British Journal of Pharmacology (2004) 141, 517–525
www.nature.com/bjp

PPARγ ligands, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and rosiglitazone regulate human cultured airway smooth muscle proliferation through different mechanisms

*,[1]Jane E. Ward, [1]Haslinda Gould, [1]Trudi Harris, [1]John V. Bonacci & [1]Alastair G. Stewart

[1]Department of Pharmacology, University of Melbourne, Melbourne, Victoria 3010, Australia

1 The influence of two peroxisome proliferator-activated receptor γ (PPARγ) ligands, a thiazolidinedione, rosiglitazone (RG) and the prostaglandin D_2 metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on the proliferation of human cultured airway smooth muscle (HASM) was examined.

2 The increases in HASM cell number in response to basic fibroblast growth factor (bFGF, 300 pM) or thrombin (0.3 U ml^{-1}) were significantly inhibited by either RG (1–10 μM) or 15d-PGJ_2 (1–10 μM). The effects of RG, but not 15d-PGJ_2, were reversed by the selective PPARγ antagonist GW9662 (1 μM).

3 Neither RG nor 15d-PGJ_2 (10 μM) decreased cell viability, or induced apoptosis, suggesting that the regulation of cell number was due to inhibition of proliferation, rather than increased cell death.

4 Flow-cytometric analysis of HASM cell cycle distribution 24 h after bFGF addition showed that RG prevented the progression of cells from G1 to S phase. In contrast, 15d-PGJ_2 caused an increase in the proportion of cells in S phase, and a decrease in G2/M, compared to bFGF alone.

5 Neither RG nor 15d-PGJ_2 inhibited ERK phosphorylation measured 6 h post mitogen addition. The bFGF-mediated increase in cyclin D1 protein levels after 8 h was reduced in the presence of 15d-PGJ_2, but not RG.

6 Although both RG and 15d-PGJ_2 can inhibit proliferation of HASM irrespective of the mitogen used, only the antiproliferative effects of RG appear to be PPARγ-dependent. The different antimitogenic mechanisms of 15d-PGJ_2 and synthetic ligands for PPARγ may be exploited to optimise the potential for these compounds to inhibit airway remodelling in asthma.
British Journal of Pharmacology (2004) **141,** 517–525. doi:10.1038/sj.bjp.0705630

Keywords: Airway smooth muscle; ciglitazone; 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; GW9662; peroxisome proliferator-activated receptor γ; PPARγ antagonist; proliferation; rosiglitazone; thiazolidinedione

Abbreviations: ASM, airway smooth muscle; bFGF, basic fibroblast growth factor; Cdk, cyclin-dependent kinase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; DMEM, Dulbecco's modified Eagles medium; DMSO, dimethyl sulphoxide; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorter; FCS, foetal calf serum; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage-colony-stimulating factor; HASM, human airway smooth muscle; IL-1β, interleukin-1β; ITS, insulin, transferrin, selenium; MEK1, mitogen-activated ERK kinase; PBS, phosphate-buffered saline; PI, propidium iodide; PPARγ, peroxisome proliferator-activated receptor γ; RA, retinoic acid; RG, rosiglitazone; RXR, retinoid X receptor

Introduction

Airway wall remodelling is a major contributor to airway hyperresponsiveness in asthma. The structural changes are characterised by hyperplasia and/or hypertrophy of the airway smooth muscle (ASM) (Ebina *et al*., 1993), which may be a consequence of elevated levels of mitogens such as thrombin or basic fibroblast growth factor (bFGF) in the asthmatic airway (Gabazza *et al*., 1999; Redington *et al*., 2001). However, ASM also releases the anti-inflammatory mediator prostaglandin E_2 (PGE_2) (Delamere *et al*., 1994), which inhibits ASM DNA synthesis (Florio *et al*., 1994; Tomlinson *et al*., 1995) and potentially the proliferation of surrounding cells (McAnulty & Laurent, 1995). Drugs that target airway wall remodelling may provide additional benefit in the treatment of asthma, when used in conjunction with current therapies which inhibit inflammation and bronchoconstriction.

The peroxisome proliferator-activated receptor γ (PPARγ) is a widely expressed ligand-activated nuclear receptor present in vascular tissue (Hupfeld & Weiss, 2001), monocytes and macrophages (Ricote *et al*., 1998), lung epithelial cells (Wang *et al*., 2001a) and ASM (Benayoun *et al*., 2001; Pang *et al*., 2003; Patel *et al*., 2003). The expression of both PPARγ mRNA and protein expression has been demonstrated in cultured human ASM (HASM) cells, with the receptor localised in the perinuclear region and within the nucleus (Patel *et al*., 2003). PPARγ forms a heterodimer with the retinoid X receptor (RXR) after ligand binding of these distinct nuclear receptors (Kliewer *et al*., 1992). The RXR is activated by 9-*cis* retinoic acid (RA), an endogenous stereo-

*Author for correspondence; E-mail: janeew@unimelb.edu.au
Advance online publication: 12 January 2004

isomer of RA (Allenby *et al*., 1993). Heterodimerisation stimulates translocation of the receptor complex to the nucleus where it modulates the transcription of target genes. The putative endogenous PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) activates PPARγ at concentrations in the micromolar range (Kliewer *et al*., 1995). The thiazolidinediones, a class of synthetic antidiabetic agents, also activate PPARγ. Rosiglitazone (RG) activates PPARγ at concentrations in the nanomolar range; other thiazolidinediones such as ciglitazone or pioglitazone are less potent (Lehmann *et al*., 1995). The large binding pocket of PPARγ enables it to accommodate both natural and synthetic PPARγ ligands, despite their structural diversity (Nolte *et al*., 1998). A selective and irreversible antagonist for PPARγ has also been recently described (Leesnitzer *et al*., 2002).

