

PPARs: Regulators and Translational Targets in the Lung

Guest Editors: Raju C. Reddy, Virender K. Rehan, Jesse Roman, and Patricia J. Sime





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PPAR Research

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Editorial

PPARs: Regulators and Translational Targets in the Lung

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In this special issue, we present a selection of review articles and new studies that cover many aspects of PPAR biology and their potential translational implications. These papers provide cogent examples of the importance of PPARs in the lung and its diseases, focusing mainly on PPAR- γ . They illustrate the wide variety of functions served by this receptor and indeed all PPARs.

For example, PPAR- γ in epithelial cells is essential for normal development of fetal and neonatal lungs, suggesting that PPAR- γ agonists might be useful for treating bronchopulmonary dysplasia of prematurity and similar conditions. Another prominent role of PPAR- γ and other PPARs is regulation of inflammatory responses. The influence of PPARs on inflammation is exerted through many different pathways, including effects on migration of inflammatory cells from the bloodstream into affected tissues. Effects of PPARs on eosinophils are somewhat complex, however, as high concentrations of PPAR- γ activators inhibit migration while low concentrations stimulate it, probably by upregulating non-directional cell movement (chemokinesis) rather than directed chemotaxis. PPAR- γ is also a key regulator of fibroblast transdifferentiation, promoting differentiation into adipocytes while inhibiting transformation of fibroblasts to myofibroblasts. These findings suggest that PPAR- γ agonists might have therapeutic utility for fibrosis. Interestingly, new findings provide evidence that intracellular mycobacterial pathogens including *M. tuberculosis* can activate PPAR- γ , exploiting its immunosuppressive actions to subvert the

immune response while promoting an intracellular environment favorable for mycobacterial survival by stimulating accumulation of lipid droplets.

Early research identified PPAR- α and PPAR- γ as metabolic regulators, but later discoveries revealed far broader regulatory roles for all three PPARs. Their anti-inflammatory roles may be particularly important in the lung, which is constantly exposed to infectious agents and noxious stimuli and yet depends on the integrity of delicate structures such as the alveolar-capillary interface for its crucial gas-exchange function. All three PPARs exert anti-inflammatory effects and are widely distributed in the lung. PPAR- γ in particular is highly expressed in lung epithelium and endothelium as well as in alveolar macrophages, acting in these cells to limit inflammation and promote its resolution. Activated PPARs act through several mechanisms, including transrepression of proinflammatory transcription factors such as NF- κ B. In this way, they limit the production of cytokines and other mediators that drive inflammation. Together, these findings point to potential utility of PPARs as targets for treatment of selected inflammatory lung diseases.

A key challenge is to discover and clearly define the roles of endogenous PPAR agonists, and whether inadequate upregulation of PPAR activity by endogenous agonists contributes to pathogenesis of certain diseases. No endogenous ligands are currently known for PPAR- β/δ , but recent discoveries demonstrated two distinct endogenous PPAR- α agonists that are physiologically relevant in different cell

types: a specific phospholipid in hepatocytes and leukotriene B₄ in cells of the immune system. Such utilization of different PPAR- α agonists presumably permits regulation by molecules relevant to key functions of the respective cells (phospholipids in hepatocytic lipid metabolism; leukotriene B₄ in immune cell responses to infection and inflammatory stimuli). Cell-type selective effects of relevant PPAR agonists represent a potentially important mechanism for pleiotropic physiological regulation via common and widely distributed receptors. It will be interesting to discover whether other PPARs follow a similar pattern.

The search for endogenous ligands of PPAR- γ has been a major focus of research. Several candidates have been identified, although the physiological relevance of most of them remains questionable. Perhaps the strongest current candidates are nitrated fatty acids (NFAs), which are produced by nonenzymatic reactions of NO (and its products) with unsaturated fatty acids. NFAs activate all three PPARs, but are most potent for PPAR- γ . NO production is often upregulated during inflammation, which would tend to raise local NFA concentrations and thereby trigger anti-inflammatory effects of PPAR activation, thus presumably mitigating inflammatory tissue damage. The total concentrations of NFAs in the bloodstream significantly exceed their EC₅₀ for PPAR- γ activation. However, most circulating NFAs are either esterified or bound to plasma proteins. A portion of the esterified NFAs could be released due to inflammation-induced upregulation of cholesterol ester hydrolases, thus providing a further basis for physiological, feedback-driven inflammatory modulation by NFAs. Nevertheless, whether concentrations of free endogenous NFAs are sufficient for efficacious PPAR- γ activation remains unknown. Furthermore, like PPAR- α , PPAR- γ may prove to utilize different endogenous agonists for different, functionally diverse, cell types in which it is expressed. Accordingly, efforts are underway to develop synthetic PPAR- γ agonists that effectively activate it in a targeted, cell type-selective manner. Successful development of such ligands could reduce the likelihood of unwanted effects on non-target tissues.

Synthetic PPAR agonists, both novel and established, are a focus of translational and therapeutic interest, although many of these have been found to also exert significant PPAR-independent effects. Recent years have seen the development, although not yet the clinical use, of “triple” agonists that activate all three PPARs. The PPAR- γ -activating thiazolidinediones and PPAR- α -activating fibrates alike have been used clinically for well over a decade. Some of these drugs can cause adverse effects during long-term use, such as cardiovascular events that led to restrictions on rosiglitazone use. However, such adverse effects might be reduced by shorter periods of use, or by using lower doses made possible by synergy with other therapeutic agents. Preclinical studies have demonstrated such synergy in several diseases including certain cancers. In this connection, a preliminary clinical study of pioglitazone as potential neoadjuvant therapy in non-small-cell lung cancer is currently underway, while two phase I studies of a novel PPAR- γ agonist in combination with the established chemotherapeutic agents carboplatin/paclitaxel

or erlotinib in patients with non-small-cell lung cancer have recently been completed.

In asthma, preclinical studies have strongly supported the beneficial effects of PPAR- γ agonists. Two studies of pioglitazone as asthma therapy are currently in progress, one of which is restricted to patients whose severe asthma is not adequately controlled by currently available treatments. Such patients would clearly benefit if pioglitazone or other PPAR- γ agonists prove helpful. There are no clinical trials of PPAR- γ agonists in other pulmonary diseases. However, considering available preclinical evidence for the benefits and relative safety of these drugs, along with the current absence of satisfactory therapy in many instances, it would be reasonable to conduct such trials in diseases such as COPD. It is also likely that we will see clinical trials of PPAR- β/δ agonists, which to date have not found therapeutic application.

In clinical trials, as in current clinical practice, PPAR agonists are administered orally. A promising alternative delivery route for treatment of lung diseases is via inhalation, which is advantageous because it achieves maximum drug concentrations at the intended target while limiting systemic side effects. Inhalation can also circumvent problems pertaining to oral bioavailability and first-pass metabolism. This is already the usual route of administration for anti-inflammatory glucocorticoids in treatment of asthma and other lung diseases, as it minimizes systemic adverse effects. There is currently no inhalable formulation of any PPAR agonist, but development of such formulations seems feasible and might enhance the range of therapeutic applications of PPAR agonists in lung disease.

We appreciate the opportunity to share exciting research on the varied roles that PPARs play in lung biology and disease and hope that the papers presented here will stimulate further important research and translational progress.

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Research Article

TGF β 1 Controls PPAR γ Expression, Transcriptional Potential, and Activity, in Part, through Smad3 Signaling in Murine Lung Fibroblasts

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Transforming growth factor β 1 (TGF β 1) promotes fibrosis by, among other mechanisms, activating quiescent fibroblasts into myofibroblasts and increasing the expression of extracellular matrices. Recent work suggests that peroxisome proliferator-activated receptor γ (PPAR γ) is a negative regulator of TGF β 1-induced fibrotic events. We, however, hypothesized that antifibrotic pathways mediated by PPAR γ are influenced by TGF β 1, causing an imbalance towards fibrogenesis. Consistent with this, primary murine primary lung fibroblasts responded to TGF β 1 with a sustained downregulation of PPAR γ transcripts. This effect was dampened in lung fibroblasts deficient in Smad3, a transcription factor that mediates many of the effects of TGF β 1. Paradoxically, TGF β 1 stimulated the activation of the PPAR γ gene promoter and induced the phosphorylation of PPAR γ in primary lung fibroblasts. The ability of TGF β 1 to modulate the transcriptional activity of PPAR γ was then tested in NIH/3T3 fibroblasts containing a PPAR γ -responsive luciferase reporter. In these cells, stimulation of TGF β 1 signals with a constitutively active TGF β 1 receptor transgene blunted PPAR γ -dependent reporter expression induced by troglitazone, a PPAR γ activator. Overexpression of PPAR γ prevented TGF β 1 repression of troglitazone-induced PPAR γ -dependent gene transcription, whereas coexpression of PPAR γ and Smad3 transgenes recapitulated the TGF β 1 effects. We conclude that modulation of PPAR γ is controlled by TGF β 1, in part through Smad3 signals, involving regulation of PPAR γ expression and transcriptional potential.

1. Introduction

Transforming growth factor β 1 (TGF β 1) is a pleomorphic growth factor with anti-inflammatory and profibrotic properties that has been implicated in many forms of natural and experimental tissue fibrosis [1]. In lung, TGF β 1 is produced by epithelial cells, alveolar and tissue macrophages, and fibroblasts after exposure to injurious agents such as silica, bleomycin, hyperoxia, and paraquat among others [2]. A key role for TGF β 1 in lung fibrosis has been confirmed in studies showing the development of lung fibrosis in animals transfected with TGF β 1-producing adenovirus, and by work demonstrating inhibition of experimental lung fibrosis by interventions targeting TGF β 1 or its downstream signals [3, 4].

