smooth muscle cells of asthmatics, and its intensity was negatively related to the lung function decline, a marker of asthma severity (Benayoun *et al*., 2001). The expression of PPAR-γ was confirmed in a human airway epithelial cell line and its activation was shown to antagonise proinflammatory events in this system (Wang *et al*., 2001).

Based on the above, PPAR agonists may be predicted to have the potential to inhibit the inflammatory component found in asthma. To test this hypothesis, we have used synthetic PPAR agonists, and study their potential anti-inflammatory role in a murine model of asthma. Moreover, since published works postulate that PPARs exert their anti-inflammatory effects via antagonism of the nuclear factor-κB (NF-κB) pathway (Chung *et al*., 2000; Delerive *et al*., 2001), we have tested this hypothesis using a human airway epithelial cell line, A549, *in vitro*.

Methods

Reagents

All PPAR agonists were synthesised by the Department of Chemistry (Novartis Pharmaceuticals UK Ltd, Horsham, U.K.). 2-(2-Benzoyl-phenylamino)-3-{4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl}-propionic acid; GI 262570. 2-(4-{2[3(2,4-Difluoro-phenyl)-1-heptyl-ureido]-ethyl}-phenoxy)-2-methyl-butyric acid; GW 2331. {2-Methyl-4-[4-methyl-2-(4-trifluoromethyl-phenyl)-thiazol-5-ylmethylsulphanyl]-phenoxy}-acetic acid; GW 501516. 2-(4-{2-[3-(2,4-Difluorophenyl)-1-heptyl-ureido]-ethyl}-phenylsulphanyl)-2-methyl-propionic acid; GW 9578. Dexamethasone was obtained from Sigma. Recombinant interleukin 1 beta (IL-1β), TNF-α and inter feron gamma (IFN-γ) were obtained from R&D Systems. Cell culture media were obtained from Gibco.

Cell culture and cytokine stimulation

A549 cells were obtained from ATCC (Rockville, MD, U.S.A.) and cultured in F12 K nutrient mix supplemented with 10% foetal calf serum (FCS) and 1% penicillin and streptomycin. A549-κBtkluc cells were kindly provided by Dr Robert Newton, Department of Biological Sciences, University of Warwick, and cultured in DMEM, 2 mM glutamine, 10% FCS, 0.5 mg ml^{-1} geneticin. A549 cells were plated, pretreated with PPAR agonist for 2 h and then treated with either cytokine mix (10 ng ml^{-1} IL-1β, 10 ng ml^{-1} TNF-α and 100 U ml^{-1} IFN-γ) for 24 h and assayed for IL-8 secretion, or treated with 2 ng ml^{-1} recombinant human TNF-α for 24 h and assayed for intracellular adhesion molecule-1 (ICAM-1) expression. A549-κBtkluc cells were pretreated with PPAR agonist for 2 h and then treated with either IL-1β (10 ng ml^{-1}) or TNF-α (2 ng ml^{-1}) before assaying for NF-κB activation.

Animals

Female BALB/c mice (4 weeks old) were obtained from Charles River (U.K.). All animals were housed in plastic cages in air-conditioned room at 24°C in a 12 h light – dark cycle. Food and water were available *ad libitum*. All animals were acclimated for a period of at least 7 days upon arrival before any experimental work began. The studies reported here conformed to the U.K. Animals (scientific procedures) Act 1986.

Ovalbumin-induced lung inflammation

All the procedures used below have been previously described by this laboratory (Trifilieff *et al*., 2000). In brief, mice were immunised with ovalbumin on days 0 and 14, exposed to an aerosol challenge of ovalbumin or phosphate-buffered saline (PBS) on day 21 and killed on day 23 for measurement of inflammatory cells into the bronchoalveolar lavage. Animals were intranasally treated with compounds an hour before the aerosol exposure using PBS containing 2% dimethyl sulphoxide as vehicle.

LPS-induced lung inflammation

Mice were challenged intranasally with 0.3 mg kg^{-1} of LPS (*Salmonella typhosa*) in 50 μl of sterile PBS or with sterile PBS alone and killed 3 h later for bronchoalveolar lavage, as described previously (Corteling *et al*., 2002). TNF-α and KC levels were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, U.K.). The limit of sensitivity of the assay was 2 – 5 pg ml^{-1}. Animals were intranasally treated with compounds an hour before the aerosol exposure using PBS containing 2% dimethyl sulphoxide as vehicle.

