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Activation of Peroxisome Proliferator-Activated Receptors in Human Airway Smooth Muscle Cells Has a Superior Anti-inflammatory Profile to Corticosteroids: Relevance for Chronic Obstructive Pulmonary Disease Therapy

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Airway smooth muscle is actively involved in the inflammatory process in diseases such as chronic obstructive pulmonary disease and asthma by 1) contributing to airway narrowing through hyperplasia and hypertrophy and 2) the release of GM-CSF and G-CSF, which promotes the survival and activation of infiltrating leukocytes. Thus, the identification of novel anti-inflammatory pathways in airway smooth muscle will have important implications for the treatment of inflammatory airway disease. This study identifies such a pathway in the activation of peroxisome proliferator-activated receptors (PPARs). PPAR ligands are known therapeutic agents in the treatment of diabetes; however, their role in human airway disease is unknown. We demonstrate, for the first time, that human airway smooth muscle cells express PPARα and -γ subtypes. Activation of PPARγ by natural and synthetic ligands inhibits serum-induced cell growth more effectively than does the steroid dexamethasone, and induces apoptosis. Moreover, PPARγ activation, like dexamethasone, inhibits the release of GM-CSF. However, PPARγ ligands, but not dexamethasone, similarly inhibit G-CSF release. These results reveal a novel anti-inflammatory pathway in human airway smooth muscle, where PPARγ activation has additional anti-inflammatory effects to those of steroids. Hence, PPAR ligands might act as potential treatments in human respiratory diseases. The Journal of Immunology, 2003, 170: 2663–2669.

I

t is now known that human airway smooth muscle (HASM) is actively involved in the inflammatory process. Indeed, it is well accepted that remodeling due to the processes of hyper trophy and hyperplasia of the airway smooth muscle contributes significantly to the narrowing that is characteristic of asthma (1). Airway smooth muscle remodeling is also seen in the bronchioles of chronic obstructive pulmonary disease (COPD) patients (1) and contributes to the obstruction of the lower airways, which characterizes this disease. In addition, under inflammatory conditions, HASM cells release a variety of pro- and anti-inflammatory mediators, such as the chemokines IL-8 (2) and eotaxin (3), PGs (4), and NO (5). HASM cells also release the cytokines GM-CSF (6) and G-CSF (7), which are important survival and activation factors for eosinophils (8) and neutrophils (9), respectively. These activated cells then release further inflammatory mediators that perpetuate the inflammatory response commonly associated with diseases such as asthma and COPD, respectively. Hence, agents that inhibit proliferation of HASM cells as well as CSF release would represent potential new therapies to treat asthma and COPD.

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear hormone receptors belonging to the steroid receptor superfamily (10, 11). To date, three different PPAR subtypes have been identified: PPARα, -γ, and -δ (also referred to as NUC-1, PPARβ, or FAAR). PPARs were first identified for their role in lipid and glucose regulation (energy balance), and until recently, their actions were thought to be limited to specific tissue types. PPARα is highly expressed in tissues exhibiting high carbolic rates of fatty acids such as the liver, heart, kidney, and intestinal mucosa (12). PPARγ is expressed at high levels in adipose tissue, where it plays a critical role in adipocyte differentiation (13). PPARδ is almost ubiquitously expressed (12, 14, 15); however, its role is relatively unknown.

Recently, however, PPARα and PPARγ have been suggested to be important immunomodulators (16). PPARα activation inhibits inflammatory mediator release from vascular smooth muscle cells (17) and is consistent with the finding that PPARα knockout mice exhibit exacerbated inflammatory responses (18). PPARγ ligands have been shown to inhibit the release of proinflammatory cytokines from activated macrophages (19) and airway epithelial cells (20). Furthermore, PPARγ ligands have been shown to inhibit vascular smooth muscle cell proliferation (21) and induce apoptosis in endothelial cells (22), vascular smooth muscle cells (23), T lymphocytes (24, 25), and macrophages (26). However, the potential anti-inflammatory properties of PPAR ligands on airway smooth muscle have not been investigated. Furthermore, a comparison between the anti-inflammatory efficacy of PPAR ligands and that of glucocorticoids, currently one of the major treatment options for both asthma and COPD, has never been made.
Therefore, in this study, we have investigated whether HASM cells express PPARs and whether activation of these receptors can inhibit 1) the release of survival factors under proinflammatory conditions and 2) serum-induced growth. Moreover, we have investigated whether PPAR activation can induce apoptosis in HASM cells.