The profibrotic effects of TGF β 1 are mostly, but not entirely, mediated by intracellular signals triggered by the transcription factor Smad3 [5]. TGF β 1/Smad3 signaling stimulates connective tissue expression and epithelial-mesenchymal transition, events considered key to the development of lung fibrosis [6]. In fibroblasts, TGF β 1/Smad3 signaling stimulates their transdifferentiation into myofibroblasts and their expression of matrix genes like fibronectin and collagens [7]. Importantly, knockdown of the TGF β 1/Smad3 signaling pathway inhibits experimental lung fibrosis [8]. In a model of immune-mediated airway fibrosis, we demonstrated inhibition of myofibroblast transdifferentiation and matrix deposition in animals deficient in Smad3 [9].

Considering its importance in the development of lung fibrosis, research directed at investigating the factors

that control TGF β 1/Smad3 signaling has intensified. This research has led to the exploration of *peroxisome proliferator-activated receptor γ* (PPAR γ), a member of the ligand-activated nuclear hormone receptor superfamily of transcription factors that is known for its ability to regulate glucose and lipid metabolism and that has been implicated in insulin sensitivity, atherosclerosis, and inflammation [10, 11]. Preliminary studies in our laboratory suggested that PPAR γ inhibits the effects of TGF β 1 through direct interactions with Smad3 (Ramirez et al., unpublished observations). Furthermore, others have demonstrated protection against TGF β 1-induced myofibroblast transdifferentiation in cells treated with PPAR γ activators [12].

These observations suggest a promising role for PPAR γ activators in the treatment of fibrotic lung disorders. However, we wondered if TGF β 1 influences PPAR γ expression and/or activation in tissues. To test this idea, we examined the effects of TGF β 1 on PPAR γ in murine primary lung fibroblasts and found that TGF β 1, in part through Smad3 signaling, differentially controls PPAR γ expression levels, transcription, and activation. These observations suggest that TGF β 1/Smad3 signaling triggers profibrotic events, while concomitantly influencing the expression of PPAR γ .

2. Materials and Methods

2.1. Cell Culture. NIH/3T3 fibroblasts were purchased from ATCC. Primary murine lung from Smad3-deficient mice and wildtype C57BL/6 were generated and maintained as previously described [7, 9]. Primary lung fibroblasts were used between passages 2–10 in all experimental conditions. Where indicated, serum-starved fibroblasts were first pretreated for 1 hour in the presence or absence of TGF β 1 (10 ng/mL) (R&D Systems, Minneapolis, MN, USA) followed by incubation with 10 μ M of troglitazone (Cayman Chemical, Ann Arbor, MI, USA) for the specified time. The studies were approved by the institutional animal research review committee.

2.2. Western Blots. Whole cell extracts were processed and analyzed as described [7, 9] with antibodies to phospho-PPAR γ (Millipore, Billerica, MA, USA) and PPAR γ protein (Cell Signaling Technology). β -Actin was used as control (Sigma, St. Louis, MO, USA).

2.3. Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated and tested as previously described [7]. Murine forward and reverse primers for PCR reactions were based on GenBank published sequences and are as follows: PPAR γ (5'-GAC CAC TCG CAT TCC TTT-3'; 5'-CCA CAG ACT CGG CAC TCA-3') and 28s rRNA (5'-TTG AAA ATC CGG GGG AGA-3'; 5'-ACA TTG TTC CAA CAT GCC AG-3'). Amplicons were resolved on 1% agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator.

2.4. Immunofluorescence Microscopy. A phospho-PPAR γ antibody was applied to paraformaldehyde-fixed, Triton X-100-permeabilized cells at 4°C overnight followed by an Alexa Fluor 555-labeled anti-rabbit secondary antibody (Invitrogen). Slides were cover-slipped with ProLong Gold mounting medium (Invitrogen) and viewed under epifluorescence microscopy (Olympus BX41, Melville, NY, USA). Images were captured using MagnaFire 2.1 digital image acquisition software (Goleta, CA, USA).

2.5. Plasmids. Murine pCMX-PPAR γ [13] and the human PPAR γ promoter [14] were generous gifts from L. Jameson, (Northwestern University, Chicago, IL, USA) and C.M. Hart (Emory University, Atlanta, GA, USA), respectively. Flag-Smad3, AP2 (PPRE)-Luc, and T β RI (CA) were purchased from Addgene (numbers 14827, 8858, and 14833, resp., Cambridge, MA, USA).

2.6. Transfection and Reporter Studies. A calcium-phosphate transient transfection protocol was followed [15]. Briefly, at 50% confluence, cells were exposed to fresh growth media for 1 hour. DNA precipitate was applied for 24 hours. Cells were then washed, cultured for 6 hours in complete serum-free media, and treated as indicated. For reporter assays, treated cells were lysed using 5x Passive Lysis Buffer (Promega, Madison, WI, USA) and exposed to luciferase reagent, which was prepared according to Dyer et al. [16]. Luminescence was measured with a Thermo Luminoskan Ascent luminometer (Waltham, MA, USA) and normalized to Renilla activity [16].

2.7. Data Analysis. Western blotting and RT-PCR experiments were performed in duplicates and repeated at least three times to ensure consistency. Reporter and proliferation data were also repeated thrice each with 3-4 replicates per experiment. All results are presented as mean \pm SE. GraphPad Prism v3.0 was used to analyze data by one-way ANOVA computation with Tukey's multiple comparisons test. A *P* value of 0.05 was considered significant.

3. Results

3.1. Effects of TGF β 1 on PPAR γ Expression. To begin to evaluate the effects of TGF β 1 on PPAR γ expression, we first tested for PPAR γ gene transcription in lung fibroblasts. After a small and nonsignificant increase in PPAR γ expression, primary lung fibroblasts displayed a dramatic downregulation of PPAR γ mRNA expression beginning after an hour of exposure to TGF β 1 and persisting for at least 48 hours (Figure 1(a)). To determine a mechanism by which TGF β 1 might regulate PPAR γ expression, we examined the role of the transcription factor and TGF β 1 intracellular transducer, Smad3. For this, primary lung fibroblasts were harvested from the lungs of Smad3-deficient mice and wildtype mice from the same genetic background and were cultured in the presence or absence of TGF β 1 (Figure 1(b)). In wildtype primary lung fibroblasts, TGF β 1 downregulated PPAR γ mRNA expression as previously demonstrated. However,