Recent studies suggest that both synthetic and natural PPARγ ligands have cell cycle-regulatory effects. The antiproliferative effects of PPARγ ligands have been demonstrated in a number of vascular cell types, including endothelial (Gralinski *et al*., 1998) and smooth muscle cells (Benson *et al*., 2000b). PPARγ ligands have been shown to inhibit mitogen-induced DNA synthesis (Law *et al*., 1996; Asano *et al*., 1999), cyclin D1 protein levels and gene expression (Miwa *et al*., 2000), and to induce G1-phase arrest by inhibiting retinoblastoma phosphorylation in vascular smooth muscle (Wakino *et al*., 2000).

These *in vitro* studies show the potential of PPARγ ligands to target vascular remodelling, and have been extended to animal models, where synthetic PPARγ ligands inhibit neointimal thickening after endothelial injury in Wistar rats, and vascular hypertrophy in spontaneously hypertensive rats (Yoshimoto *et al*., 1999; Igarashi *et al*., 2001).

It has recently been demonstrated that activation of PPARγ in human ASM can also inhibit serum-induced cell growth, and interleukin-1β (IL-1β)-induced release of the inflammatory mediators granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-colony-stimulating factor (G-CSF) (Patel *et al*., 2003), Thus, PPARγ has the potential to regulate both ASM proliferation and cytokine production, each of which may contribute to asthma pathogenesis.

The current study explores the influence of PPARγ in human ASM, by examining the effects of 15d-PGJ_2 and RG on thrombin- and bFGF-mediated proliferation. The mechanism of these effects is explored for the first time in ASM, by utilising the recently described selective PPARγ antagonist (Leesnitzer *et al*., 2002), and by examining cell cycle distribution and markers of cell cycle progression. Characterisation of their mechanism(s) of action is essential to assess their potential as a novel therapy targetting the remodelling component of asthma pathology. Our studies reveal that RG regulates cell cycle progression by a PPARγ-dependent mechanism that is not associated with cytotoxicity or apoptosis.

Methods

Cell culture

HASM cultures were generated from bronchi (0.5–1.5 cm diameter) obtained from macroscopically normal airways, resected from lung transplant recipient or donors and from pneumonectomy specimens. Smooth muscle microdissected from the bronchus wall was enzymatically digested with collagenase (1 mg ml^{-1}) and elastase (0.5 mg ml^{-1}), as previously described (Stewart *et al*., 1997).

The cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) (supplemented with 2 mM L-glutamine, 0.25% BSA, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 2 μg ml^{-1} amphotericin B) with foetal calf serum (FCS) (10% v v^{-1}). Cells were passaged weekly at a 1 : 4 split ratio by 10 min exposure to trypsin (0.5% w v^{-1} in phosphate-buffered saline (PBS) containing 1 mM EDTA. HASM, subcultured into six-well plates (Falcon) at approximately 10^4 cells cm^{-2}, were allowed to grow to monolayer confluency over 72 h in DMEM containing 10% FCS, and then serum-deprived for 24 h in FCS-free medium (with 0.25% w v^{-1} BSA). All experiments reported were carried out on cultures between passage numbers 4 and 16, since during this period there was no relationship between passage number and responsiveness to growth factors or inhibitors, and the expression of smooth muscle α-actin was maintained (Stewart *et al*., 1997).

Drug treatment protocol

All experiments were carried out in the presence of the RXR ligand 9-*cis* RA (1 μM), as this concentration has been shown to enhance the antiproliferative effects of PPARγ ligands in vascular smooth muscle (Benson *et al*., 2000a). In addition, all cells were incubated with the progression factors insulin (1 μg l^{-1}), transferrin (0.6 μg l^{-1}) and selenium (0.5 ng l^{-1}) (ITS), necessary for mitogenesis.

In some experiments, the selective PPARγ antagonist GW9662 (0.1–1 μM) was added 30 min prior to the addition of the PPARγ agonists. Cells were then pretreated with RG (1–10 μM), 15d-PGJ_2 (1–10 μM) or ciglitazone (10 and 30 μM), added 30 min prior to mitogen addition. Proliferation was elicited with a maximally effective concentration of mitogen (thrombin 0.3 U ml^{-1} or bFGF 300 pM) (Stewart *et al*., 1995).

Cell enumeration and analysis of cell cycle distribution

Cells were incubated for 24 or 48 h after the addition of mitogen for fluorescence-activated cell sorter (FACS) analysis or cell enumeration, respectively. Cells were washed once in PBS and detached by the addition of trypsin (0.5% w v^{-1} in PBS with 1 mM EDTA). The enzymatic reaction was stopped with FCS (2% v v^{-1} in PBS) and the cell suspension was isolated by centrifugation (12,000 × *g*, 5 min). Cells were resuspended in 2% FCS for total cell enumeration with a haemocytometer, and to establish the number staining positive with trypan blue (0.2% w v^{-1} in PBS) as a measure of nonviable cells. Alternatively, cells were fixed in ethanol (70% v v^{-1} in PBS) and stored at 4°C. Cell-cycle status was then measured by staining permeabilised cells with propidium iodide (PI, 50 μg ml^{-1} in PBS containing 0.1% v v^{-1} Triton X-100) at 4°C. After at least 24 h of staining, approximately 10,000 total events (stained cells) were counted using FACS (Becton Dickinson, U.S.A.) and analysed using ModFit (version 2, Verity Software House, U.S.A.), as previously described (Hughes *et al*., 2002).

Immunoblot analysis

Cells seeded onto six-well culture plates were treated with bFGF (300 pM), according to drug-treatment protocols described above, for 6 h to measure extracellular signal-regulated kinase (ERK) protein phosphorylation, or for 8 h to measure cyclin D1 protein. Total cell protein was then harvested for Western blotting, performed by a previously described procedure (Fernandes *et al*, 1999). Following incubation in reagents for enhanced chemiluminescence detection, bands were detected and quantified using a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY, U.S.A.).