RT – PCR

Total RNA was extracted from unstimulated A549 cells using the RNeasy Mini Kit (QIAGEN). RNA (400 ng) was reverse transcribed into complementary DNA using random hexamers in a total volume of 20 μl using the TaqMan Reverse Transcription kit (Applied Biosystems). Complememtary DNA (20 ng) was PCR-amplified in a 10 μl reaction using the HotStarTaq DNA polymerase kit (QIAGEN), polymerase chain reaction (PCR) nucleotide mix (Amersham Pharmacia Biotech Inc.) and 30 ng of each primer (custom synthesised by Sigma-Genosys; see Figure 5 legend for primer details). PCR cycling conditions were as follows: 95°C for 15 min; 36 cycles

at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; and a final extension step at 72°C for 10 min.

NF-κB reporter gene assay

A549-κBtkluc cells were seeded onto 24-well culture plates (Costar) at a density of 1.5×10^5 cells in 0.5 ml culture medium and incubated at 37°C for 5 – 6 h to allow attachment to the plate. The medium was then replaced with fresh serum-free medium and the plate was incubated overnight at 37°C. Cells were preincubated with compound at the appropriate concentration in 200 μl serum-free medium for 2 h. The medium in all the wells was replaced with 200 μl fresh serum-free medium containing IL-1β (10ng ml^{-1}) or TNF-α (2ng ml^{-1}) and the required concentration of compound. The plates were then incubated for a further 6 h. Promega Steady Glo buffer/substrate (200 μl) was added to each well, mixed thoroughly, and left to stand for 10 min at room temperature. The supernatant was mixed further and 200 μl from each well was transferred to an opaque-walled 96-well plate (Costar). Luminescence was measured on a Beckman Topcounter with a 2 s read per well.

ICAM-1 expression in A549 cells

A549 cells (American Type Culture Collection) were cultured in F12 K nutrient mix supplemented with 10% FCS and 1% penicillin and streptomycin (Gibco) and seeded into six-well plates (Costar) at a density of 6×10^5 cells well^{-1}. The cells were allowed to adhere overnight before the addition of PPAR agonists. PPAR agonists were tested in triplicate at a range of concentrations from 0.1 nM to 10 μM. Compounds were diluted in PBS and added to the culture medium in the appropriate wells. Cells were preincubated with the agonists for 2 h at 37°C before stimulation with 2ng ml^{-1} recombinant human TNF-α (R&D Systems). The cells were incubated at 37°C for a further 24 h to allow stimulation of ICAM-1 expression. Cells were washed with PBS, trypsinised and transferred to fluorescence-activated cell sorter tubes (Becton-Dickinson). The cells were pelleted, resuspended in No-Zyme buffer (JRH Biosciences) and incubated at 4°C for 1 h with 1/100 dilution of anti-ICAM-1-fluorescein isothiocyanate labelled antibody (Sigma). Labelled cells were washed in 2 ml No-Zyme buffer, resuspended in 500 μl of the same buffer and analysed using a FACSCaliber (Becton-Dickinson).

IL-8 release from A549 cells

Nunc immuno-Maxisorb 96-well enzyme-linked immunosorbent assay plates were coated with an anti-human IL-8 monoclonal antibody (R&D Systems) at a dilution of 5 μg ml^{-1} in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), 100 μl per well, overnight at 4°C. Plates were washed three times with 400 μl of wash buffer (1 × PBS, 0.05% Tween-20) per well. Supernatants (100 μl per well diluted in wash buffer plus 2% FCS) from A549 cells treated with PPAR agonist and stimulated with cytokine mix were added to the plates in duplicate and incubated at 37°C for 2 h. The plates were washed three times with 400 μl of wash buffer per well, and 100 μl of biotinylated anti-human IL-8 polyclonal antibody (R&D Systems) diluted to 0.05 μg ml^{-1} in wash buffer was applied to each well. After incubation for 2 h at 37°C, the plates were washed as described above. Avidin – peroxidase conjugate (Sigma; diluted 1 : 50,000 in wash buffer plus 2% FCS) was added to each well and incubated at 37°C for 30 min. The plates were again washed as described above and 100 μl per well of BM blue POD substrate was added to the plates. After incubating at room temperature for 10 min, the reaction was quenched by adding 50 μl per well of 1 M H_2SO_4. Absorbance was measured at 450 – 650 nm using a SpectraMax 250 plate reader (Molecular Devices Inc.)

Data analysis

Results are expressed as individual data and mean or mean ± s.e.mean. ED_{50} values were calculated from dose-dependent curves by nonlinear regression analysis. Statistical significance ($P < 0.05$) was determined using a Mann – Whitney test.

Results

In vivo *anti-inflammatory effect*

In order to minimise the influence of the pharmacokinetic properties of the compounds, when given orally, PPAR agonists were dosed locally into the lung via the intranasal route. We used specific agonists for PPAR-α (GW 9578), PPAR-γ (GI 262570) and PPAR-δ (GW 501516). A dual α/γ agonist (GW 2331) was also used. The published selectivity profile of the compounds is shown in Table 1.