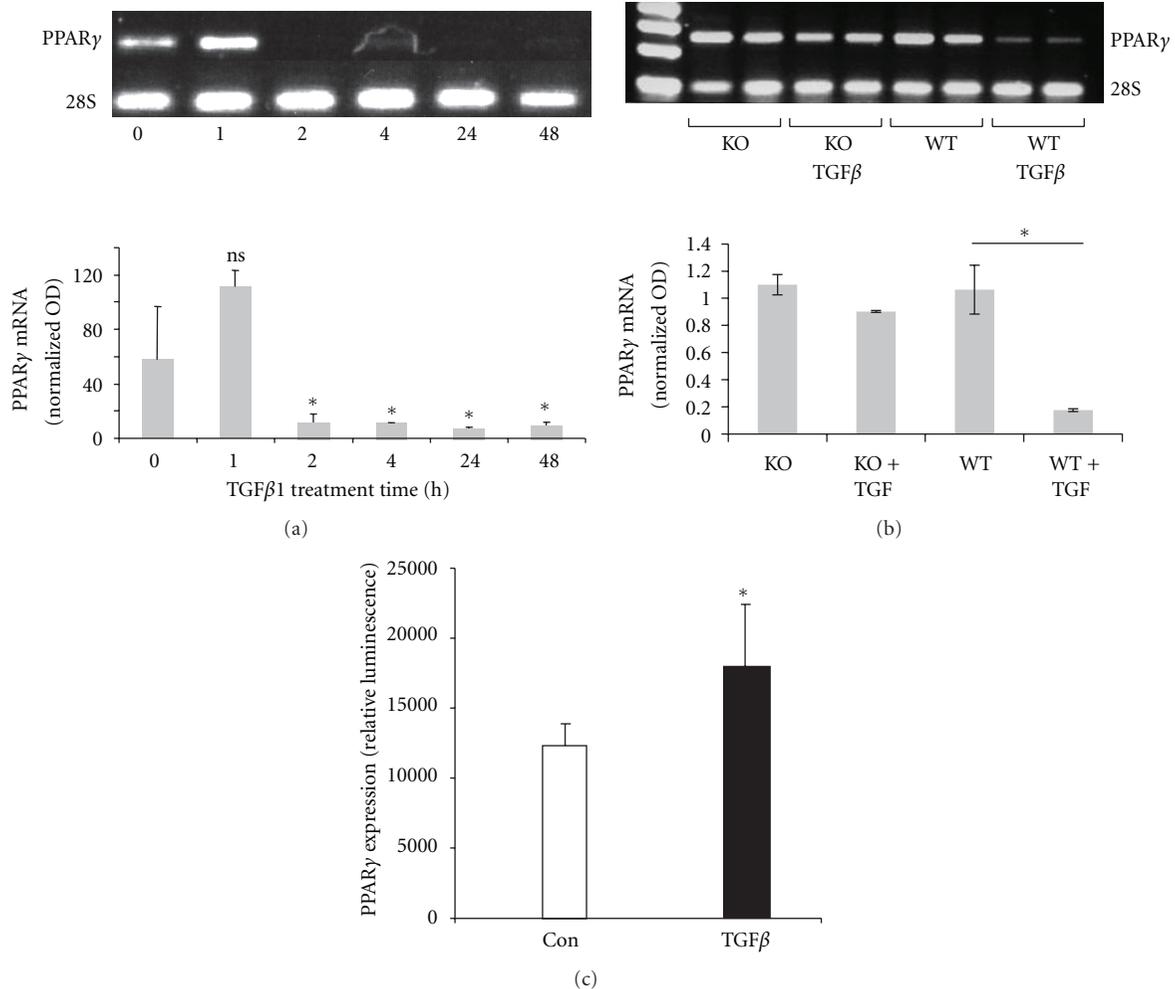


FIGURE 1: TGFβ1 modulates PPARγ expression, in part, via Smad3 signaling. (a) TGFβ1 and PPARγ mRNA expression. Murine primary lung fibroblasts were stimulated with TGFβ1 (10 ng/mL) for the indicated times. The treated cells were then submitted for analysis by RT-PCR for PPARγ. (b) Role of Smad3. Primary lung fibroblasts were isolated from the lungs of Smad3-deficient and wildtype mice and incubated with TGFβ1 (10 ng/mL) for 24 hours. Transcripts of PPARγ mRNA were detected using RT-PCR. **P* < 0.05. (c) Regulation of PPARγ gene transcription. NIH/3T3 fibroblasts were transiently transfected with a luciferase reporter driven by the full-length PPARγ promoter and incubated with TGFβ1 (10 ng/mL). Cellular extracts were analyzed after 24 hours for luminescence. Luciferase activity was normalized to Renilla with data shown as fold change ± SE relative to control. **P* < 0.05.

this effect was greatly blunted in cells lacking Smad3, suggesting that Smad3 signaling is responsible for much of the inhibition of PPARγ gene expression observed in TGFβ1-treated fibroblasts.

To test if the inhibition of PPARγ expression by TGFβ1/Smad3 signaling occurred at the level of the gene promoter, NIH/3T3 fibroblasts were transfected with full-length human PPARγ promoter ligated to a luciferase reporter (Figure 1(c)). However, instead of inhibiting PPARγ expression, we observed that TGFβ1 stimulated activity of the PPARγ gene promoter.

3.2. Effect of TGFβ1 on PPARγ Phosphorylation. Next, we determined if TGFβ1 could induce PPARγ posttranslational modifications, namely, phosphorylation, given TGFβ1's known ability to activate intracellular signaling molecules. To

this end, primary lung fibroblasts were treated with TGFβ1 at several different time points and examined for phospho-PPARγ with an antibody that specifically detects phosphorylation at serine 82 of PPARγ1 (corresponding to serine 112 of PPARγ2). By Western blot, phosphorylation of PPARγ was detectable within 3 hours of TGFβ1 exposure, with maximal effects at 6 hours, but persistent up to 24 hours after stimulation when the experiment ended (Figure 2(a)). In parallel experiments, phospho-PPARγ was visualized by immunofluorescence in the nuclear compartment one hour after initiation of TGFβ1 treatment, peaked three hours later, but was mostly absent by six hours (Figure 2(b)).

3.3. Effects of TGFβ1 on PPARγ-Dependent Gene Expression. We then examined the functional effects of TGFβ1 signaling on the transcriptional capability of PPARγ after activation

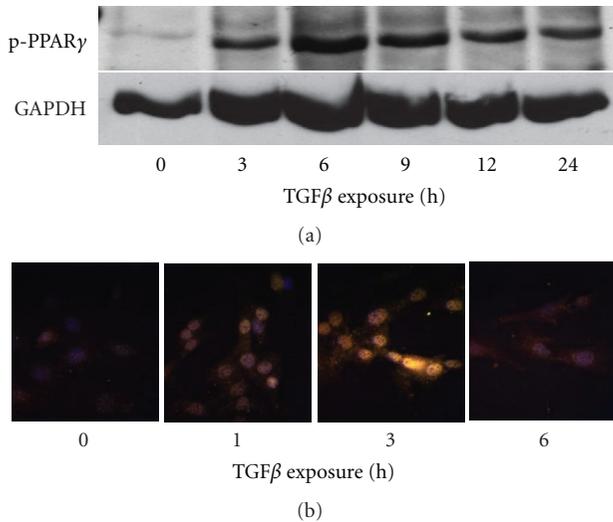


FIGURE 2: TGF β 1 induces the phosphorylation of PPAR γ . Primary lung fibroblasts were treated with TGF β 1 (10 ng/mL) for the indicated times. Phosphorylation of PPAR γ was detected by Western blot (a) and immunofluorescence microscopy (b) using a phospho-specific antibody.

by the PPAR γ ligand, troglitazone. These experiments were conducted in NIH/3T3 fibroblasts containing a luciferase reporter driven by a PPAR γ response element. In other words, luciferase induction indicates stimulation of PPAR γ -dependent gene expression. As shown in Figure 3(a), troglitazone stimulated PPAR γ activation as demonstrated by the ability of troglitazone to induce PPAR γ -dependent gene transcription. This effect was blunted by the overexpression of a constitutively-active type I TGF β receptor, that is, T β RI or activin-like kinase 5 (ALK5), suggesting a role for TGF β 1 receptor activation in the TGF β 1-dependent repression of gene transcription by PPAR γ . However, the inhibition of transcriptional ability of PPAR γ by TGF β 1 signaling was overcome by the forced overexpression of the full-length PPAR γ gene (Figure 3(b)). As expected, when a Smad3 transgene was cotransfected with the PPAR γ transgene in stoichiometrically equivalent amounts, TGF β 1 was again able to partially offset activation of a PPAR γ -responsive gene promoter induced by troglitazone (Figure 3(c)). These data indicate that stimulation of TGF β 1-dependent pathways, via activation of TGF β 1 receptors and Smad3 signaling, regulates the activity of PPAR γ on gene transcription.

4. Discussion

Our studies suggest that TGF β 1 influences the expression and activity of PPAR γ with potential implications to lung inflammation and fibrosis. PPARs are recognized as versatile members of the ligand-activated nuclear hormone receptor superfamily of transcription factors that includes receptors for steroids, thyroid hormone, retinoic acid, and vitamin D among others [10, 11]. PPARs are considered to play key roles in diverse physiological processes ranging from lipid

metabolism to inflammation and have been implicated in diseases such as cancer, atherosclerosis, and lung injury [17]. Three subtypes of PPARs have been identified and cloned: PPAR α , PPAR β/δ , and PPAR γ . Of the three PPARs identified to date, PPAR γ represents the most promising PPAR target in lung diseases in view of emerging reports implicating this molecule in various pulmonary processes [17]. Importantly, PPAR γ has been described as a negative regulator of macrophage function since its activation suppresses the production of inflammatory cytokines, chemokines, metalloproteinases, and nitric oxide [18]. These PPAR γ -mediated anti-inflammatory effects are not restricted to monocytic cells as treatment with PPAR γ agonists results in inhibition of cytokine and chemokine production in other cells [18–20]. More recently, it has been reported that PPAR γ activators inhibit TGF β 1-induced myofibroblast transdifferentiation [12].

In diseased tissues, PPAR γ expression has been shown to relate inversely with that of TGF β 1 [21]. Thus, it appears that the balance between TGF β 1 and PPAR γ may determine, among other factors, whether fibrogenesis predominates after tissue injury. However, in many patients and in experimental models, endogenous PPAR γ is unable to counter the effects of TGF β 1. We reasoned that tissue injury results in the expression of factors capable of inhibiting PPAR γ expression or of blunting its antifibrotic effects. We further hypothesized that TGF β 1 itself could directly influence PPAR γ expression. The observations reported here suggest that this is indeed the case. We observed that TGF β 1 has an early, but transient, inductive effect on PPAR γ mRNA expression in primary lung fibroblasts; this effect was likely caused by the ability of TGF β 1 to induce PPAR γ gene transcription (Figure 1). This early effect was associated with PPAR γ mRNA translation into protein and phosphorylation of the PPAR γ protein. However, this effect was later associated with profound inhibition of PPAR γ mRNA accumulation. The exact mechanisms responsible for this late effect are unknown, but increased mRNA degradation is likely. Based on these observations, we postulate that TGF β 1 expression and/or activation in injured tissues is associated with early induction of a counterregulatory factor, PPAR γ . However, persistence of TGF β 1 expression/activation results in late inhibition of PPAR γ mRNA accumulation. It should be highlighted that this “biphasic” effect of TGF β 1 on PPAR γ (early stimulation and late repression) has been observed by others when studying the actions of TGF β 1 on PPAR γ expression in vascular smooth muscle cells [22]. When studying these effects, one must consider cell type and other factors that could potentially affect the responses observed. For example, others have demonstrated that TGF β increases PPAR γ expression in H460 cells, but not in Ch27 cells, whereas nuclear accumulation of p-Smad3 was only observed in CH27 cells [23].

Interestingly, despite late inhibition of PPAR γ mRNA accumulation, we observed persistent PPAR γ phosphorylation at least 24 hours after TGF β stimulation (Figure 2). This, however, did not correlate with persistent nuclear localization of PPAR γ as demonstrated by cytochemistry (Figure 2(b)). The mechanisms responsible for these events remain unclear.