Materials and drug preparation

The materials used were of analytical grade or higher and include the following:

RG and GW9662 (generous gifts from GlaxoSmithKline, U.K.), 15d-PGJ_2 and ciglitazone (Biomol, U.S.A.), 9-*cis* RA, thrombin and ITS (Sigma, U.S.A.), DMEM and amphotericin B (Gibco), FCS, trypsin, penicillin G and streptomycin (Commonwealth Serum Laboratories, Australia), bFGF (Promega, U.S.A.), elastase and collagenase (Worthington Biochemical, U.S.A.), phospho-specific ERK rabbit polyclonal antibody (New England Biolaboratories) and cyclin D1 anti-rabbit polyclonal antibody (Upstate Biotechnology Inc., U.S.A.).

Stock solutions of RG (10 mM), GW9662 (10 mM), 15d-PGJ_2 (3.13 mM), ciglitazone (30 mM) and 9-*cis* RA (1 mg ml^{-1}) were prepared in 100% dimethyl sulphoxide (DMSO).

Statistical analysis of results

All data are expressed as the mean of the response ± s.e.m. of n cultures. Statistical significance was measured by one-way ANOVA followed by Bonferroni *post hoc* tests for comparison of selected pairs of data, using GraphPad Prism (GraphPad, San Diego, CA, U.S.A.). Cell count data are expressed as a percentage of the control value (in the absence of mitogen) for the same culture. FACS data are expressed as the percentage of events in the different phases of the cell cycle. Densitometry data from Western blots are expressed as a percentage of the amounts of the relevant protein in the presence of mitogen.

Results

Effects of PPARγ ligands on HASM proliferation

The effects of RG and 15d-PGJ_2 (1–10 μM) on bFGF- (300 pM) or thrombin- (0.3 U ml^{-1}) mediated HASM proliferation were examined in incubations of 48 h duration, in the presence of the RXR ligand 9-*cis* RA (9-cis RA, 1 μM). Control cell numbers in the absence of mitogen ($2.25 \pm 0.36 \times 10^5$ cells $well^{-1}$, $n = 13$) were unaffected by preincubation with 9-*cis* RA (1 μM) ($2.09 \pm 0.31 \times 10^5$ cells $well^{-1}$, $P > 0.05$ cf. control). 9-*cis* RA also had no effect on the increase in control cell number in response to thrombin (thrombin alone 47 ± 5%, thrombin + 9-*cis* RA 36 ± 11%, $n = 9$, $P > 0.05$ cf. thrombin).

bFGF increased the cell number by 36 ± 7% (Figure 1a, $n = 9$). This increase was prevented by either 10 μM RG

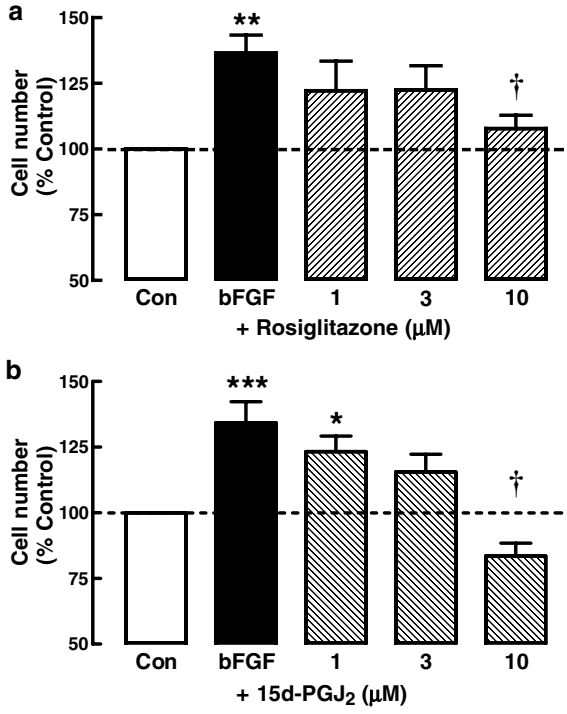

Figure 1 Effects of RG and 15d-PGJ_2 on bFGF-induced proliferation of HASM. Serum-deprived cells were incubated in the presence of the PPARγ ligand (1–10 μM) for 30 min prior to the addition of bFGF (300 pM). Cell enumeration data at 48 h are expressed as mean percentage ± s.e.m. of the control cell number (no mitogen, $2.34 \pm 0.46 \times 10^5$, $n = 9$, upper panel, and $2.40 \pm 0.54 \times 10^5$ cells, $n = 7$, lower panel). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ cf. control, †$P < 0.001$ cf. bFGF.

(Figure 1a, $n = 9$, $P < 0.001$ cf. bFGF) or 10 μM 15d-PGJ_2 (Figure 1b, $n = 7$ $P < 0.001$ cf. bFGF). At 10 μM, RG also prevented the increase in cell number in response to thrombin (Figure 2a, $n = 7$, $P < 0.05$ cf. thrombin). At the same concentration, 15d-PGJ_2 also prevented the thrombin response (Figure 2b, $n = 6$, $P < 0.05$ cf. thrombin). The degree of inhibition of the increase in cell number by both mitogens was greater for 15d-PGJ_2 than RG.

The effects of ciglitazone (30 μM), a thiazolidinedione structurally related to RG, on the thrombin-induced increase in cell number were also tested. Ciglitazone also prevented the thrombin response (thrombin 136 ± 6%, thrombin + ciglitazone 104 ± 8% control, $n = 6$, $P < 0.05$ cf. thrombin).

At the highest concentrations tested, none of the ligands had any effect on control cell number in the absence of mitogen (% control cell number: RG (10 μM) 105 ± 9% ($n = 10$), 15d-PGJ_2 (10 μM) 96 ± 8% ($n = 9$) and ciglitazone (30 μM) 111 ± 7% ($n = 6$), all $P > 0.05$ cf. control). In addition, the percentage of cells stained with trypan blue was below 5%, indicating that cell viability was unaffected by the ligands.