Ovalbumin challenge induced an influx of eosinophils, neutrophils and lymphocytes into the bronchoalveolar lavage. Macrophage numbers were not affected by the challenge (Figures 1 – 4. The PPAR-α (Figure 1), PPAR-γ (Figure 2) and the dual PPAR-α/γ (Figure 3) agonists dose-dependently inhibited eosinophil and lymphocyte influx with similar potency (Table 2). In contrast, the PPAR-δ agonist had no effect against the ovalbumin-induced eosinophil and lymphocyte influx. However a possible effect on the lymphocyte influx could have been masked because of the low numbers of this

Table 1 Published activities of synthetic PPAR agonists in cell-based assays

	Murine receptor EC_{50} (μM)			*Human receptor EC_{50} (μM)*		
Compounds	*PPAR-α*	*PPAR-γ*	*PPAR-δ*	*PPAR-α*	*PPAR-γ*	*PPAR-δ*
GW 9578	0.005	1.5	2.6	0.05	1	1.4
GI 262570	> 10	0.00035	> 10	0.45	0.00034	> 10
GW 501516	> 10	> 10	0.024	> 10	> 10	0.001
GW 2331	0.010	0.2	> 10	0.050	0.3	> 10

Data from Willson *et al.* (2000), Oliver *et al.* (2001) and Kliewer *et al.* (1997).

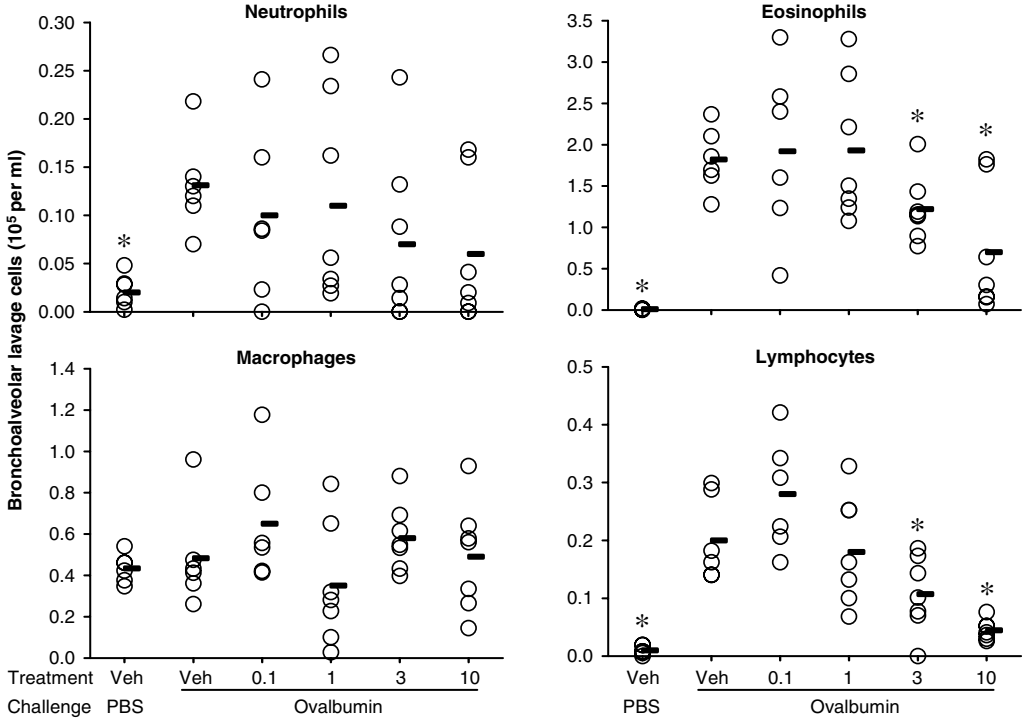

Figure 1 Effect of a PPAR-α agonist (GW 9578) on ovalbumin-induced bronchoalveolar lavage inflammatory cells infiltration. Animals were treated an hour before the challenge with either compound or vehicle (Veh) given intranasally and killed at 2 days postchallenge. Individual data ($n=6-7$) and means (horizontal mark) are shown. Significance, indicated as $^*P<0.05$, was determined *versus* vehicle-treated and ovalbumin-challenged animals. Doses are expressed in mg kg^{-1}.