Materials and Methods

Isolation of HASM cells

As described previously (4, 27), tracheal rings, from either lung/heart or lung transplantation donors (two female and three male; aged 17–45 years), were dissected under sterile conditions in HBSS (in mM: NaCl 136.8; KCl, 5.4; MgSO4 0.8; NaHPO4·2H2O, 0.4; CaCl2·2H2O, 1.3; NaHCO3, 4.2; and glucose, 5.6) supplemented with the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) and the antifungal amphotericin B (2.5 μg/ml). The smooth muscle layer was dissected free of adherent connective tissue and cartilage, and the epithelial layer was removed using a rounded scalpel blade. The smooth muscle section was then incubated for 30 min at 37°C in 5% CO2-air in HBSS containing 10 mg/ml BSA and the enzymes collagenase (type XI; 1 mg/ml) and elastase (type I; 3.3 U/ml). After the removal of any remaining connective tissue, the smooth muscle was chopped finely and incubated for a further 150 min in the enzyme solution outlined above with the elastase content increased to 15 U/ml. Cells were centrifuged (100 g, 5 min) at 4°C and resuspended in DMEM containing heat-inactivated FCS (10% v/v), sodium pyruvate (1 mM), l-glutamate (2 mM), nonessential amino acids (1X), and antimicrobial agents as previously described.

Primary culture of HASM cells

The HASM cellular suspension was placed in a tissue culture flask (75 cm2) with 6 ml of supplemented DMEM and incubated at 37°C in 5% CO2-air. The cells adhere after ∼12 h, and the culture medium was replaced after 4–5 days (12 ml) and subsequently every 3–4 days. After 10–14 days, the cells reach confluence and are identified by their typical hill-and-valley appearance and positive immunostaining for α-actin. Cells were plated onto either 96-well plates (Costar, High Wycombe, U.K.) at an initial seeding density of 2000 cells/well, 8-well chamber slides (Life Technologies, Rockville, MD), for receptor localization experiments and experiments using Hoechst stain) at an initial seeding density of 4000 cells/well, or 6-well plates at an initial seeding density of 20,000 cells/well for the experiments described below. At subconfluence, the cells were treated by being placed in DMEM containing apotransferrin (5 μg/ml), insulin (1 μM), ascorbate (100 μM), and BSA (0.1%) for 24 h. The medium was then replaced with DMEM containing 3% FCS and the treatment drugs or appropriate controls as described below.

RT-PCR for PPAR isoforms

Total RNA was prepared from cells grown under basal conditions (3% FCS) on 6-well plates using TRIzol reagent according to the manufacturer’s protocol. Reverse-transcribed DNA, was prepared using MLV-RT (Promega, Southampton, U.K.), and PCR was performed using Taq DNA polymerase (Promega), according to the manufacturer’s recommended protocols. PCR parameters were the following: initial denaturing for 3 min at 94°C, followed by 25 cycles of denaturing for 20 s at 94°C, annealing for 20 s at 55°C, followed by elongation for 30 s at 72°C, followed by termination for 7 min at 72°C. PCR primers (28) were the following: for human PPARα, sense, AAG TTC TTC AAG TAG GCC TCG, and antisense, GGA GTA TTT AGG AGG CTT TCG; and for PPARγ, sense, TCT CTC CGT AAT GGA AGA CC, and antisense, GCA TTA TGA GAC TTC CCC AC. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. For positive controls, the pBSCompPPAR contains homologous modified sequences of cDNA encoding hPPARα and -γ. The control contains an internal deletion, hence producing a smaller product than the native PPARα and -γ (28). Primers produce a 492-bp product for hPPARα mRNA (positive control, 422 bp) and a 474-bp product for hPPARγ mRNA (positive control, 400 bp). Localization of PPAR isoforms in HASM cells by confocal microscopy