Effects of a PPARγ antagonist on regulation of HASM proliferation by PPARγ ligands

The effect of preincubation with the selective PPARγ antagonist GW9662 (Leesnitzer *et al*., 2002) on HASM cell number was examined. Control cell numbers ($2.31 \pm 0.11 \times 10^5$ cells $well^{-1}$, $n = 5$) were unaffected by 1 μM

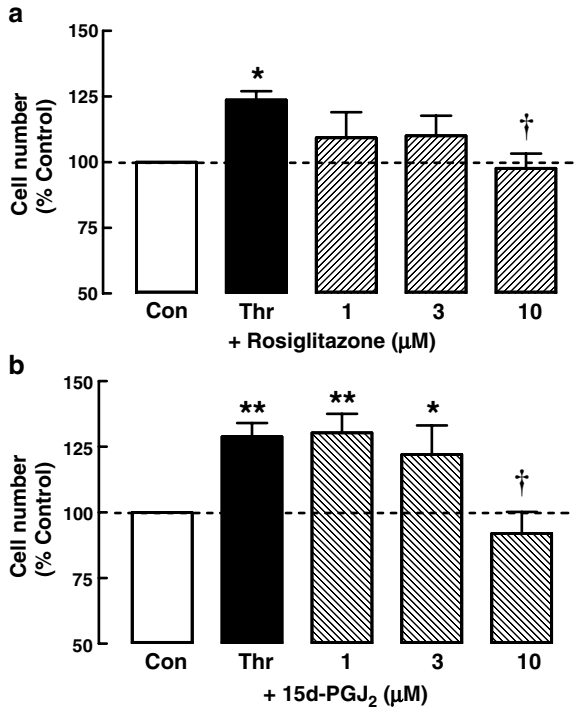

Figure 2 Effect of RG and 15d-PGJ_2 on thrombin-induced proliferation of HASM. Serum-deprived cells were incubated in the presence of the PPARγ ligand (1–10 μM) for 30 min before the addition of thrombin (0.3 U ml^{-1}). Cell enumeration data at 48 h are expressed as the mean percentage ± s.e.m. of the control cell number (no mitogen) for $n=9$ (RG) or $n=7$ (15d-PGJ_2). $^{*}P<0.05$, $^{**}P<0.01$ cf. control, $^{\dagger}P<0.05$ cf. thrombin.

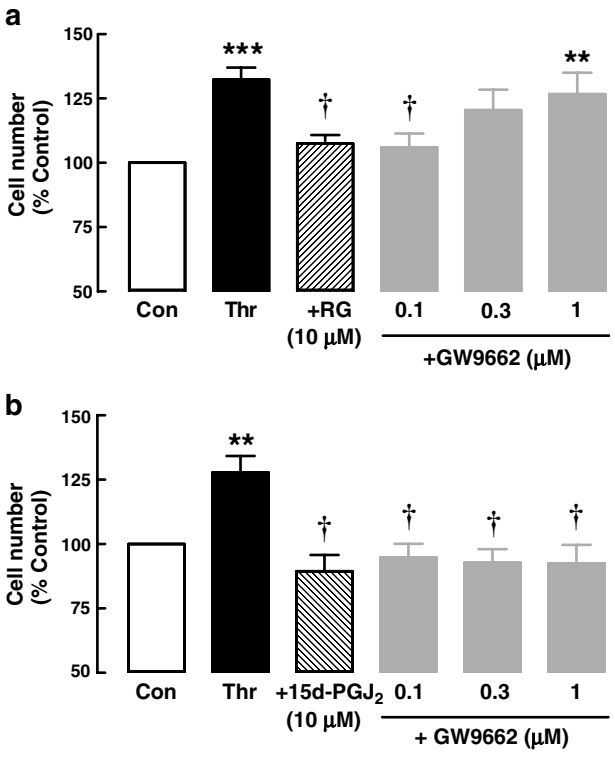

Figure 3 Effect of the selective PPARγ antagonist GW9662 on the ability of RG and 15d-PGJ_2 to decrease thrombin-mediated proliferation. Serum-deprived cells were incubated with either: vehicle (control); thrombin (0.3 U ml^{-1}); thrombin and PPARγ ligand (RG or 15d-PGJ_2, 10 μM, added 30 min prior to mitogen); or thrombin, PPARγ ligand and GW9662 (0.1–1 μM, added 30 min prior to RG or 15d-PGJ_2). Cell enumeration data at 48 h are expressed as mean percentage ± s.e.m. of control for $n=7$ (RG) or $n=5$ (15d-PGJ_2). $^{**}P<0.01$, $^{***}P<0.001$ cf. control, $^{\dagger}P<0.05$ cf. thrombin.

GW9662 ($2.18\pm0.11\times10^5$ cells $well^{-1}$, $P>0.05$ cf. control). The increase in control cell number in response to thrombin (21.5 ± 6.0%, $n=5$) was also unaffected by the antagonist (thrombin + 1 μM GW9662 28.5 ± 4%, $P>0.05$ cf. thrombin). However, GW9662 (0.1–1 μM) reversed the inhibition of the thrombin-induced increase in cell number by 10 μM RG in a concentration-dependent manner (Figure 3a). Inhibition of the proliferative response to thrombin by 10 μM RG was completely prevented in the presence of 1 μM GW9662 (thrombin alone 132 ± 2%, thrombin + 10 μM RG + 1 μM GW9662 127 ± 3% control, $n=6$, $P>0.05$ cf. thrombin) However, GW9662 did not affect inhibition by 15d-PGJ_2 (Figure 3b). At the highest concentration of GW9662 tested (1 μM), the response to thrombin in the presence of 10 μM 15d-PGJ_2 was still prevented ($n=5$, $P<0.05$ cf. thrombin alone).