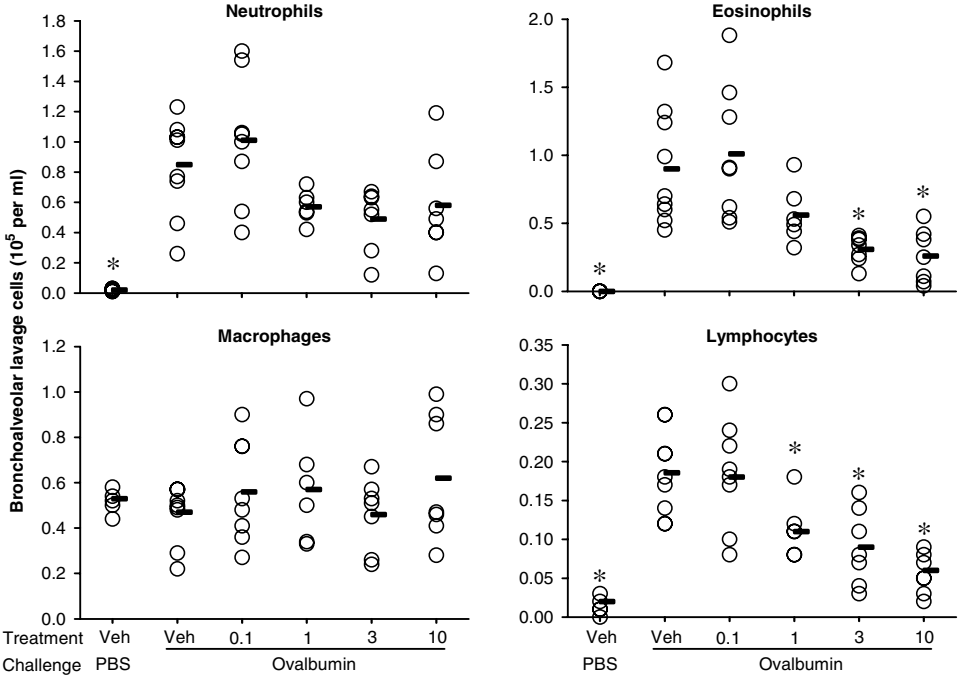

Figure 2 Effect of a PPAR-γ agonist (GI 262570) on ovalbumin-induced bronchoalveolar lavage inflammatory cells infiltration. Animals were treated an hour before the challenge with either compound or vehicle (Veh) given intranasally and killed at 2 days postchallenge. Individual data ($n=5-9$) and means (horizontal mark) are shown. Significance, indicated as $^*P<0.05$, was determined *versus* vehicle-treated and ovalbumin-challenged animals. Doses are expressed in mg kg^{-1}.

cell type following ovalbumin challenge (Figure 4). Together, these results suggest a role for PPAR-α and -γ, but not PPAR-δ, in the regulation of airway eosinophil and lymphocyte influx following allergen challenge.

Since the PPAR-α, -γ and α/γ agonists, which demonstrated anti-inflammatory activity against the ovalbumin-induced airway eosinophil and lymphocyte influx, did not affect the neutrophil influx, they were tested in a model of LPS-induced

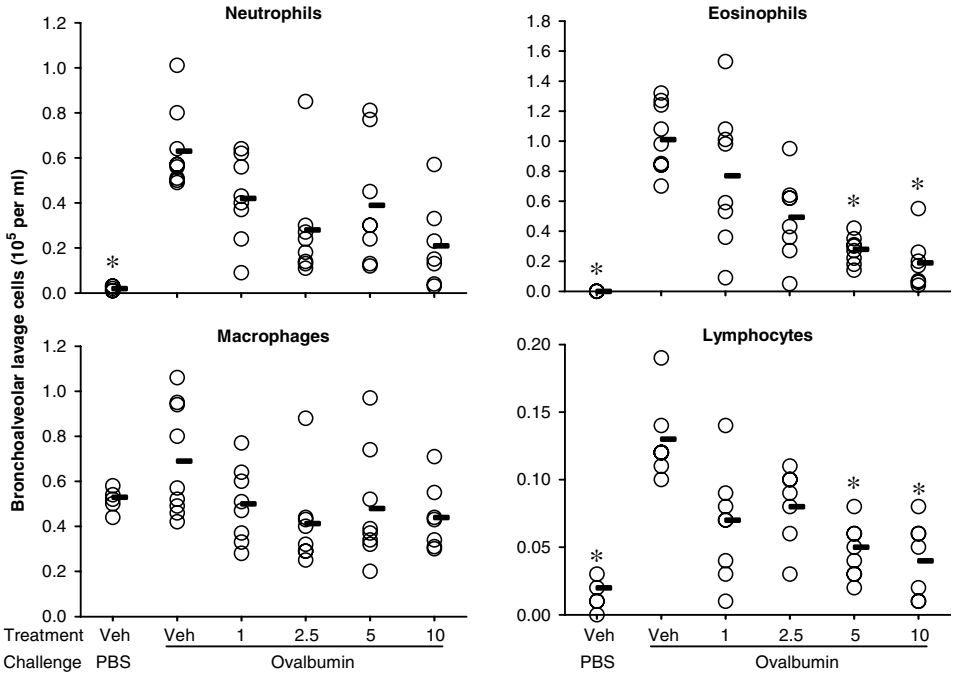

Figure 3 Effect of a dual PPAR-α/γ agonist (GW 2331) on ovalbumin-induced bronchoalveolar lavage inflammatory cells infiltration. Animals were treated an hour before the challenge with either compound or vehicle (Veh) given intranasally and killed at 2 days postchallenge. Individual data ($n = 5 - 9$) and means (horizontal mark) are shown. Significance, indicated as $^*P < 0.05$, was determined *versus* vehicle-treated and ovalbumin-challenged animals. Doses are expressed in mg kg^{-1}.