Cells were fixed in methanol/acetic (1:1, −20°C) after 24-h treatment with the PPARα- and PPARγ-synthetic ligands (1, 10, and 100 μM) in the absence/presence of 3% FCS and/or in the absence/presence of IL-1β (10 ng/ml). Cells were then washed with PBS and blocked with 1% BSA in PBS for 30 min. Cells were then incubated with primary Ab directed against human PPARα, PPARγ, or PPARδ subtypes (1:250 dilution; 0.1% BSA in PBS) at 4°C overnight, followed by two washes with PBS. Secondary FITC-conjugated Ab (1:500 dilution; 0.1% BSA in PBS) was then added for 30 min, after which the cells were further washed three times with PBS and viewed by fluorescence microscopy (MRC600 confocal microscope; Bio-Rad, Hercules, CA). In some experiments, primary Ab was excluded, or primary Ab was preincubated for 1 h with a 50-fold excess of blocking peptide before the combination was added to the cells.

PPARγ expression by Western blot analysis

HASM cells cultured on 6-well plates were treated with 3% FCS for 24 h. The medium was then removed, and the cells were washed with PBS. Proteins were extracted with Tris buffer (50 mM, pH 7.4) containing EDTA (10 mM), Triton X-100 (1% v/v), and PMSF (1 mM). Extracts were boiled at a 1:1 ratio with Tris (50 mM, pH 6.8; 4% w/v SDS; 10% w/v glycerol; 4% v/v 2-ME; and 2 mg/ml bromophenol blue). Western blot for PPARγ was performed as previously described (29) using rabbit polyclonal anti-PPARγ (1:1000; Biomol, Mannheim, Exter, U.K.).

Measurement of GM-CSF and G-CSF release

Cells were treated with the synthetic PPARα ligand WY-14643 (0.01–100 μM), the synthetic PPARγ ligand ciglitazone (0.01–100 μM), or the prostanoid dehydration product PPARγ activator 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2; 1–100 μM) for 24 h in the presence of IL-1β (0.1 ng/ml) and dexamethasone (10 μM) to block endogenous cyclooxygenase activity, which has opposing effects on GM-CSF and G-CSF release (7, 30). In parallel experiments and under the same conditions, cells were also treated with the glucocorticosteroid dexamethasone (0.1 nM to 1 μM). Supernatants were either assayed directly or stored at −80°C until required. Samples were assayed for CSFs by ELISA constructed in-house from commercially available constituents according to the manufacturer’s recommendations. The limits for detection for GM-CSF and G-CSF were 16–2000 and 7.8–100 pg/ml, respectively.

Measurement of DNA synthesis

Cells were treated with WY-14643 (0.01–100 μM), ciglitazone (0.01–100 μM), 15d-PGJ2 (0.001–100 μM), or dexamethasone (0.1 nM to 10 μM) diluted in DMEM containing 3% FCS. In preliminary studies, we found that 3% FCS produced a submaximal (50% max) proliferative response; hence, this concentration of serum was used to stimulate proliferation for all experiments. We have previously established that serum stimulation results in a typical S-phase of proliferation in these cells, which is linear between 24 and 32 h (31). Thus, at 24 h after serum/drug treatment, [3H]thymidine was added to the cells and incubated for 6 h and then frozen. When required, freeze-thawed cells were harvested onto glass fiber filters and washed three times to remove any excess unbound [3H]thymidine. Cells were assayed for proliferation as measured by thymidine incorporation by scintillation spectrometry (TopCount; Packard Instrument, Meriden, CT). We have previously shown that changes in thymidine uptake are representative of changes in differentiation of HASM cells (5, 31).