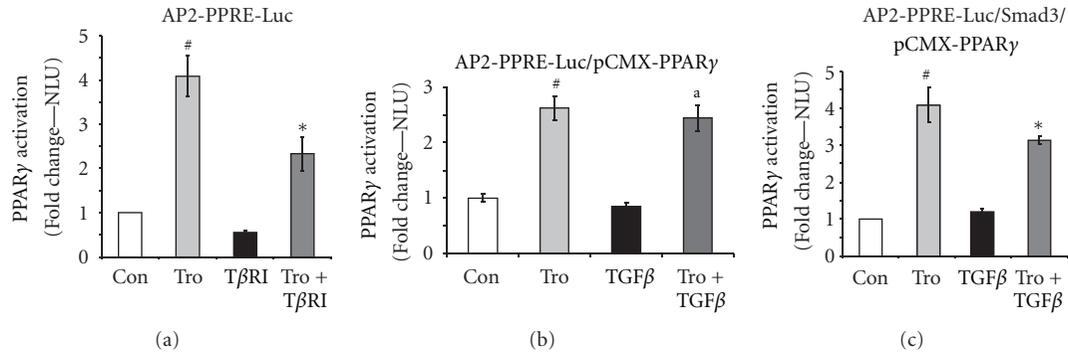


FIGURE 3: TGF β 1 signaling inhibits the transcriptional activity of PPAR γ . PPAR γ transcriptional activity was determined with luciferase assays in NIH/3T3 fibroblasts bearing a PPAR γ response element-luciferase reporter (AP2-PPRE-Luc). Luciferase activity was normalized to Renilla with data shown as fold change \pm SE relative to control. # $P < 0.001$ compared to control. * $P < 0.001$ compared to troglitazone. ^a $P < 0.001$ compared to troglitazone. (a) Effects of troglitazone and TGF β RI. PPRE-containing NIH/3T3 fibroblasts were treated with troglitazone (Tro 10 μ M) and/or cotransfected with a constitutively active type I TGF β receptor (TGF β RI). At 24 hours, cells were analyzed for luciferase activity. (b) Overexpression of PPAR γ gene. The full-length PPAR γ gene and a PPRE were cotransfected into NIH/3T3 fibroblasts, which were then pretreated with TGF β 1 (10 ng/mL) for 1 hour, followed by troglitazone (10 μ M) for 24 hours. (c) Role of Smad3. Smad3 and PPAR γ were coexpressed in NIH3T3 fibroblasts with a PPRE. Cells were exposed to TGF β 1 (10 ng/mL) for 1 hour and then stimulated with troglitazone (10 μ M) for 24 hours.

We also studied the role of Smad3 signaling in the effects observed. We found that Smad3 appears to mediate, at least in part, the effects of TGF β 1 since cells deficient in Smad3 showed blunting of the TGF β 1 downregulatory effect on PPAR γ . This was not surprising considering that Smad3 mediates many of the effects of TGF β 1 in tissues. These data are consistent with observations made in dermal fibroblasts [21]. In other work, we reported that activation of P38 MAPK plays a role in TGF β 1-induced myofibroblast transdifferentiation [7], and others have documented the interplay between TGF β 1 and MAPKs in other systems [24]. Lin et al. (2011) reported that TGF β -induced expression of PPAR γ was related to activation of p38, but this was tested in H460 carcinoma cells [23]. Interestingly, they also showed that PPAR γ 1 can bind Smad3 and p-Smad3. Zheng and Chen, on the other hand, reported that exogenous TGF β inhibits PPAR γ expression in activated hepatic stellate cells and this appeared mediated through Smads [25]. Furthermore, and consistent with our data, blocking TGF β signaling by dominant negative type II TGF β receptor increased PPAR γ . Choy and Derynck (2003) reported that TGF β inhibits adipogenesis by signaling through Smad3 which, in turn, interacts with C/EBPs leading to transcriptional inhibition of the PPAR γ 2 promoter [26].

The studies described above suggest that TGF β 1 controls PPAR γ transcription, mRNA accumulation, and protein phosphorylation in differential ways. Considering the many points at which TGF β 1 could influence PPAR γ expression and activation, these studies did not allow us to predict the overall effects of TGF β 1 on PPAR γ function. This critical issue was addressed by testing cells transfected with a PPAR γ -responsive element that is transcribed only when activated PPAR γ enters the nucleus and interacts with target genes. To test the system, we first showed that troglitazone, a PPAR γ activator, stimulated PPAR γ -dependent gene expression (Figure 3(a)). Interestingly, expression of a constitutively

active TGF β 1 receptor, TGF β 1RI, reduced the effect of troglitazone implicating this receptor in mediating the effects of TGF β 1. Together, TGF β R1 and TGF β R2 form an activated ligand-receptor complex capable of stimulating downstream signals like Smads [27].

As expected, the inhibition of transcriptional ability of PPAR γ by TGF β 1 was overcome by the forced expression of the full-length PPAR γ gene. This is an important observation because it suggests that targeting the TGF β 1/PPAR γ balance by enhancing PPAR γ activation may have potential therapeutic relevance. Several natural and synthetic compounds have been identified as activators of PPAR γ . The insulin-sensitizing antidiabetic drugs known as thiazolidinediones were the first compounds identified as PPAR γ agonists [28]; troglitazone, used in this report, is a thiazolidinedione, but it is no longer available commercially due to liver toxicity [29]. However, other thiazolidinediones are available and are being tested in clinical trials in different areas.

Finally, we tested the role of Smad3 by cotransfecting cells with the Smad3 and PPAR γ transgenes in stoichiometrically equivalent amounts. As before, TGF β 1 was able to partially offset activation of a PPAR γ -responsive gene promoter induced by troglitazone. These data indicate that stimulation of TGF β 1-dependent pathways, via activation of TGF β 1 receptors and Smad3 signaling, regulates the activity of PPAR γ on gene transcription.

In conclusion, modulation of PPAR γ is intricately controlled by TGF β 1, in part through Smad3 signals, involving tight regulation of PPAR γ expression levels and transcriptional potential. These mechanisms begin to explain how TGF β 1 is able to overcome the anti-inflammatory and anti-fibrotic effects of PPAR γ and may have implications *in vivo*. The commercial availability of PPAR γ activators capable of tilting the TGF β 1/PPAR γ balance to reduce, delay, and even reverse fibrosis raises the possibility of targeting PPAR γ in humans with fibrotic lung disease.

Conflict of Interests

The authors declare that they have no conflicts of interests.

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Review Article

PPAR γ Ligands Regulate Noncontractile and Contractile Functions of Airway Smooth Muscle: Implications for Asthma Therapy

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In asthma, the increase in airway smooth muscle (ASM) can contribute to inflammation, airway wall remodeling and airway hyperresponsiveness (AHR). Targetting peroxisome proliferator-activated receptor γ (PPAR γ), a receptor upregulated in ASM in asthmatic airways, may provide a novel approach to regulate these contributions. This review summarises experimental evidence that PPAR γ ligands, such as rosiglitazone (RGZ) and pioglitazone (PGZ), inhibit proliferation and inflammatory cytokine production from ASM *in vitro*. In addition, inhaled administration of these ligands reduces inflammatory cell infiltration and airway remodelling in mouse models of allergen-induced airways disease. PPAR γ ligands can also regulate ASM contractility, with acute treatment eliciting relaxation of mouse trachea *in vitro* through a PPAR γ -independent mechanism. Chronic treatment can protect against the loss of bronchodilator sensitivity to β_2 -adrenoceptor agonists and inhibit the development of AHR associated with exposure to nicotine *in utero* or following allergen challenge. Of particular interest, a small clinical trial has shown that oral RGZ treatment improves lung function in smokers with asthma, a group that is generally unresponsive to conventional steroid treatment. These combined findings support further investigation of the potential for PPAR γ agonists to target the noncontractile and contractile functions of ASM to improve outcomes for patients with poorly controlled asthma.

1. Introduction

Asthma is a chronic inflammatory lung disease affecting over 300 million people worldwide, with 250,000 deaths per year attributed to the disease [1]. Asthma is characterized by inflammation, airway wall remodeling, and airway hyperresponsiveness (AHR), whereby airways are more sensitive to a variety of stimuli and subsequently contract too easily and too much [2].

A major feature of airway remodeling in asthma is an increase in airway smooth muscle (ASM) mass. This thickened ASM layer can act as both a source and target of inflammatory cytokines and extracellular matrix (ECM) proteins, contributing to persistent inflammation and increased airway narrowing. Proliferative, synthetic, and contractile functions of ASM can therefore play distinct roles in both the pathogenesis of asthma and perpetuation of disease symptoms (Figure 1) [3, 4].

In current asthma therapy, inhaled β_2 -adrenoceptor agonists are used to reverse ASM contraction while the frequency and severity of asthma attacks can be reduced by combined therapy with β_2 -adrenoceptor agonists and glucocorticoids (GCS). However, a significant proportion of patients have poorly controlled symptoms, with variable responses to β_2 -adrenoceptor agonists and persistent AHR despite optimal anti-inflammatory treatment. Cigarette smoking in asthma patients also contributes to increased severity of symptoms, with an impaired response to both inhaled and oral corticosteroids [5].

This resistance to therapy is likely to be associated with significant structural changes to the airways, including ASM accumulation, fibrosis, and increased vascularity. These changes have been mechanistically associated with disease severity and accelerated lung function decline [6] but may be difficult to reverse in established asthma. In this context

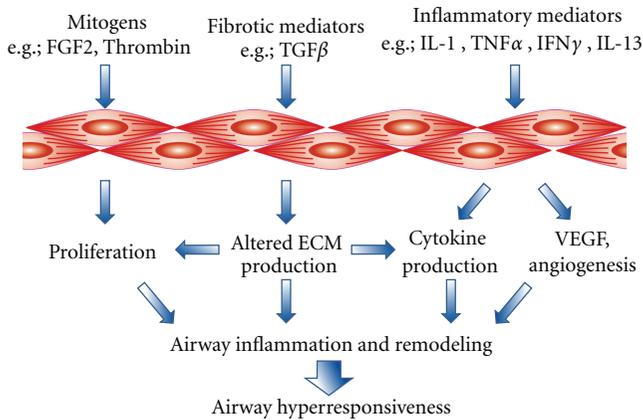


FIGURE 1: Potential targets for the regulation of noncontractile (proliferative and synthetic) and contractile functions of airway smooth muscle contributing to airway hyperresponsiveness.

therefore, it is crucial to identify alternative drugs that inhibit the development of AHR, as well as the contribution of ASM to inflammation and remodeling to limit contraction or directly enhance the relaxation of the increased ASM in the airways [7, 8].