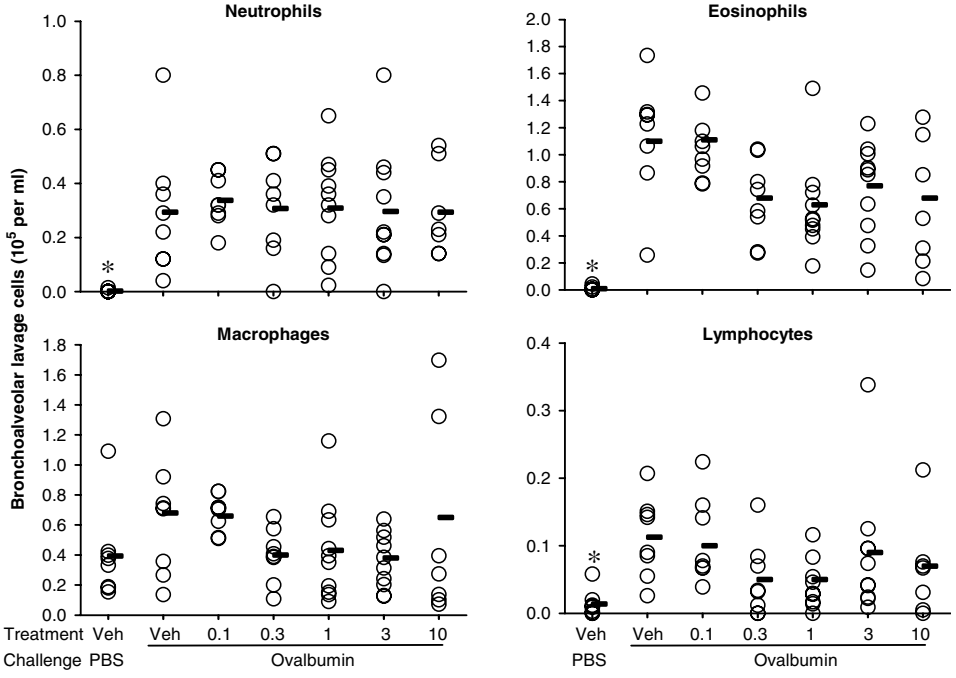

Figure 4 Lack of effect of a PPAR-δ agonist (GW 501516) on ovalbumin-induced bronchoalveolar lavage inflammatory cells infiltration. Animals were treated an hour before the challenge with either compound or vehicle (Veh) given intranasally and killed at 2 days postchallenge. Individual data ($n = 8 - 10$) and means (horizontal mark) are shown. Significance, indicated as $^*P < 0.05$, was determined *versus* vehicle-treated and ovalbumin-challenged animals. Doses are expressed in mg kg^{-1}.

neutrophil infiltration. At 3 h after the challenge, LPS induced an increase in airway neutrophil numbers and KC and TNF-α levels. At doses of 3 and 10 mg kg^{-1}, none of the compounds had a significant effect on the neutrophil influx and the TNF-α production (Table 3).

In vitro *studies*

Published work postulates that PPARs exert their anti-inflammatory effects via antagonism of the NF-κB pathway (Chung *et al*., 2000; Delerive *et al*., 2000). In order to study this

Table 2 ED_{50} values for the PPAR agonists against ovalbumin-induced eosinophils and lymphocytes influx into the airways

	ED_{50} (mg kg^{-1})	
Compounds	*Eosinophils*	*Lymphocytes*
PPAR-α (GW 9578)	5.9±0.9	3.1±1.4
PPAR-γ (GI 262570)	1.5±0.4	1.9±0.4
PPAR-δ (GW 501516)	NA	NA
PPAR-α/γ (GW 2331)	2.4±0.1	1.3±0.6

Data are expressed as mean±s.e.mean. NA: not active at 10 mg kg^{-1}.