Effects of PPARγ ligands on cell cycle distribution

The mechanism of action of PPARγ ligands was further explored by investigating their effects on cell cycle distribution, using concentrations which were maximally effective at inhibiting mitogen-induced increases in cell number. Results from flow cytometry 24 h after the addition of RG (10 μM) or 15d-PGJ_2 (10 μM) showed no change in the proportion of cells with a sub-G0/G1 DNA content, compared to controls, either in the absence or presence of mitogen, indicating the absence of apoptosis at 24 h (see Figure 4 for FACS profiles).

Under control conditions, the greatest proportion of cells was in G0/G1 phase of the cell cycle (Figures 4a, 5a). There was a small increase in the proportion of cells in G0/G1 in the presence of 15d-PGJ_2 (Figure 5a, 13 ± 4% above control $n=5$, $P<0.05$ cf. control). However, neither 15d-PGJ_2 nor RG changed the proportion of unstimulated cells in S or G2/M phase (Figures 4, 5b, c).

Addition of bFGF (300 pM) decreased the proportion of HASM in G0/G1 phase, with an associated increase in S phase events (Figure 4d). The decrease in events in G0/G1 induced by bFGF (Figure 5a, from 63 ± 2 to 51 ± 2% total events, $n=5$, $P<0.05$ cf. control) was prevented by RG (Figure 5a, $P<0.05$ cf. bFGF), but not by 15d-PGJ_2. The bFGF-mediated increase in S-phase cells (Figure 5b, from 22 ± 3 to 36 ± 1%, $n=5$, $P<0.05$ cf. control) was also prevented by RG (Figure 5b, $P>0.05$ cf. control). In contrast, the proportion of cells in S phase in the presence of 15d-PGJ_2 and bFGF was higher than with bFGF alone (Figure 5b, $P<0.05$ cf. bFGF). 15d-PGJ_2 also decreased the G2/M proportion of proliferating cells in the presence of bFGF from 13 ± 2 to 5 ± 2% (Figure 5c, $P<0.01$ cf. bFGF).

Effects of PPARγ ligands on mitogen-induced ERK activation and cyclin D1 levels

The effects of 15d-PGJ_2 and RG on cell cycle proteins were also investigated. The increase in ERK phosphorylation induced by bFGF (300 pM) (399 ± 72% control, $n=6$) at 6 h

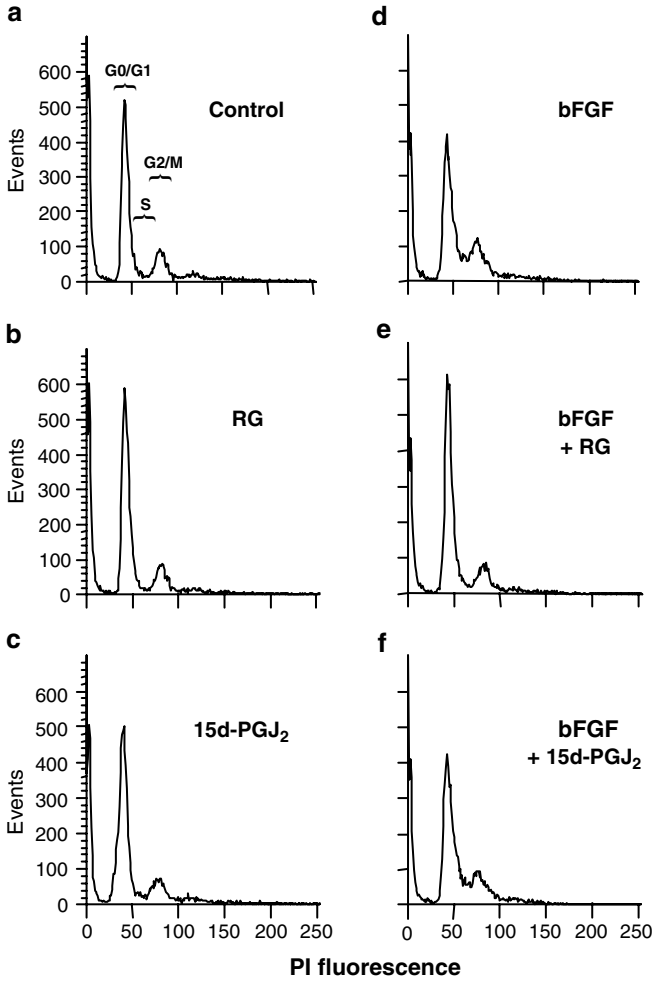

Figure 4 Effect of PPARγ ligands on the distribution of events in the cell cycle of HASM. (a) shows the representative FACS profile of the distribution of cells detected in G0/G1, S and G2/M phases of the cell cycle under control conditions (no PPARγ ligand or mitogen). The histogram shows DNA staining by PI, with an increase in PI fluorescence linearly related to an increase in DNA content. The frequency of events with each level of PI fluorescence is shown. The greatest proportion of events is detected in G0/G1 phase. Cells with approximately twice the PI fluorescence of G0/G1 cells represent those cells in G2/M phase of the cell cycle. Cells with intermediate staining represent cells with varying DNA content, that is, those in S phase. (b, c) show the effects of 10 μM RG or 15d-PGJ_2 on control cell cycle distribution, respectively. The distribution profile 24 h after mitogen addition (bFGF, 300 pM) in the absence (d) and presence of 10 μM RG (e) or 10 μM 15d-PGJ_2 (f) is also shown. All representative profiles have been obtained from experiments performed on one culture, and are consistent with the results from the five cultures examined.

was unaffected by 10 μM RG or 15d-PGJ_2 (data not shown). At 8 h after mitogen addition, bFGF significantly increased cyclin D1 levels (Figure 6, $n=5$, $P<0.01$ cf. control). Neither RG nor 15d-PGJ_2 affected basal cyclin D1 levels. RG had no effect on the increase in cyclin D1 levels in response to bFGF (Figure 6a, 130±11% bFGF, $P>0.05$ cf. bFGF). However, in the presence of 15d-PGJ_2, there was no significant increase in bFGF-mediated cyclin D1 levels (bFGF + 15d-PGJ_2, $P>0.05$ cf. control) (Figure 6b).