A potential novel approach to regulate ASM function in asthma is to target peroxisome proliferator-activated receptor γ (PPAR γ). It has been suggested that downregulation of PPAR γ signaling may be a contributing factor to the development of AHR in asthma following *in utero* nicotine exposure [9], while the expression of PPAR γ in ASM is upregulated in the airways of asthmatic patients [10].

This paper provides a brief overview of PPAR γ pharmacology and describes the contribution of ASM to inflammation, remodeling, and hyperresponsiveness in asthma. Its major focus is to outline the increasing experimental and clinical evidence that PPAR γ ligands can regulate ASM function, through both PPAR γ -dependent and PPAR γ -independent mechanisms.

2. PPAR γ Structure and Ligands

PPAR γ is a member of the nuclear hormone receptor (NHR) family of ligand-activated transcription factors, which also includes glucocorticoid receptors (GRs) and thyroid hormone receptors. PPAR γ is one of three PPAR isoforms designated PPAR α (NR1C1), PPAR β (PPAR δ , NR1C2), and PPAR γ (NR1C3).

Like other NHR, PPAR γ possesses a multidomain structure. This includes a DNA binding domain (DBD) containing two zinc finger motifs that recognise specific PPAR response elements (PPREs) sequences. These PPREs consist of hexameric direct repeats of AGGTCA recognition sequences separated by one or two random nucleotide. The DBD is linked via a hinge region to the large ligand binding domain (LBD) that occupies the C-terminal half of the receptor and an activator function (AF)-1 domain is present at the N-terminus [11–13].

PPAR γ possesses an unusually large T-shaped ligand-binding pocket that enables interaction with a structurally diverse library of ligands [13]. The most widely studied synthetic agonists are the thiazolidinedione class of drugs, rosiglitazone (RGZ, BRL 49653), pioglitazone (PGZ), troglitazone (TGZ), and ciglitazone (CGZ). RGZ binds the receptor with high affinity (K_d 43 nM), whereas PGZ and CGZ are less potent [14]. Alternative nonglitazone agonists include GW262570 [15] and novel triterpenoid compounds derived from oleanic acid such as 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) [16]. Despite binding affinities in the nM range, most biological effects of these synthetic PPAR γ agonists have been observed at μ M concentrations.

Potential natural ligands for PPAR γ also show marked structural diversity and include prostaglandin D₂ (PGD₂) and its metabolites PGJ₂ and 15-deoxy- $\Delta^{12,14}$ -prostaglandin-J₂ (15dPGJ₂) [17]. 15dPGJ₂ in particular has been widely used for comparisons with glitazones in experimental settings [18]. However, these agonists, and other putative PPAR γ ligands such as the oxidised lipids 9- and 13-hydroxyoctadecadienoic acid (HODE) and 12- and 15-hydroxyeicosatetraenoic acid (HETE) [19], have multiple additional sites of action, suggesting that demonstrating their PPAR γ -dependent actions is likely to be particularly challenging.

3. Mechanisms of Action of PPAR γ and Its Ligands

3.1. PPAR γ Activation. Cytosolic PPAR γ exists as a monomer, with both the LBD and AF-1 domain regulating interactions with coactivators and corepressors that control activation of the receptor [11–13]. PPAR γ does not form homodimers, but can associate with multiple partners to form heterodimers. Its most preferential binding partner is the retinoid X receptor (RXR), with 9-*cis* retinoic acid acting as its natural ligand [20]. Translocation of the ligand-activated PPAR γ -RXR complex to the nucleus and binding to PPRE in the promoter region of target genes can result in either the upregulation or inhibition of gene expression. Multiple PPAR γ -responsive genes involved in diverse cellular processes including adipogenesis, insulin sensitivity, and inflammation have been identified [21, 22].

Alternatively, PPAR γ can cause the transrepression of transcriptional factors such as NF κ B, CAAT/enhancer binding protein (C/EBP), signal transducers and activators of transcription (STAT), or activator protein- (AP-) 1. This transrepression may occur either by direct binding to the transcription factors, by sequestration of shared coactivators of these factors or by Small Ubiquitin-like Modifier (SUMO)ylation of PPAR γ and subsequent PPAR γ binding to a corepressor complex [23]. These actions also have the potential to inhibit inflammatory responses in the lung.

Given the marked differences between the reported PPAR γ binding affinities of the glitazones and the concentrations required to elicit their cellular effects, multiple approaches are required to support claims for PPAR γ -dependency. These include confirming PPAR γ expression in cells of interest and the use of pharmacological antagonists,

with GW9662 being the most commonly used. GW9662 irreversibly inhibits PPAR γ by covalently binding to Cys²⁸⁵ in the ligand binding pocket and prevents heterodimerisation with RXR as well as interactions with coactivator and corepressor molecules [24]. GW9662 has been used to confirm the PPAR γ -dependence of known PPAR γ ligands both *in vitro* [18] and *in vivo* [25]. Additional molecular techniques such as the use of dominant-negative constructs or adenoviral PPAR γ (AdPPAR γ) have been employed to implicate PPAR γ in the regulation of cellular responses both *in vitro* and *in vivo* [26, 27].

3.2. PPAR γ -Independent Mechanisms. The mechanistic complexity underlying responses to PPAR γ ligands occurring via PPAR γ activation is further complicated by evidence of PPAR γ -independent pathways. This may involve PPAR γ ligands binding directly to alternative receptors, regulating transcription factor activity, or altering signalling through enzymes or ion channels to mediate their cellular responses.

Glitazones have been shown to activate free fatty acid receptors (FFA1, also known as GPR40) causing phosphorylation of the ERK1/2 mitogen-activated protein (MAP) kinases [28]. RGZ and CGZ can also bind directly to GR independently of PPAR γ , evidenced by their stimulation of GR nuclear translocation in a PPAR γ -deficient cell line [29], and defining a potential alternative anti-inflammatory mechanism for these ligands.

In addition, 15dPGJ₂ has been demonstrated to directly inhibit the activity of the enzyme I κ B kinase, thereby reducing the phosphorylation of I κ B and its subsequent dissociation from the proinflammatory transcription factor NF κ B [30, 31]. Actions of PPAR γ ligands may also be mediated by increasing PGE₂ levels, subsequent to inhibition of its metabolism via 15-hydroxyprostaglandin dehydrogenase [32].

Both 15dPGJ₂ and CDDO have been shown to induce heme-oxidase by PPAR γ -independent, glutathione-dependent mechanism, although this antioxidant action was restricted to PPAR γ ligands possessing an electrophilic centre [33]. Additional evidence of nongenomic, rapid regulation of enzyme activity by PPAR γ ligands includes activation of mitogen-associated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K), and adenosine monophosphate-activated protein kinase (AMPK) pathways [34–36] with implications for regulation of cell proliferation and inflammatory cytokine production.

Some of these nongenomic effects may be mediated through altered calcium signalling. TGZ and PGZ have been shown to mobilize calcium from intracellular stores [37, 38]. Although RGZ rapidly inhibited the activity of sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA)-2b [39], chronic treatment with PGZ has been shown to increase SERCA activity via a PPAR γ -dependent mechanism [40], suggesting that regulation of calcium homeostasis by PPAR γ ligands is likely to depend on temporal and cellular contexts.

These diverse PPAR γ -dependent and PPAR γ -independent actions define numerous mechanisms whereby PPAR γ ligands could regulate the altered proliferative, secretory and contractile functions of ASM contributing to asthma.

4. PPAR γ Is Increased in Airway Smooth Muscle in Asthma

Although PPAR γ was originally characterised as a regulator of adipocyte differentiation, this receptor is also widely expressed in the lung, in both inflammatory and structural cells implicated in asthma pathophysiology [41].

Regulation of PPAR γ expression can occur in response to *in vitro* exposure to inflammatory cytokines, with acute upregulation occurring in response to interleukin-4 (IL-4) in airway epithelial cells and macrophages [42, 43] during macrophage differentiation and activation [44, 45] or following antigen exposure in sensitised mast cells [46].

PPAR γ may provide a target to overcome chronic inflammation and increased airway reactivity *in vivo*. Higher levels of PPAR γ were evident in total lung extracts from mouse models of established allergen-induced inflammation [27, 47] and could be localised to ASM and epithelium, mast cells, and some inflammatory cells [25]. In contrast, perinatal exposure to nicotine appears to decrease PPAR γ expression and signaling, with increased alveolar interstitial fibroblast-to-myofibroblast differentiation contributing to the development of an asthma-like phenotype in newborn rats [48, 49].

In human airway biopsies, expression levels of PPAR γ in ASM, epithelium, and mucosal eosinophils and macrophages were elevated in asthmatic patients compared with controls [10]. In the same study, asthmatics treated with GCS had lower levels of PPAR γ expression compared with untreated asthmatics. Although ASM from asthmatics had lower PPAR γ levels compared to healthy controls *in vitro*, this was reversed in the presence of a mitogenic stimulus [50]. These results suggest that increased PPAR γ expression observed *in situ* may be a product of the inflammatory and mitogenic pathways and may also be sensitive to steroid therapy.