Morphological detection of HASM apoptosis

DNA chromatin morphology was assessed using Hoechst staining (32). For these experiments, cells were grown onto 8-well chamber slides. After incubation with treatment drugs (100 μM) or vehicle for 24 h, the cells were washed with PBS and fixed in freezing cold methanol (−20°C) for 5 min. Hoechst dye no. 33258 (100 ng/ml) was added for 5 min, and then the cells were washed a further three times with PBS. Cells were examined for the morphological features of apoptosis such as condensation of DNA and rounding and shrinkage of the cells by fluorescence microscopy as previously described (32).

Detection of apoptosis by determination of DNA fragmentation

HASM cells cultured on 96-well plates were treated for 24, 48, and 72 h with the synthetic PPARγ ligand ciglitazone (1–100 μM). Apoptosis was assessed using an ELISA method based on the specific determination of histone-associated DNA fragments in the cytoplasmatic fraction of cell lysates, according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany).
Materials

[1H-methyl]Thymidine was obtained from Amersham (Bucks, U.K.). Amphotericin B, nonessential amino acids, and sodium pyruvate were purchased from Life Technologies. Ciglitazone, WY-14643, and 15d-PGJ2 were purchased from Biomol. IL-1β and human G-CSF matched Ab pairs were bought from R&D Systems (Abington, Oxfordshire, U.K.). Matched Ab pairs for human GM-CSF ELISA was purchased from BD PharMingen were bought from R&D Systems (Abington, Oxfordshire, U.K.). Matched

Statistical analysis

Results are shown as the mean ± SEM of n determinations from HASM cells obtained from at least two to five patients. Data was normalized as a percentage of the stimulus control response and analyzed by one-sample t test. All treatments were compared with control values, and p < 0.05 was considered to be significant. EC50 values were calculated by iterative curve fitting using Graphpad Inplot (Graphpad, San Diego, CA.)

Results

Detection of mRNA of PPAR isoforms in HASM cells by RT-PCR

HASM cells used in this study were found to express mRNA for both PPARα and PPARγ under control conditions. Primers produced a 492-bp product for hPPARα mRNA and a 474-bp product for hPPARγ mRNA. The positive control contains an internal deletion and therefore produced a smaller product of 422 bp for hPPARα and -400 bp for hPPARγ than the native PPARα and γ (Fig. 1). No bands were observed in experiments where MLV-rRT, showing specificity of the Abs used for PPARs.

Localization of PPAR isoforms in HASM cells

Using specific antisera for the PPAR subtypes, immunofluorescence was detected for PPARγ and -α in the perinuclear region and within the nucleus of HASM cells (Fig. 2, A and B, respectively). There was no difference in the localization of these receptors in either the presence/absence of 3% FCS or in the presence/absence of IL-1β (data not shown). No immunofluorescence was detected under any conditions for the PPARδ subtype. There was no immunofluorescence where the primary Ab was preincubated with an excess of blocking peptide for PPARα (data not shown) or PPARγ (Fig. 2C) or where the primary Ab was absent (Fig. 2D) showing specificity of the Abs used for PPARs.

PPARγ expression by Western blot analysis

A single band of ~55 kDa corresponding to PPARγ, was detected in protein extracts from HASM cells cultured under basal conditions using an Ab specific for PPARγ (Fig. 3). The band was consistently detected in cells cultured from three different donors.