Table 3 Lack of effect of PPAR-α, -γ and α/γ agonists on LPS-induced bronchoalveolar lavage inflammation

	Neutrophils (10^5 *cells ml*$^{-1}$)	*KC* (ng ml^{-1})	*TNF-α* (ng ml^{-1})
PBS	0.02±0.01*	0.04±0.03*	0.01±0.01*
LPS	2.39±0.17	12.54±1.46	16.46±1.54
PPAR-α (GW 9578)			
3 mg kg^{-1}	1.91±0.24	12.17±2.03	15.07±1.40
10 mg kg^{-1}	2.16±0.14	10.68±2.35	16.77±1.50
PPAR-γ (GI 262570)			
3 mg kg^{-1}	2.03±0.20	12.77±3.49	17.76±2.97
10 mg kg^{-1}	2.59±0.27	12.75±1.35	17.21±1.52
PPAR-α/γ (GW 2331)			
3 mg kg^{-1}	2.07±0.42	12.09±1.12	15.43±1.54
10 mg kg^{-1}	2.19±0.33	12.96±2.94	15.71±2.45

Animals were treated an hour before the challenge with either compound or vehicle given intranasally and killed at 3 h postchallenge. Data are expressed as mean±s.e.mean of six mice per group. Significance, indicated as $*P<0.05$, was determined versus vehicle-treated and lipopolysaccharide-challenged animals.

hypothesis, the specific agonists for PPAR-α (GW 9578), PPAR-γ (GI 262570) and PPAR-δ (GW 501516) were tested in a human bronchial epithelial cell line (A549) using three assays that monitored the activation of the NF-κB pathway. Expression of PPAR-α, -β and -δ in A549 cells was confirmed at the mRNA level using PCR (Figure 5).

Although the three compounds inhibited IL-8 release from A549 cells following stimulation with a mixture of IL-1β, TNF-α and INF-γ, effects were only seen at micromolar concentrations, well above those required to bind to the receptors, suggesting that these effects were not PPAR-mediated (Figure 6a). When we studied ICAM-1 expression following stimulation with TNF-α, none of the PPAR agonists were observed to have an inhibitory effect, even at 10 μM (Figure 6b). Finally, using an NF-κB reporter gene assay, and following stimulation with TNF-α, all the three PPAR agonists had an IC_{50} of more than 10 μM (Figure 6c). Similar results were found when IL-1β was used as a stimulus instead of TNF-α (data not shown). IL-4 has been reported to induce PPAR-γ in A549 cells (Wang *et al*., 2001). Therefore, A549 cells were preincubated for 24 h, with 10 ng ml^{-1} of this cytokine. However, this did not enhance the ability of any of the three compounds to inhibit TNF-α-induced ICAM-1 expression (not shown). A similar lack of effect was seen with IL-8 release. As a positive control, dexamethasone inhibited the response with subnanomolar potency, in all the assays (not shown).

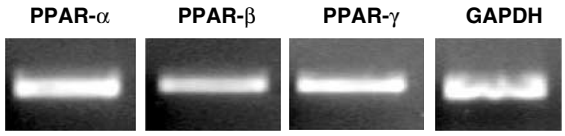

Figure 5 PPAR mRNA expression in A549 cells by RT – PCR. Total RNA was extracted from A549 cells, reverse-transcribed into complementary DNA and PCR-amplified as described in Methods. PCR products (2 μl) were analysed on a 2% agarose gel. The PCR primers used were 5′-GCGATTCGTTTTGGACGAAT-3′ and 5′-TCCAAGTTTGCGAAGCCTG-3′ (395 base pair amplicon for PPAR-α); 5′-TGGTGTGGAAGCAGTTGGTGA-3′ and 5′-GGCGTTGAACTTGACAGCAAAT-3′ (344 base pair amplicon for PPAR-δ); 5′-TCCAACTCCCTCATGGCAA-3′ and 5′-AGCTGGTCGATATCACTGGAGA-3′ (305 base pair amplicon for PPAR-γ); and 5′-CATCACTGCCACCCAGAAGA-3′ and 5′-CTGGTGCTCAGTGTAGCCCA-3′ (301 base pair amplicon for GAPDH). GenBank accession numbers NM_005036, NM_006328, L40904 and J04038 were used as the reference sequences for the PPAR-α, -β, -γ and GAPDH mRNAs, respectively.

Discussion

There is significant interest in the role of PPARs in the regulation of inflammation. So far, *in vitro* and *in vivo* data have led to contradictory results, probably because of the use of nonspecific ligands or the use of specific ligands at concentrations far exceeding those required to bind to the receptors (Clark, 2002). However, recent observations suggest the possible utility of PPAR-γ activation in the treatment of chronic asthma. PPAR-γ expression is increased in the airway mucosa of asthmatics when compared with healthy subjects (Benayoun *et al*., 2001). Moreover, activation of PPAR-γ inhibited proinflammatory events in a human airway epithelial cell line (Wang *et al*., 2001). In this study, we show that activation of PPAR-α and -γ but not PPAR-δ using specific synthetic agonists down-regulates allergen-induced bronchoalveolar lavage inflammatory cell infiltration in mice. Furthermore, our *in vitro* data generated in a human epithelial cell line suggest that this anti-inflammatory effect is not related to the NF-κB pathway.