Discussion

Recent studies describing the potential role of the peroxisome proliferator-activated receptor γ (PPARγ) as a transcription factor in the control of cellular growth and cytokine release are of interest in the regulation of asthma. PPARγ agonists have been shown to inhibit vascular smooth muscle proliferation (Asano *et al*., 1999) and the cytokine-induced expression of inflammatory mediators in airway epithelial cells (Wang *et al*., 2001a). Recently, PPARγ expression has been shown to be increased in ASM from asthmatic patients, with higher levels of expression being observed in steroid-treated asthmatics than in controls (Benayoun *et al*., 2001). Furthermore, PPARγ expression has been demonstrated in cultured HASM, where the serum-induced incorporation of [^{3}H]-thymidine was inhibited by PPARγ ligands (Patel *et al*., 2003).

In the present study, we show that 15d-PGJ_2, RG and ciglitazone decreased mitogen-induced increases in HASM cell number. The mechanism of this reduction in cell number was explored to determine whether these agents were causing cell death or inhibiting HASM proliferation. At the concentrations tested in this study (up to 10 μM of RG and 15d-PGJ_2 and 30 μM ciglitazone), there was no evidence of cytotoxicity using trypan blue exclusion as an indicator of HASM cell viability. It has been demonstrated that the extracellular matrix provides strong signals for cell survival of HASM in culture, and a low background rate of apoptosis (Freyer *et al*., 2001). In the

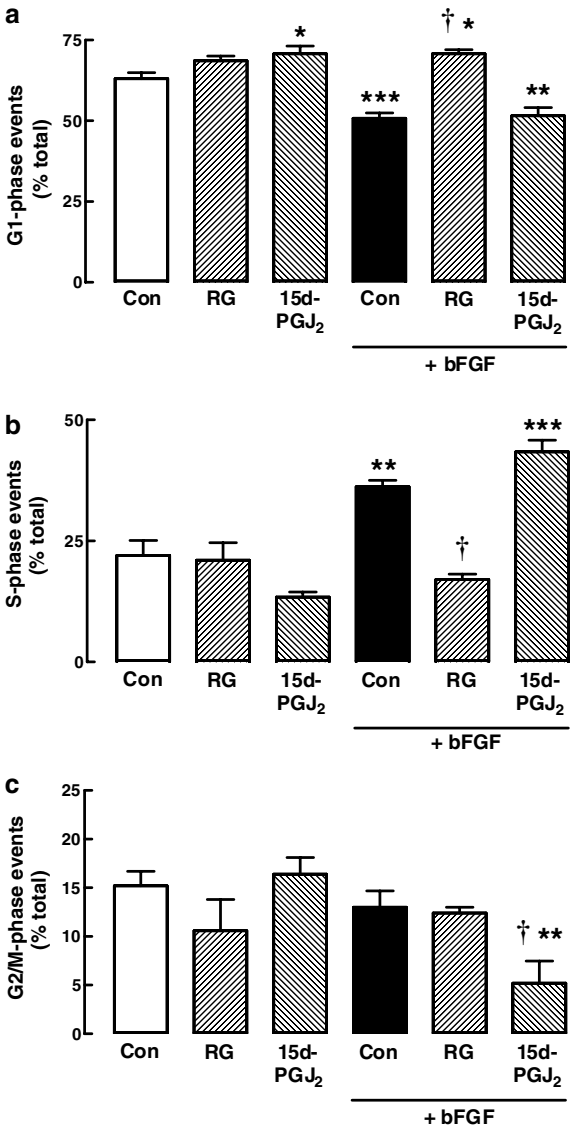

Figure 5 Effect of PPARγ ligands on the cell cycle distribution of HASM. Cells were incubated for 24 h in the absence or presence of bFGF (300 pM), 30 min after addition of vehicle control, 10 μM RG or 10 μM 15d-PGJ_2. The frequency of events detected by fluorescence of PI in (a) G0/G1, (b) S and (c) G2/M phases of the cell cycle are shown as mean ± s.e.m. for $n=5$ cultures. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ cf. control, $^{\dagger}P<0.05$ cf. bFGF.

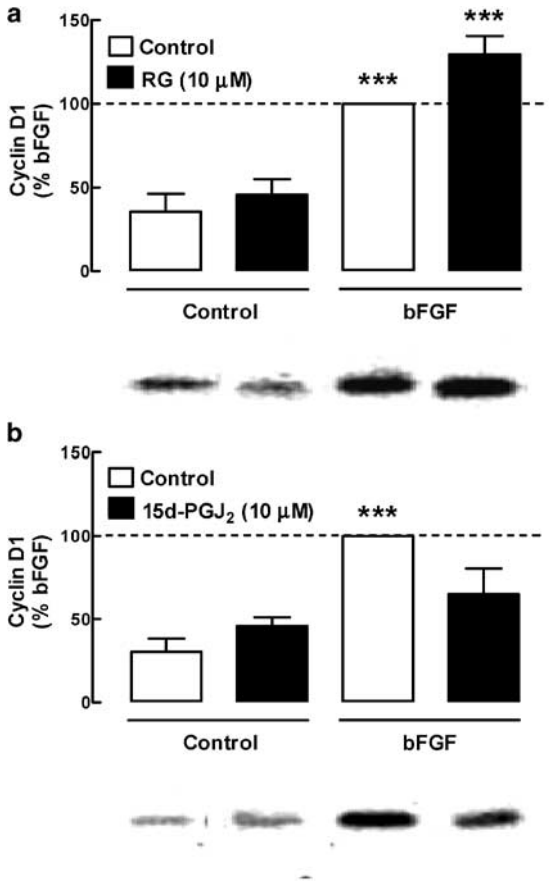

Figure 6 Effects of (a) 10 μM RG and (b) 10 μM 15d-PGJ_2 on cyclin D1 protein levels at 8 h after stimulation with bFGF (300 pM). Western blots are representative of experiments in six different HASM cultures. Representative blots of the effects of 15d-PGJ_2 and RG on cyclin D1 levels were obtained using 60 μg of protein. The densitometry results are shown as mean percentage ± s.e.m. of bFGF. $^{***}P<0.001$ cf. control.