Effect of PPAR ligands and dexamethasone on IL-1β-induced release of GM-CSF and G-CSF from HASM cells

Under control cell culture conditions, HASM cells released low or undetectable levels of GM-CSF (less than the lower limit of detection for 16 pg/ml) and G-CSF (less than the lower limit of detection for 7.8 pg/ml). However, after stimulation with IL-1β (0.1 ng/ml), the levels of both GM-CSF (531.8 ± 79.9 pg/ml) and G-CSF (1435 ± 281.7 pg/ml) were greatly increased. The synthetic PPARα ligand WY-14643 (0.01–100 μM) had no significant effect on IL-1β-induced release of GM-CSF or GC-SF (Fig. 4A). In contrast, the synthetic PPARγ ligand ciglitazone (0.01–100 μM) completely inhibited the release of both CSFs at the highest concentration tested (100 μM; Fig. 4A). In these experiments, ciglitazone appeared to have an all-or-nothing effect, and therefore, it was not possible to accurately model for a representative IC50 value for ciglitazone on CSF release. In contrast, 15d-PGJ2 inhibited both GM-CSF and G-CSF release in a concentration-dependent manner (Fig. 4B). 15d-PGJ2 produced maximum inhibition of GM-CSF and G-CSF release at 30 μM with EC50 values of 5.1 and 5.4 μM, respectively. Under the same conditions, dexamethasone (0.1 nM to 1 μM) inhibited GM-CSF release in a concentration-dependent manner, with complete inhibition at 0.01 μM (Fig. 4C). In contrast, dexamethasone produced only minor reductions in G-CSF release with a maximum effect of only 35.1 ± 12.2% inhibition (Fig. 4C).

FIGURE 1. Detection of mRNA of PPARα and γ isoforms in HASM cells. Example of the detection of PPARα and PPARγ mRNA by RT-PCR in HASM cells under control conditions. Standard markers (dX174 DNA-HaeIII, lanes 1 and 6). Positive control (10 amol cDNA, lane 2). The positive control contains an internal deletion, hence producing a smaller product than native PPARα and γ. Primers produce a 492-bp product for hPPARα mRNA (positive control, 422bp) and a 474-bp product for hPPARγ mRNA (positive control, 400 bp). HASM cells from two different donors (lanes 3 and 4). No bands were observed in experiments where MLV-rRT (used to prepare reverse-transcribed DNA) was absent (lane 5).

FIGURE 2. Expression of PPARγ and PPARα in HASM cells. Representative immunofluorescence micrographs showing PPARγ (A) and PPARα (B) subtypes localized to the nuclear and perinuclear regions of HASM cells under control conditions. There is no immunofluorescence under conditions where the primary Ab for PPARα (data not shown) or PPARγ was preincubated with an excess of blocking peptide (C) or where the primary Ab was absent (D) showing specificity of the Abs used for PPARs.

FIGURE 3. PPARγ expression in HASM cells by Western blot analysis. A single band of 55 kDa corresponding to PPARγ, was recognized in protein extracts from HASM cells cultured from three different donors (lanes 1, 2, and 3).
Effect of PPAR ligands and dexamethasone on HASM cell proliferation

[^3]HThymidine incorporation was increased when cells were stimulated with culture medium containing 3% FCS (13,200 ± 1,898 cpm/well) compared with cells treated with culture medium without FCS (1,291 ± 160.9 cpm/well). 15d-PGJ$_2$, at concentrations of 0.01–1 μM, modestly increased DNA synthesis; however, at concentrations >1 μM, 15d-PGJ$_2$ induced a profound reduction in serum-induced proliferation (Fig. 5A). Similarly, ciglitazone blocked serum-induced DNA synthesis (Fig. 5A). The PPAR$_{γ}$ ligand WY-14643 had no effect on proliferation at concentrations from 0.01 to 1 μM. However, at 10 μM, WY-14643 had a proproliferative effect (42.3 ± 16.4% increase over control). This proproliferative effect was abolished at a higher concentration of WY-14643 (at 100 μM, 2.6 ± 11.5% inhibition), and in contrast to results with either of the PPAR$_{γ}$ ligands (at concentrations of up to 100 μM), WY-14643 had no inhibitory effect on serum-induced proliferation. Moreover, in contrast to the PPAR$_{γ}$ ligands, dexamethasone (0.1 nM to 10 μM) poorly inhibited serum-induced growth with a maximum effect of only 54.1 ± 3.2% inhibition (Fig. 5B).