These combined findings suggest that PPAR γ expression is increased in response to acute or chronic inflammation in multiple cell types including ASM, and that PPAR γ could be targeted to limit inflammation, airway remodeling, and increased ASM contraction in asthma.

5. *In Vitro* Regulation of ASM Function by PPAR γ Ligands

Because of the capacity of ASM to perpetuate airway inflammation, orchestrate airway wall remodelling, and modulate airway tone, it has been suggested that targeting ASM is critical for effective asthma treatment [4, 7, 8]. Accumulating *in vitro* evidence now supports the efficacy of PPAR γ ligands in the regulation of ASM cytokine production, proliferation, and contraction, while their direct effects on the potential contribution of ASM to fibrosis and angiogenesis have yet to be confirmed.

5.1. Regulation of ASM Inflammatory Cytokine Production. In response to inflammatory stimuli, ASM can secrete various cytokines and chemokines contributing to the pathophysiology of asthma [51–53]. These mediators include factors such as granulocyte/macrophage-colony-stimulating factor (GM-CSF) [54], granulocyte-colony stimulating factor (G-CSF)

[55], eotaxin [56], regulated on activation, normal T cells expressed and secreted (RANTES) [57], IL-6 [58], monocyte chemoattractant protein-1 (MCP-1) [59], and vascular endothelial growth factor (VEGF) [60]. Thus, inhibition of ASM cytokine production has the potential to regulate inflammatory cell growth, survival, and recruitment as well as the autocrine influence of these cytokines to induce ASM proliferation and hyperresponsiveness in the inflamed airways in asthma [4, 61].

The anti-inflammatory efficacy of PPAR γ ligands has been established in human-cultured ASM cells, with various glitazones inhibiting the release of multiple cytokines irrespective of stimulus used. IL-1 β -induced increases in GM-CSF and G-CSF release were both attenuated by CGZ and to a lesser extent by 15dPGJ₂ [55]. Although dexamethasone also completely abolished the increase in GM-CSF release, G-CSF induction was only partially inhibited, suggesting that PPAR γ ligands may target steroid-resistant pathways in ASM [55]. Induction of eotaxin and MCP-1 by tumour necrosis factor α (TNF α) was also inhibited by PPAR γ agonists, and the expression of these chemokines was further decreased when 15dPGJ₂ and TGZ were used in the presence of GCS and/or a long-acting β -adrenoceptor agonists [62], supporting potential benefit when these agents are used in combination.

In a more recent study, TGZ inhibited IL-1 β -induced release of IL-6 and VEGF, TNF α -induced release of eotaxin and RANTES, and IL-4-induced release of eotaxin, while RGZ also inhibited TNF α -stimulated release of RANTES. These anti-inflammatory effects were not prevented by the PPAR γ antagonist GW 9662 or by PPAR γ knockdown using short hairpin RNA [63].

Additional PPAR γ -independent mechanisms have been considered. Although PPAR γ ligands each caused the activation of AMPK, their effects on cytokine release were not prevented by AMPK inhibitors [63]. Since CGZ increased the IL-1 β -induced expression of COX-2 [22], this potentially proinflammatory effect could also contribute to increased PGE₂ levels to provide negative feedback to inhibit cytokine release [64]. However, induction of PGE₂ synthesis was not a requirement for the anti-inflammatory effect of PPAR γ ligands, since CGZ reduced GM-CSF and G-CSF in the presence of indomethacin [55]. Inhibition of NF κ B has also been excluded, since CGZ did not regulate NF κ B (p65) nuclear translocation in the absence or presence of IL-1 β [22].

The qualitative importance of ASM-derived cytokines remains to be clearly established *in vivo* and in asthmatic subjects. Nevertheless, evidence of the diverse anti-inflammatory actions of PPAR γ ligands in ASM, consistent with their reported actions in other cell types [41], supports their therapeutic potential for the treatment of asthma.

5.2. Regulation of ASM Proliferation. A key feature in airway remodeling in asthma is the increased ASM layer associated with increases in both size (hypertrophy) and number (hyperplasia, migration) of myocytes [65] with ASM cell migration also playing a potential role. To assess the potential efficacy of antiremodeling agents, ASM proliferation can be

induced *in vitro* in response to the cocktail of mitogens present in serum, and to specific stimuli such as thrombin or fibroblast growth factor 2 (FGF2), known to be increased in the asthmatic airway [66, 67].

PPAR γ ligands have now been shown to inhibit proliferation of human ASM in culture. The increase in [³H]-thymidine incorporation in response to serum was completely abolished by both CGZ and 15dPGJ₂ [55], while RGZ and 15dPGJ₂ significantly attenuated both FGF2 and thrombin-stimulated increase in ASM cell numbers [18], demonstrating that the antiproliferative effects are mitogen-independent. Unlike GCS, inhibition of proliferation was not associated with reduced cyclin D1 levels [18, 68]. Responses were mediated by both PPAR γ -dependent and PPAR γ -independent mechanisms, as the PPAR γ antagonist GW9662 inhibited the antiproliferative effects of RGZ but not 15dPGJ₂ [18], with cell cycle analysis suggesting that neither mediated ASM apoptosis [18]. Although CGZ and 15dPGJ₂ had previously been reported to cause nuclear condensation, a characteristic morphological change associated with apoptosis [55], this single finding was not consistent with the known resistance of ASM to apoptosis [69].

Cultured ASM derived from asthmatic patients has been shown to proliferate faster than cells from nonasthmatic patients [70]. Since GCS can only inhibit the *in vitro* proliferation of ASM from subjects without asthma [68, 71], alternative therapeutic approaches are required to target this steroid-resistant mitogenic response. In a recent study, the effects of CGZ were assessed in cells from nonasthmatic and asthmatic patients cellular proliferation in response to serum by measuring bromodeoxyuridine uptake [50]. Further studies are required to explain why CGZ failed to inhibit serum-induced proliferation in either group [50], since this finding contradicts the previously reported antiproliferative effects of both CGZ [55] and RGZ [18]. CGZ did upregulate PPAR γ expression in ASM cells derived from both asthmatic and nonasthmatic subjects, and in ASM from asthmatics in the presence of serum [50], however the functional significance of these changes and their potential impact on ASM in remodeled airways remain to be determined.

5.3. Regulation of Extracellular Matrix Production and Turnover. Airway remodeling in asthma is also characterized by alterations in the amount and composition of ECM proteins, including increases in collagen I and fibronectin deposition [72]. Subepithelial fibrosis is associated with increased transforming growth factor β (TGF β), with this profibrotic cytokine present at relatively higher levels in BAL fluid from asthmatic subjects compared to healthy subjects [73]. Although fibroblasts are considered the major resident cells contributing to the increased collagen deposition in the asthmatic airway, ASM is also known to produce ECM proteins and to regulate their turnover by secreting matrix modifying enzymes.

In this context, it is important to consider that the ECM exists not only as a structural scaffold in the airways but as a partner in bidirectional interactions with ASM, influencing proliferation and cytokine release as well as contractility [74]. Since *in vitro* secretion of collagen and fibronectin from ASM

derived from asthmatic patients is increased by GCS, and TGF β -induced ECM protein synthesis is unaffected by GCS, this aspect of remodeling appears to be resistant to steroids [75] and alternative strategies to minimize the impact of the altered ECM on ASM function need to be identified.

Confirmation of the ability of PPAR γ ligands to inhibit TGF β -induced collagen synthesis from ASM would suggest that these agents have the capacity oppose proasthmatic changes associated with increased ASM-ECM interactions. To date, the effects of PPAR γ ligands have only been assessed in human lung fibroblasts which express PPAR γ , and respond to TGF β treatment by differentiating into myofibroblasts expressing α smooth muscle actin (α SMA), and increasing their synthesis of fibrillar collagen I [26]. Both differentiation and collagen I secretion were abrogated by treatment with RGZ, CGZ, or 15dPGJ₂. These antifibrotic effects of the PPAR γ ligands were shown to be at least partially mediated by PPAR γ receptor activation as inhibition was attenuated by transfection of TGF β -treated fibroblasts with a dominant negative PPAR γ receptor [26]. Similar antifibrotic properties have also been described for RGZ and CGZ in the regulation of epithelial-mesenchymal transition in alveolar epithelial cells [76].

An alternative way to regulate ASM-ECM interactions would be by regulating the activity of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Activation of PPAR γ by RGZ or PGZ in human bronchial epithelial cells reduced TNF α -induced MMP-9 gelatinolytic activity via inhibition of NF- κ B, but did not alter the expression of its endogenous inhibitor TIMP-1 [77]. These results suggest that limiting the expression of MMP-9 by PPAR γ activation might have therapeutic potential in the treatment of chronic inflammatory diseases of the respiratory system. However, the effects of PPAR γ ligands on ASM-derived MMPs and TIMPs in the asthma context have yet to be directly assessed.

5.4. Regulation of Angiogenesis. Significant increases in the number and size of blood vessels supplying the remodeled airway wall are seen in asthma [6, 78]. This expanded vascular compartment is likely to contribute to asthma symptoms through tissue swelling and amplification of inflammatory cell trafficking [79]. ASM has the potential to promote angiogenesis as cultured ASM has been shown to constitutively release factors such as VEGF, which can be further increased in response to inflammatory mediators such as IL-1 β , TNF α , and TGF β [80]. Of note, these proangiogenic responses have recently been shown to be further elevated in ASM from asthmatics [81].