As demonstrated by the use of the PPAR-α, -γ and dual -α/γ agonists in the ovalbumin model, activation of PPAR-α or PPAR-γ induced a selective inhibition of eosinophil and lymphocyte influx, without affecting the neutrophil influx. In a similar model, it was demonstrated that neutrophil infiltration following ovalbumin challenge is dependent on the release of TNF-α and CXC chemokines from $CD4^+$ T cells (Canetti *et al*., 2001; Knott *et al*., 2001), whereas the eosinophil infiltration is associated with Th2 cytokine production such as IL-4 and IL-5 (Tomkinson *et al*., 2001). This has been confirmed using adoptive transfer of ovalbumin-specific T cells. In this model and following ovalbumin challenge of the airways, transfer of Th1 cells induced a neutrophilia via production of CXC chemokines, whereas Th2 cells induced an eosinophilia via the production of IL-5 (Takaoka *et al*., 2001). Our results suggest that PPAR-α and -γ agonists might exert their anti-inflammatory activities by interfering specifically with the Th2 pathways, thereby directly inhibiting airway eosinophil and lymphocyte influx following ovalbumin challenge. The inability of PPAR-α and -γ agonists to inhibit airway neutrophil influx was further studied using an LPS challenge model. LPS is a macromolecule cell surface antigen

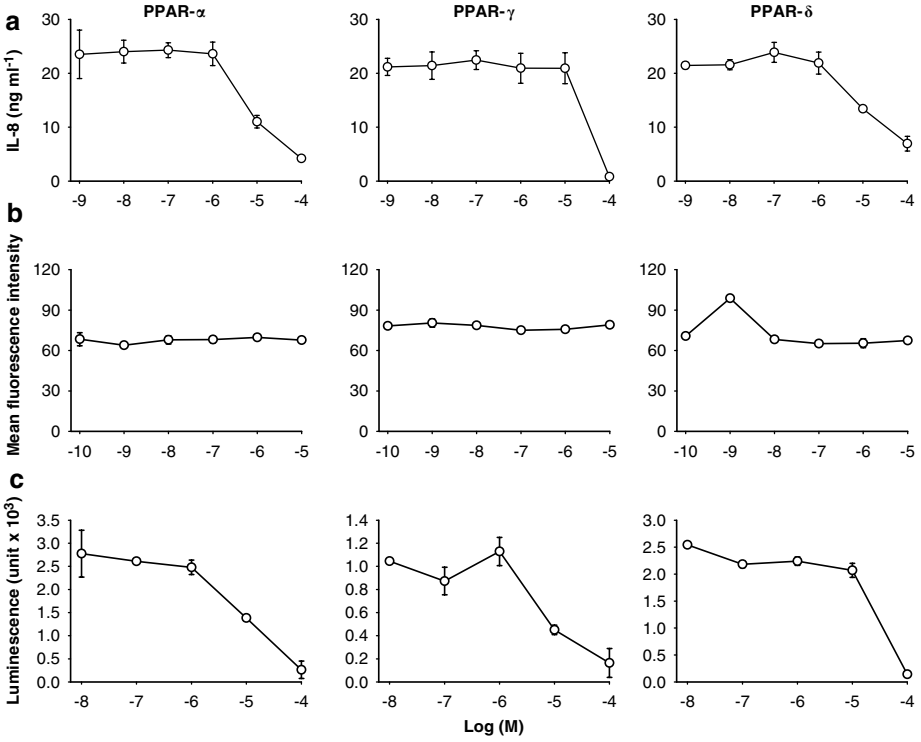

Figure 6 Effect of selective PPAR agonists in A549 cells. A549 and A549-κBtkluc cells were treated with the PPAR-α (GW 9578), -γ (GI 262570) or -δ (GW 501516) agonists, stimulated with cytokines and assayed for IL-8 production (a), NF-κB activation (b) and ICAM-1 expression (c) as described in Methods. Data are presented as mean + s.e.mean of two independent experiments. Each condition was performed in triplicate.