Morphological identification of apoptosis

The apoptotic effects of the PPAR$_{γ}$ ligands ciglitazone and 15d-PGJ$_2$ (both at 100 μM) on HASM cells were clearly visible as evidenced by cell shrinkage and nuclear condensation (Fig. 6, B and C, respectively). The morphology of these cells was clearly different to that of control cells, which displayed characteristic hill-and-valley morphology under light microscopy and no nuclear condensation (Fig. 6A). There was no evidence of apoptosis in cells treated with WY-14643 (100 μM; Fig. 6D).

FIGURE 4. Effect of PPAR ligands and dexamethasone on cytokine-induced GM-CSF and G-CSF release from HASM cells. A, 15d-PGJ$_2$ and ciglitazone completely inhibit IL-1β-induced CSF release at 100 μM. WY-14643, at the same concentration, has no significant effect. B, 15d-PGJ$_2$ inhibits both GM-CSF and G-CSF in a concentration-dependent manner and with equal potency. C, Dexamethasone completely inhibits GM-CSF release in contrast to its effects on G-CSF release, where there is only partial inhibition. Results are shown as the mean ± SEM of n = 9 determinations from three patients. Data are normalized to stimulus control and analyzed by a one-sample t test; *, p < 0.05.

FIGURE 5. Effect of PPAR ligands and dexamethasone on HASM cell proliferation. A, Ciglitazone concentration-dependently inhibits DNA synthesis in HASM cells stimulated with 3% FCS. The effects of 15d-PGJ$_2$ on serum-induced DNA synthesis were biphasic. Profound inhibitory effects were seen at concentrations ≥1 μM. B, In contrast, dexamethasone, only inhibited proliferation by a maximum of 54.1 ± 3.2%. Results are shown as the mean ± SEM of n = 6 determinations from three patients. Data are normalized to stimulus control and analyzed by a one-sample t test. Value of p < 0.05; #, Increased; *, Decreased compared with control.
Detection of apoptosis by determination of DNA fragmentation

Ciglitazone (1–100 μM) induces DNA fragmentation in HASM cells in a concentration-dependent manner after 72 h of treatment (Fig. 7). There was no effect of ciglitazone at either 24 or 48 h of treatment (data not shown). The highest concentration of ciglitazone after 72 h of treatment induced a 62.0 ± 6.7% increase over basal control (OD reading of 0.11 ± 0.004).

Discussion

This study demonstrates for the first time that PPARα and -γ, but not PPARδ, are expressed on HASM cells and that activation of the PPARγ subtype has profound anti-inflammatory effects. Moreover, activation of PPARγ can inhibit a steroid-insensitive proinflammatory response in these cells at the level of both survival factor release and proliferation. Our data adds to the growing body of evidence that these ligands may provide an alternative to the currently used steroids for inflammation control and specifically identifies COPD and asthma as new therapeutic areas.

Steroids are ineffective in the long-term management of COPD and only provide limited relief in acute exacerbations (33), and although the current first-line therapy for the management of COPD are bronchodilators, such as anticholinergics, β₂-adrenoceptor agonists, and methylxanthines, they provide only limited symptomatic relief to patients. Although steroids are highly effective in controlling asthma (34), there is some concern with long-term and high-dose use, because they cause unwanted side effects, such as cortisol suppression, bone density and growth effects, oral candidiasis, and skin bruising. Furthermore, a small proportion of patients are resistant to the anti-inflammatory effects of steroids. Hence, there is an urgent need to identify novel anti-inflammatory therapies for the treatment of these diseases.

Asthma is an allergic eosinophilic-driven disease and is associated with elevated levels of eosinophil survival factors including GM-CSF in bronchoalveolar lavage fluid (35), serum (36), and lung tissue of asthmatic patients (37, 38). Furthermore, using a mouse model of allergic inflammation, it has been shown that GM-CSF significantly contributes to the development of allergic airway inflammation through potentiating and prolonging inflammatory infiltration induced by cytokines (39). In contrast, COPD is predominantly associated with neutrophilia. Thus, survival factors for neutrophils are likely to be more important than those for eosinophils in the progression of COPD. Interestingly, GM-CSF is also a potent survival factor for neutrophils (40). In addition, neutrophil survival can be increased by the related survival factor G-CSF, which is often attributed to neutrophil-related inflammatory lung diseases (41). Hence, a similar scenario may exist in COPD where these factors may promote the survival and activation of neutrophils and thus prolong the inflammatory response.