Studies examining the effects of PPAR γ ligands on this aspect of remodelling are lacking; however, conflicting reports show that the generation of VEGF from vascular smooth muscle cells is increased by CGZ and PGJ₂ [82, 83], while TGZ has been shown to inhibit VEGF-induced angiogenic signaling in endothelial cells [84]. Further investigations are required to explore the potential of PPAR γ ligands to regulate the contribution of ASM to angiogenesis.

5.5. Regulation of ASM Contraction. The increased contractile response of asthmatic airways which defines AHR is likely

to be due to multiple factors (recently reviewed in [4]), including the presence of higher levels of contractile mediators and reduced levels of relaxant mediators. Critically, the increased ASM bulk displays alterations in contractile protein expression that favour contraction [85, 86]. In this context, it is of interest that RGZ and other PPAR γ ligands can inhibit the increase in α -smooth muscle actin and calponin associated with both epithelial-mesenchymal transition of alveolar epithelial cells [76] and alveolar interstitial fibroblast-to-myofibroblast differentiation [87].

Increased excitation/contraction coupling may also occur through disruption of calcium homeostasis [88]. Indeed, increased contraction of ASM cells from asthmatic patients has been associated with downregulation in their expression and function of SERCA2 [89]. PPAR γ ligands have recently been reported to increase SERCA expression and activity in pancreatic islet cells and platelets [40, 90], with PGZ inhibiting cytokine-induced increases in intracellular calcium by facilitating its reuptake into the SR [40]. In ASM, calcium plays a key role not only in enhancing ASM contractile function, but also in promoting cell proliferation, migration and the secretion of proinflammatory cytokines and chemokines [88]. It will therefore be of particular interest to determine if acute or chronic treatment with PPAR γ ligands can also restore SERCA levels and activity in ASM to inhibit the diverse proasthmatic functions that could be driven by elevated intracellular calcium.

There is now evidence that acute treatment with PPAR γ ligands may exert direct effects on ASM contractility. In a single study, RGZ has been reported to cause relaxation of mouse tracheal preparations precontracted with carbachol [91]. Since this response was evident within minutes and required μ M concentrations, it was likely to be occurring independently of PPAR γ activation. Relaxation to RGZ in the static organ bath setting was indomethacin-sensitive and was attributed to accumulation of the dilator prostanoid PGE₂ through inhibition of its breakdown rather than an increase in PGE₂ synthesis. This interpretation is consistent with the previously reported finding that RGZ can inhibit its metabolism by 15-hydroxyprostaglandin dehydrogenase [32].

Further studies are required to explore acute dilator responses to RGZ and other PPAR γ ligands, to compare their efficacy with β_2 -adrenoceptor agonists in current clinical use for the relief of asthma symptoms and to test their actions in the disease context when ASM responsiveness is altered.

6. *In Vivo* Regulation of ASM Function by PPAR γ Ligands

6.1. PPAR γ Ligands Have Efficacy in Rodent Models of Allergic and Nicotine-Induced Airways Disease. The reported effects of PPAR γ ligands on ASM functions *in vitro*, namely inhibition of proliferation and production of cytokines from human ASM cells as well as regulation of contractile protein expression and direct relaxation intracheal preparations, has provided an impetus for considering their effects in animal models of airways disease, using perinatal exposure to maternal nicotine or chronic ovalbumin (OVA) challenge to trigger asthma-like changes in the airways.

TABLE 1: Effects of PPAR γ ligands in mouse OVA models of allergic airways disease.

Strain	Ligand	Inflammation	Remodeling	AHR	Mechanism	References
Balb/C	CGZ	↓ IL-4, IL-5, IL-6, IL-13 ↓ eosinophils	↓ mucus ↓ collagen ↓ wall thickness	↓	↓ GATA-3	[25, 94]
Balb/C	CGZ	↓ IFN γ , IL-2, IL-4 ↓ eosinophils	↓ mucus	N.D.		[100]
Balb/C	CGZ	↓ eosinophils	↓ ASM thickness	↓		[95]
Balb/C	CGZ RGZ	↓ eosinophils		N.D.	↑ IL-10	[99]
Balb/C	RGZ PGZ	↓ IL-4, IL-5, IL-13, ECP ↓ eosinophils		↓	↑ PTEN ↑ IL-10	[27, 47]
Balb/C	GI 262570	↓ eosinophils		N.D.		[92]
C57Bl/6	RGZ PGZ	↓ IL-4, IL-5, IL-13, ↓ VEGF, eotaxin, RANTES ↓ eosinophils		↓	↑ IL-17 via NF κ B	[93, 97]
C57Bl/6	RGZ	↔ eosinophils	↔ mucus ↔ wall thickness	↓		[98]

ASM: airway smooth muscle; CGZ: ciglitazone; ECP: eosinophil cationic protein; IL: interleukin; N.D.: not determined; PGZ: pioglitazone; PTEN: phosphatase and tensin homologue deleted on chromosome ten; RANTES: regulated upon activation, normal T-cell expressed, and secreted; RGZ: rosiglitazone; VEGF: vascular endothelial growth factor.

It is well known that cigarette smoking during pregnancy increases the incidence and severity of childhood asthma, and has been associated with increased generation of contractile myofibroblasts in the developing lung (reviewed in [9]). A recent study of newborn rats following *in utero* nicotine exposure has revealed that increases in airway resistance under basal conditions and reactivity to acetylcholine both *in vivo* and *in vitro* are sensitive to RGZ treatment [48].

Chronic allergen challenge models mimic key features of asthma including inflammatory cell infiltration predominantly not only by eosinophils, but also by neutrophils and lymphocytes [92]. Increased inflammatory Th₂ cytokines and chemotactic factors can also be detected in bronchoalveolar lavage (BAL) fluid [27, 93, 94]. Airway wall remodeling, with variable changes in goblet cell hyperplasia, thickening of the ASM layer and increased collagen deposition in the lamina propria is also evident [25, 95]. A common feature is the development of AHR to methacholine (MCh), demonstrated both with noninvasive, but now largely discredited [96] measurement of the heuristic variable enhanced pause (Penh) in conscious mice [25, 27, 94, 95] and by measurements of increased airways resistance using invasive plethysmography in anesthetized mice [47, 97, 98].

The efficacy of PPAR γ ligands in these OVA challenge models in mice has been confirmed in multiple studies, assessing the effect of chronic treatment with glitazones or other PPAR γ selective agonists such as GI 262570, administered either by inhaled, oral or intraperitoneal (i.p.) routes [25, 27, 47, 92–95, 97–100] or the response to transient overexpression of PPAR γ via adenoviral delivery (Table 1) [27, 47, 93]. However, since the overall changes in phenotype in these models can vary with the type of OVA used [101], the duration of the challenge protocol and the species of mice in which the model is applied [102], the reported effects of pharmacological intervention with different PPAR γ ligands administered by various routes must be considered

and interpreted in context. Nevertheless, these models provide compelling evidence that PPAR γ ligands can regulate inflammatory cell infiltration, BAL cytokines, airway remodeling particularly ASM thickening and fibrosis, and altered reactivity to MCh.

6.2. Regulation of Airway Inflammation. Assessment of airway inflammation has consistently shown that RGZ or CGZ treatment attenuated the increase in total and eosinophil cell numbers in BAL fluid in OVA-treated C57Bl/6 or Balb/C mice in a PPAR γ -dependent manner (Table 1) [27, 93, 94]. Similar results were observed with GI 262570 administration to Balb/C mice, where eosinophil and lymphocytes, but not neutrophils, were reduced [92]. Although RGZ reduced eosinophilic airway inflammation when administered to Balb/C mice by oral gavage [99], it was ineffective in C57Bl/6 mice administered i.p. [98]. The reason for this discrepancy is therefore more likely to be due to differences in challenge protocols and the administration methods (route, dose, and duration) of different compounds, rather than the mouse strain used.

Regulation of cytokine production in the lung has also been assessed. OVA-induced increases in IL-4, IL-5, IL-13, eosinophil cationic protein (ECP), and eotaxin in lung tissue and BAL fluid were inhibited by administration of RGZ, PGZ, or by PPAR γ overexpression [27, 93]. Similar changes were seen with nebulized CGZ, although eotaxin levels were not affected [94], while oral CGZ has been shown to reduce IL-2, IL-4, and interferon γ (IFN γ) [100]. Since cytokine release from ASM is also inhibited by PPAR γ ligands *in vitro*, it is likely that the glitazones can reduce the contribution of ASM-derived cytokines to the levels measured in this *in vivo* setting.

Several potential mechanisms have been proposed to explain the anti-inflammatory effects of PPAR γ ligands in these models. Regulation of NF κ B has been considered since

PPAR γ activation inhibits the function of the proinflammatory transcription factor *in vitro* [103, 104]. Treatment of OVA-sensitized mice with RGZ, PGZ, or AdPPAR γ also reduced the nuclear translocation of NF κ B in response to OVA, evidenced by inhibition of increases in NF κ B p65 protein in lung extracts [93], suggesting a direct action of PPAR γ ligands on NF κ B. Inhibition of NF κ B activity by PPAR γ agonists has also been associated with decreased IL-17 protein and mRNA expression. Since the effects of RGZ or PGZ could be abrogated by coadministration of rIL-17, this implicates a novel mechanism whereby PPAR γ agonists regulate NF κ B activity by reducing IL-17 to limit inflammation [97].