of bacteria that, when applied *in vivo*, triggers a network of inflammatory responses. One of the primary events is the activation of macrophages through a receptor-mediated process, leading to the release of a number of proinflammatory cytokines (Yang *et al*., 1998) which in turn lead to a massive neutrophil infiltration in the pulmonary space (Corteling *et al*., 2002). Among the cytokines produced, TNF-α (Goncalves de Moraes *et al*., 1996) and KC (Huang *et al*., 1992; Frevert *et al*., 1995) have been shown to play a major role in neutrophil influx. In agreement with the results obtained in the ovalbumin model, the PPAR-α and -γ agonists as well as the dual PPAR-α/γ agonist were unable to inhibit the LPS-induced neutrophil influx in the airways. Moreover, the high levels of TNF-α and KC induced by LPS challenge were also unaffected by the compounds. Although PPAR-γ ligands have been reported to inhibit LPS-induced murine macrophage activation *in vitro* (Ricote *et al*., 1998), the ligands used have been shown to have anti-inflammatory effects when using PPAR-γ-deficient macrophages (Chawla *et al*., 2001), indicating that these compounds do not require PPAR-γ for their anti-inflammatory effects in macrophages. *In vivo*, PPAR-α agonists have been shown to be proinflammatory agents in a mouse model of endotoxaemia. Following intraperitoneal injection of LPS, mice chronically treated with PPAR-α agonist had five-fold higher TNF-α plasma levels when compared with nontreated animals. This was shown to be mediated by PPAR-α using deficient mice (Hill *et al*., 1999). In the same study, however, PPAR-α overexpression was shown to inhibit the activity of a human TNF-α promoter-luciferase construct *in vitro* (Hill *et al*., 1999). The authors proposed that other systemic effects *in vivo* could mask the possible anti-inflammatory effect of PPAR-α activators *in vitro* (Hill *et al*., 1999). In our hands, PPAR-α activation *in vivo* did not induce any proinflammatory effect following LPS challenge. However, when compared to our model, duration, route of treatment and route of challenge could explain these differences. Together, our results suggest that although activation of PPAR-α and/or PPAR-γ can inhibit a Th2-driven airway inflammation, activation of these receptors does not affect a neutrophilic airway inflammation when driven by TNF-α.

Since the compounds used in this study have nonspecific effects *in vitro* at micromolar concentrations, it might be argued that the anti-inflammatory effects of PPAR agonists seen *in vivo* are non-PPAR-mediated. We believe that this possibility is unlikely for two reasons. Firstly, if such a nonspecific effect was responsible for the *in vivo* anti-inflammatory action, all three specific agonists would be expected to be active in the ovalbumin model, but they are not, as PPAR-δ agonist was found to be inactive. Secondly, we would have expected the nonspecific inhibition of IL-8 production by PPAR agonists seen *in vitro* to be replicated *in vivo* in the LPS model. However, PPAR- α and -γ agonists had no effect on LPS-induced KC/IL-8 production. Therefore, we believe that the *in vivo* anti-inflammatory activity seen in the ovalbumin model is indeed mediated via activation of PPAR-α and -γ.

It has been suggested that PPAR activators exert their anti-inflammatory effects by antagonising the NF-κB pathway (Delerive *et al*., 2001). In contrast to these results, we could not find any evidence for effects of PPAR on the NF-κB pathway *in vitro*. However, once again, most of the published experiments demonstrating an interaction of PPAR-α activa-

tors with the NF-κB pathway have used concentrations of ligands far exceeding those necessary to recognise the receptor (Delerive *et al.*, 1999; Marx *et al.*, 1999; Chung *et al.*, 2000; Delerive *et al.*, 2000); therefore, nonselective effects cannot be ruled out. Similarly, in our hands, *in vitro* anti-inflammatory effects due to antagonism of the NF-κB pathway were demonstrated for the specific PPAR ligands tested. However, the concentrations needed to observe this effect were equivalent to 50 to 100-fold the IC_{50}. Among all the published *in vitro* work addressing the anti-inflammatory effect of PPAR-γ activation, one is of particular interest in the context of this paper. Two PPAR-γ agonists, 15-deoxy-Δ12,14-PGJ_2 and ciglitazone, have previously been shown to inhibit the IL-8 release from A549 cells (Wang *et al.*, 2001). We were able to reproduce these results, and in our hands ciglitazone and 15-deoxy-Δ12,14-PGJ_2 inhibited this response with similar IC_{50} values (9 and 25 μM, respectively; data not shown). Surprisingly though, in the same experimental set-up, the potent and selective PPAR-γ agonist (GI 262570) only inhibited IL-8 release at concentrations up to 3000 times its EC_{50}, suggesting that the effect seen with the natural ligand or the first generation of synthetic PPAR agonists might be independent of PPAR activation. The observation that 15-deoxy-Δ12,14-PGJ_2 has been shown to inhibit NF-κB activation in a PPAR-independent manner (Straus *et al.*, 2000) indicates that this is, indeed, the likely explanation (Castrillo *et al.*, 2000).

In conclusion, using synthetic selective PPAR ligands, we have shown that PPAR-α and PPAR-γ, but not PPAR-δ, activation leads to a selective inhibition of allergen-induced eosinophil and lymphocyte influx into the murine airways. None of the PPARs appear to inhibit allergen- or LPS-induced neutrophil airway influx. Finally, our *in vitro* results do not support the involvement of NF-κB pathway in the anti-inflammatory effect of PPAR activation.

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