We have previously described that HASM cells release both GM-CSF (6) and G-CSF (7) upon stimulation with the proinflammatory cytokine IL-1β. In this study, we present evidence that activation of the PPARγ subtype, by the selective synthetic ligand ciglitazone, inhibits both GM-CSF and G-CSF release, albeit at the highest concentration (100 μM). In these experiments, ciglitazone appeared to have an all-or-nothing effect, and therefore, it was not possible to accurately model for a representative IC₅₀ value for ciglitazone on CSF release. 15d-PGJ₂ also inhibited cytokine-induced GM-CSF and G-CSF release; however, in this case, 15d-PGJ₂ did so in a concentration-dependent manner and with equal potency for both survival factors. The effective concentrations used are similar to that required for functional responses in other cell types such as human lung cancer cells (42), epithelial cells (20), endothelial cells (22), and vascular smooth muscle cells (23). The inhibitory effect of 15d-PGJ₂ and ciglitazone on survival factor release is seen at concentrations that also inhibit serum-induced growth. However, the possibility that the reduced amounts of GM-CSF and G-CSF are due to reduced cell number can be excluded, because changes in DNA synthesis are not translated to changes in cell number until at least 4 days (5). Furthermore, the morphological changes that are characteristic of apoptosis were seen only at the highest concentration of 100 μM for both ciglitazone and 15d-PGJ₂.

PPARs are activated by natural ligands such as the fatty acids docosahexaenoic acid and linoleic acid and a number of eicosanoids including the prostanooids PGA₁, PGA₂, PGL₂, and PGD₂ (43). 15d-PGJ₂ has been reported to be the most potent prostanooid ligand for PPARγ yet (14) and binds directly to PPAR-γ (44). In this study, 15d-PGJ₂ and ciglitazone, which are two structurally different compounds (a prostanooid dehydration product and a thia-zolidinedione, respectively), inhibited serum-induced proliferation.
at concentrations $>1 \mu M$, with 15d-PGJ$_2$ being $\sim 10$-fold more potent than ciglitazone. This observation is consistent with other studies, which show that 15d-PGJ$_2$ is more potent than synthetic ligands (23, 45, 46). Interestingly, 15d-PGJ$_2$ has proproliferative effects on HASM cells at lower concentrations (0.001–0.1 $\mu M$), which is overridden at higher concentrations by a profound antiproliferative action; thus generating a bell-shaped response. This may be due to the actions of 15d-PGJ$_2$ on other receptor populations. In fact, our own data using WY-14643 suggest that activation of PPARs may have a small, but significant, antiproliferative effect in our cells.

In these studies, we observed an almost complete inhibition of proliferation using 10 $\mu M$ 15d-PGJ$_2$ and 100 $\mu M$ ciglitazone. These concentrations are high compared with those required by other PGs to activate typical cell surface receptors. However, the active concentrations used in our study are consistent with those used in similar systems by others (22, 23, 25, 42, 45). Until potent and selective antagonists of PPARs are characterized, we are limited to the tools that we have used.

We have previously established that IL-1$\beta$-induced release of GM-CSF and more recently G-CSF are differentially regulated by the prostanoids PGE$_2$ and PGI$_2$ (7, 30) in HASM cells and vascular smooth muscle cells, respectively. Thus, PGE$_2$, which is the major prostanoid released from HASM cells (4), inhibits cytokine-induced GM-CSF release but potentiates cytokine-induced G-CSF release (7). In the absence of any commercially available PPAR antagonists or IP/EP prostanoid receptor antagonists, we have used this phenomenon to rule out the possibility that 15d-PGJ$_2$, which is a prostanoid dehydrogen product of PGD$_2$, is mediating its inhibitory effects on G-CSF via a traditional cell surface prostanoid receptor. In the present study, we have shown that 15d-PGJ$_2$, inhibits both GM-CSF and G-CSF release, thus implying that the inhibitory effects of 15d-PGJ$_2$ on G-CSF release are not mediated through activation of a traditional cell surface prostanoid receptor.