current study, we could not find evidence for induction of HASM apoptosis by either RG and 15d-PGJ_2, since the sub-G0/G1 fraction measured by flow cytometry was unchanged in the presence of the ligands. It was recently reported that 15d-PGJ_2 and ciglitazone (a synthetic PPARγ ligand with lower PPARγ-binding affinity than RG) were able to cause nuclear condensation and shrinkage (Patel *et al*., 2003). However, this early indicator of apoptosis was seen only at 3–10-fold higher concentrations than those which completely inhibited mitogen-induced increases in cell number in the current study. A concentration-dependent increase in DNA fragmentation of HASM was also observed for ciglitazone (Patel *et al*., 2003). However, the maximal increase of approximately 60% at 100 μM ciglitazone is modest in comparison to the eight-fold increase in DNA fragmentation induced by 10 μM of the same ligand in colon cancer cells (Yang & Frucht, 2001). The only evidence of a direct effect on cell number in the current study was the tendency for 15d-PGJ_2 to decrease the HASM cell number below levels in the absence of mitogen. However, this may reflect the inhibition of the basal proliferation that occurs in the presence of the progression factors, including insulin, rather than due to 15d-PGJ_2 causing cell death. It has previously been shown that 15d-PGJ_2 is a more potent inhibitor of insulin-induced proliferation than RG in vascular smooth muscle cells (Goetze *et al*., 2000). Our results suggest that the inhibition of mitogen-induced increases in cell number is due to inhibition of proliferation by 15d-PGJ_2 and RG, rather than cytotoxicity or apoptosis.

The inhibition of thrombin-induced proliferation by 15d-PGJ_2, RG and ciglitazone in this study was similar in potency to the inhibition of serum-induced [^{3}H]-thymidine incorporation previously reported in HASM (Patel *et al*., 2003). However, there is a significant discrepancy between the concentrations required in both studies to achieve these biological effects and the reported binding affinities of these ligands (Kliewer *et al*., 1995; Lehmann *et al*., 1995). This may reflect the presence of other modifiers of affinity and efficacy in intact cells, or the poor permeability of these compounds across the cell membranes.

The effects of RG and 15d-PGJ_2 in HASM were shown to be mitogen-independent, as each ligand decreased bFGF- and thrombin-mediated proliferation with similar potency. Both bFGF and thrombin concentrations are elevated in the asthmatic airways (Gabazza *et al*., 1999; Redington *et al*., 2001). However, these established growth factors act *via* different mechanisms: bFGF is a member of a group of polypeptide growth factors that activate receptor tyrosine kinases, whereas thrombin is a serine protease that acts *via* G-protein-coupled receptors (Dery *et al*., 1998; Tran & Stewart, 2003). bFGF is a more effective inducer of HASM proliferation, S-phase entry and [^{3}H]-thymidine uptake, in comparison to thrombin (Ravenhall *et al*., 2000), and it may therefore have a greater potential to contribute to airway remodelling in asthma. PPARγ ligands have been shown to inhibit HASM proliferation in the presence of the cocktail of mitogens present in FCS (Patel *et al*., 2003). A potential mechanism for this inhibition of proliferation is *via* an increase in the levels of PGE_2. However, this is considered unlikely, since the inhibition of [^{3}H]-thymidine incorporation by 15d-PGJ_2 and ciglitazone was evident in the presence of indomethacin (Patel *et al*., 2003). The current finding that PPARγ ligands can inhibit proliferative responses to the specific mitogens elevated in the asthmatic airway further reinforces their antiremodelling potential.

It has been suggested that the antiproliferative effects of 15d-PGJ_2 may be completely independent of PPARγ, since it has been demonstrated that 15d-PGJ_2 decreases vascular smooth muscle proliferation in the presence of an antisense oligonucleotide for PPARγ (Miwa *et al*., 2000). 15d-PGJ_2 is structurally different from RG and a number of its biological effects have been attributed to inhibition of NK-κB signalling pathways rather than PPARγ activation (Straus *et al*., 2000).

The PPARγ-dependence of the effects of 15d-PGJ_2 and RG on thrombin-mediated proliferation was therefore assessed using the recently described selective and irreversible antagonist of the PPARγ receptor GW9662 (Leesnitzer *et al*., 2002). This antagonist binds covalently to the ligand-binding domain of PPARγ with an IC_{50} in the nanomolar range, and is 10- and 600-fold less potent in binding experiments with PPARα and PPARδ, respectively. Although recent studies suggest that PPARα may also have a role in regulating airway function (Trifilieff *et al*., 2003), neither 15d-PGJ_2 nor RG has been shown to have significant activity at this receptor (Forman *et al*., 1995; Kliewer *et al*., 1995). In addition, GW9662 at concentrations up to 10 μM did not cause significant activation of PPARα (Leesnitzer *et al*., 2002). GW9662 has been shown to inhibit both binding of [^{3}H]-RG to PPARγ, and RG-induced adipocyte differentiation (Leesnitzer *et al*., 2002), Therefore, we examined the potential of GW9662 to antagonise the effects of 15d-PGJ_2 or RG to determine whether their interaction with PPARγ is required for their antimitogenic action.

Our findings with the selective PPARγ antagonist GW9662, where 15d-PGJ_2 was still able to exert its antimitogenic effects against thrombin, suggest that, under these conditions, the antiproliferative action of 15d-PGJ_2 in HASM is PPARγ-independent. Both RG and another thiazolidinedione, ciglitazone, which activates PPARγ, also decreased thrombin-mediated proliferation of HASM. The effects of RG and ciglitazone observed in this study are suggestive of PPARγ dependence, since the relative potencies of their antimitogenic effects appear to relate to their relative potencies for PPARγ activation (Lehmann *et al*., 1995). The concentration-dependent reversal of the RG-mediated inhibition of thrombin-induced proliferation by the PPARγ antagonist GW9662 provides compelling additional evidence that the antimitogenic action of RG in HASM is PPARγ-dependent.

Further examination of the antiproliferative effect of these ligands was carried out by investigating proteins important in cell cycle progression. Cyclin D1 protein levels in HASM are increased by activation of ERKs, ERK1/2, by mitogen-activated ERK kinase (MEK1). A complex of cyclin D1 and cyclin-dependent kinase 4 (Cdk 4) phosphorylates retinoblastoma protein (pRb), inactivating its repression of the transcription factor E2F, enabling E2F to participate in the synthesis of proteins necessary for S-phase progression (Jones & Kazlauskas, 2001). Phosphorylation of ERK by MEK1 is elevated soon after stimulation with mitogen, and its continued activity is necessary for proliferation for 4–8 h (Fernandes & Stewart, unpublished data). bFGF-mediated ERK phosphorylation was not inhibited by RG or 15d-PGJ_2 at 6 h, indicating that their antiproliferative effects on bFGF-mediated proliferation are likely to be downstream of, or parallel to, this component of the mitogen signalling cascade.

In the presence of 15d-PGJ_2, bFGF did not increase cyclin D1 levels at 8 h. 15d-PGJ_2 has been shown to reduce cyclin D1 levels in vascular smooth muscle after stimulation with PDGF and insulin (Wakino *et al*., 2000). These results imply that 15d-PGJ_2 would inhibit the entry of cells into S phase. However, despite the lower cyclin D1 protein levels, cell cycle distribution data of HASM showed that 15d-PGJ_2 enhanced the bFGF-mediated increase in the proportion of events detected in S phase. The concurrent decrease in the proportion of events detected in G2/M phase suggests an S-phase arrest or delayed entry into G2 phase. It is possible that an earlier increase in cyclin D1 protein levels before 8 h could have facilitated S-phase entry by 15d-PGJ_2 , before the cells were retarded in their progression into G2 phase**.** The antiproliferative mechanism of action of 15d-PGJ_2 in HASM appears to be different from that of glucocorticoids. Dexamethasone decreases mitogen-stimulated cyclin D1 protein levels inducing G1-phase arrest to inhibit increases in cell number (Fernandes *et al*., 1999). The possible synergistic effects of these different drugs therefore raise interest in their therapeutic potential in combination.

Differences in the mechanism of action of 15d-PGJ_2 and RG in HASM are illustrated by the failure of RG to inhibit bFGF-mediated increases in cyclin D1 levels at 8 h. Previous studies have shown that like 15d-PGJ_2, RG inhibits both cyclin D1 gene expression and protein levels in tumour cell lines (Wang *et al*., 2001b). However, RG did not affect mitogenic induction of cyclin D1 levels in vascular smooth muscle (Wakino *et al*., 2000). Despite the lack of effect on cyclin D1 levels in HASM, RG may be inhibiting cell cycle progression in late G1 phase of the cell cycle, after 8 h, since it significantly reduced the bFGF-mediated increase in S-phase events. A late G1-phase arrest could be explained by an inhibition of the formation of an active cyclin D1/Cdk 4 complex by RG. This could occur independently of an effect on cyclin D1 levels through interference with the role of the Cdk inhibitor $p21^{cip}$, which has a dual role as both an inhibitor of cyclin/Cdk complexes and as an essential factor for formation of this complex (Wakino *et al*., 2001). Alternatively, inhibition of Rb

phosphorylation by RG may contribute to the inhibition of G1 to S transition, as has been shown in vascular smooth muscle (Wakino *et al*., 2000).

The current treatment for asthma with glucocorticoids primarily targets the inflammatory process through inhibition of inflammatory cell infiltration and cytokine release. The efficacy of these drugs may also relate in part to their antiremodelling properties, since dexamethasone has been shown to inhibit mitogen-induced HASM proliferation (Stewart *et al*., 1995) through inhibition of cyclin D1 protein levels and pRb phosphorylation (Fernandes *et al*., 1999). However, the limitations of glucocorticoid use include adverse endocrine effects, and the resistance to steroid therapy in a significant proportion of asthmatics. The modulation of cell cycle progression by synthetic PPARγ ligands in HASM occurs *via* a different mechanism to steroids, suggesting that PPARγ provides an alternative therapeutic target in asthma.

In conclusion, RG and 15d-PGJ_2 appear to have different antiproliferative mechanisms of action in HASM. RG's antimitogenic action may be common to synthetic thiazolidinediones, and appears to be dependent on PPARγ. In contrast, 15d-PGJ_2 may inhibit proliferation independently of PPARγ. RG appears to be arresting cells in late G1 phase, while 15d-PGJ_2 may cause either S-phase arrest or slow progression to G2/M. The development of optimum analogues of 15d-PGJ_2 may improve the antiproliferative potential of this drug. However, further characterisation of its site of action is critical. RG is a promising candidate for further evaluation as an antiasthma therapy, despite some similarities with dexamethasone in its antiproliferative mechanism, as it has an additional novel anti-inflammatory profile whereby IL-1β-induced release of both GM-CSF and G-CSF from ASM are inhibited (Patel *et al*., 2003). Development of analogues could prove to be valuable improvements in treatment of the chronic inflammatory and airway remodelling components of asthma.

We thank Associate Professor John Wilson and the anatomical pathologists at the Alfred Hospital for facilitating the provision of human airway smooth muscle samples. This work was funded by the National Health and Medical Research Council of Australia. Rosiglitazone and GW 9662 were generously provided by GlaxoSmithKline, U.K.

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(Received July 30, 2003
Revised October 8, 2003
Accepted November 10, 2003)