Interestingly, the glucocorticosteroid dexamethasone inhibited IL-1$\beta$-induced release of both GM-CSF and G-CSF, but to varying degrees. Dexamethasone inhibited GM-CSF release with an EC$_{50}$ of $\sim 1$ nM and maximum inhibition at 1 $\mu M$. In contrast, dexamethasone had little effect on IL-1$\beta$-induced G-CSF release where levels were reduced by only 35.1% at 1 $\mu M$.

Both ciglitazone and 15d-PGJ$_2$ maximally inhibited serum-induced DNA synthesis in a concentration-dependent manner. In marked contrast to this, dexamethasone only partially inhibited serum-induced growth. Cells that had been treated with the PPAR$\gamma$ ligands for 24 h showed nuclear condensation and shrinkage and rounding of the cells when examined by light microscopy (our unpublished observations). However, this was evident only at the highest concentration of ligand used. These morphological features were confirmed when the cells were stained with Hoechst DNA stain. Furthermore, DNA fragmentation, a commonly used marker for the apoptotic process, was increased in cells after treatment with ciglitazone for 72 h in a concentration-dependent manner. Nuclear condensation occurs before DNA fragmentation, and the fact that no DNA fragmentation was detected at 24 or 48 h may reflect the time course of the progression of apoptosis induced by this ligand in this cell type. PPAR$\gamma$ ligands have been reported to induce apoptosis in many cell types (22, 26, 42); however, this is the first description of an agent, other than Fas ligand (47), to induce apoptosis in HASM cells.

Although PPAR$\alpha$ is expressed in cultured HASM cells, the synthetic PPAR$\alpha$ ligand WY-14643 had no inhibitory effect on survival factor release, DNA synthesis, or apoptosis, suggesting that PPAR$\alpha$ does not have a role in modulating these responses in HASM cells. However, PPAR$\alpha$ activation has been shown to inhibit IL-6 and 6-keto-PGF$_{1\alpha}$ secretion from primary aortic smooth muscle cells. In addition, PPAR$\alpha$ ligands have been reported to inhibit cytokine-induced genes such as vascular cell adhesion molecule-1 and tissue factor in human endothelial cells and monocytes, respectively (48, 49). It has been suggested that PPAR$\alpha$ may have an important immunomodulatory role because PPAR$\alpha$ knockout mice exhibit an exacerbated inflammatory response (18).

It should be noted that the field of PPAR biology is currently hampered by the lack of effective and highly selective agonists and antagonists. Moreover, some groups have suggested that, where high concentrations of drugs are required to see effects in biological systems, actions on pathways other than the PPARs may also contribute to the effects seen.

The synthetic PPAR$\gamma$ ligands are already clinically used for their potent antidiabetic effects, and the anti-inflammatory effects of these agents have been demonstrated in animal models of bowel inflammation (50, 51) and arthritis (46). The present study demonstrates that PPAR$\gamma$ ligands have profound anti-inflammatory effects and may be a novel anti-inflammatory strategy that could dampen the inflammatory response by 1) limiting the recruitment and activation of infiltrating leukocytes, 2) inhibiting HASM cell growth, and 3) inducing apoptosis. Moreover, we show that PPAR$\gamma$ ligands are more potent than steroids in inhibiting smooth muscle cell growth and G-CSF release. Although this observation cannot explain the lack of effectiveness of steroids in COPD patients, PPAR$\gamma$ ligands may prove to be particularly effective in the treatment of COPD and steroid-insensitive asthma.

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