NF κ B-independent mechanisms are also likely to contribute to the anti-inflammatory effects of PPAR γ ligands [92]. Alternative mechanisms include PPAR γ -mediated inhibition of the increase in GATA-3 expression in response to OVA [94], reducing the local Th2 response elicited by this eosinophil-derived transcription factor. In addition, an increase in IL-10 in response to OVA, thought to occur as part of a negative feedback response to inhibit inflammation, could be further increased by RGZ, PGZ, or ad PPAR γ [47]. Increased IL-10 levels could explain the reported reductions in IL-4 and IL-5 as well as the inhibition of eosinophilia, since IL-10 has been shown to downregulate IL-4 and IL-5 expression by Th2 cells and reduce eosinophil survival.

In a separate study, PPAR γ expression was increased in response to OVA challenge and further enhanced by the administration of the either PPAR γ agonists or AdPPAR γ [27]. This was associated with an upregulation of phosphatase and tensin homologue deleted on chromosome ten (PTEN) PTEN expression, correlating with decreased PI3K activity as measured by a reduction in the phosphorylation of Akt. These findings demonstrate a protective role of PPAR γ in the pathogenesis of the asthma phenotype through regulation of PTEN expression [27, 93].

6.3. Regulation of Airway Remodeling. In addition to their anti-inflammatory actions in these mouse models, PPAR γ ligands have also been shown to inhibit key aspects of airway remodeling, notably fibrosis, mucus production, and thickening of the ASM layer (Table 1).

Inhaled CGZ has been shown to reduce OVA-induced increases in both collagen deposition and basement membrane thickening [25]. This was associated with reduced levels of the profibrotic cytokine TGF β [25]. Although inhaled CGZ has also been shown to decrease mucus production, based on the intensity and area of epithelial staining [25], there were no detectable effects of i.p. RGZ on goblet cell number or other aspects of airway remodeling [98], suggesting that high local concentrations may be required.

Consistent with the reported antiproliferative effects of PPAR γ ligands on human ASM *in vitro* [18, 50, 55], intranasal administration of CGZ has been shown to reduce not only eosinophilic inflammation, but also to inhibit the thickening of the ASM layer following allergen challenge [95]. This effect appeared to be independent of regulation of TGF β or VEGF levels, as the increased BAL levels of these potential mitogens were not reduced with CGZ treatment

[95]. It would be of interest to measure endogenous factors that could contribute to ASM proliferation in this setting.

6.4. Regulation of Airway Hyperresponsiveness. Studies demonstrating the inhibitory effects of PPAR γ ligands on AHR are consistent with the numerous *in vitro* findings suggesting a role for PPAR γ ligands in the regulation of ASM function in asthma (Table 1). The development of AHR to cholinergic agonists subsequent to *in utero* nicotine exposure or *in vivo* allergen exposure can be alleviated by chronic treatment with PPAR γ ligands, measured either indirectly using Penh [25, 27, 94, 95] or by assessing changes in airway resistance [47, 48, 97, 98].

A recent study has reported that coadministration of RGZ prevented the changes in lung function in rat offspring induced by perinatal nicotine exposure. Inhibition of the development of AHR as measured *in vivo* and in isolated tracheal preparations was attributed to the ability of RGZ to decrease the lipofibroblast-to-fibroblast transdifferentiation induced by nicotine, minimizing the increased expression and function of mesenchymal markers of contractility [48].

Chronic allergen studies demonstrating PPAR γ ligand efficacy suggest a PPAR γ -dependent mechanism in opposing ovalbumin-induced AHR, since the inhibitory effects could be mimicked by transient overexpression of PPAR γ via adenoviral delivery or prevented by co-treatment with GW9662 [25, 27, 47, 93, 94, 97, 98]. It would be reasonable to attribute this reduction in AHR to the inhibition of inflammation and airway remodeling mediated by the PPAR γ ligands used. However, RGZ also reduced AHR measured by invasive plethysmography in OVA-challenged C57Bl/6 mice without detectable effects on markers of inflammation or remodeling [98]. This result suggests that it is possible that PPAR γ ligands may also exert a direct effect on ASM contractile function *in vivo*.

In this context, it is notable that chronic treatment with PPAR γ ligands may not only inhibit the development of AHR, but also protect airway dilator responses. In a guinea pig model of *in vivo* homologous desensitization to salbutamol, chronic treatment with RGZ mitigated AHR to carbachol, preserved salbutamol relaxant activity, and partially restored β_2 -adrenoceptor binding sites in tracheal tissues *ex vivo* [105]. The potential for PPAR γ ligands to maintain dilator sensitivity and reverse β_2 -adrenoceptor desensitization is of particular interest since GCS can prevent cytokine-induced desensitization [106], but cannot restore sensitivity once tolerance to β_2 -adrenoceptor agonists has developed [107].

7. Potential Clinical Benefit of PPAR γ Ligands in Asthma

Although several members of the glitazone class of drugs have been used for type 2 diabetes, PGZ is the only PPAR γ agonist in current clinical use for this condition, with its potential as a treatment to reduce inflammation in rheumatoid arthritis also being assessed [108]. TGZ was the first glitazone to be marketed for diabetes, but was withdrawn because of serious hepatotoxicity in some patients [109],

while RGZ has also recently been withdrawn because of potential cardiovascular risks [110].

There is currently only limited data on the efficacy of glitazones in the treatment of respiratory diseases. Further studies characterizing the effects of PPAR γ ligands on lung development as well as nicotine-induced changes in lung function are required to determine whether these agents may provide a new therapeutic approach to minimize, or even reverse, the adverse impacts of maternal smoking that contribute to the development of paediatric asthma.

An isolated report described the effects of PGZ in two case subjects with both diabetes and established asthma [111]. One patient reported reduced wheezing when taking PGZ in addition to his asthma preventer medication, with deterioration of symptoms when PGZ was discontinued. In another, concurrent treatment with the sulfonylurea glibenclamide and PGZ effectively reduced the patient's blood glucose levels and improved pulmonary function test results, increasing both forced vital capacity and force expiratory volume in one second (FEV1).

More recently, a small single-centre trial has been conducted, assessing RGZ in a double-blind, randomised, placebo-controlled, two-period crossover study in the inhaled allergen challenge model of asthma [112]. 32 steroid naive subjects completed the study, receiving RGZ (4 mg) and placebo twice daily for 28 days in random order. The late asthmatic reaction (LAR) change from postsaline FEV1 from 4–10 hrs after allergen on day 28 was attenuated by 15% compared to the response during placebo-treatment, suggesting an inhibitory effect of RGZ on activated eosinophil recruitment. This reduction was accompanied by trends in several other markers of efficacy and anti-inflammatory activity (e.g., IL-4, IL-6, IL-13). In light of these modest changes, the authors suggested that PPAR γ agonist monotherapy is unlikely to represent a clinically useful intervention, at least in the context of relatively mild asthma.

More positive results were obtained in another recently completed exploratory clinical trial, which compared the effects of oral RGZ (8 mg) with inhaled beclometasone in a group of forty-six smokers with asthma, a group that is generally unresponsive to conventional GCS treatment [113]. In measurements taken after two and four weeks, RGZ did not significantly reduce asthma symptoms as determined by the Asthma Control Questionnaire (ACQ) scores and only produced a borderline reduction in sputum IL-8 levels compared to beclometasone-treated patients [113]. However, the patients receiving RGZ experienced significant improvements in FEV1 and forced expiratory flow over beclometasone-treated patients, which may reflect an effect of RGZ to reduce small airway obstruction.

These promising findings support the assessment of the effectiveness of long-term treatment of RGZ in a larger treatment group. The use of substantially higher oral doses may not be associated with a positive benefit/risk profile in asthma since PPAR γ agonists are associated with dose-related adverse effects such as weight gain (probably secondary to fluid retention). This suggests that a preferable alternative strategy would be to assess responses to both acute and chronic inhalation of PPAR γ agonists. This route

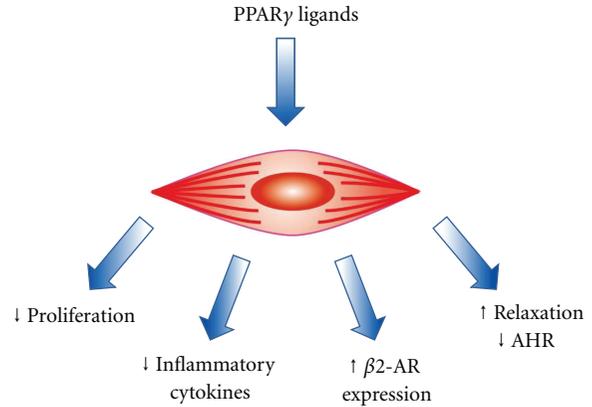


FIGURE 2: PPAR γ ligands regulate noncontractile and contractile functions of airway smooth muscle.

of administration would potentially minimize the reported adverse cardiovascular effects that have limited the systemic use of RGZ in diabetes [110]. In addition, it would achieve the higher local airway concentrations that may be required to exert direct effects on ASM contractile function to elicit acute bronchodilation as reported in mouse trachea, and chronic effects to regulate airway inflammation, remodeling, and the development of AHR.

8. Summary

An accumulating body of evidence supports the use of PPAR γ ligands for the targeting of PPAR γ receptors and other PPAR γ -independent mechanisms in ASM for the treatment of inflammatory lung diseases (Figure 2) [9, 41, 114]. *In vitro* treatment inhibits proliferation of human ASM via PPAR γ [18, 55] and also inhibits cytokine release from these cells [55, 62, 63]. Chronic *in vivo* treatment inhibits the development of nicotine-induced AHR in rat airways [48] as well as OVA-induced increases in ASM mass in mouse airways [95], part of a suite of actions involving inhibition of airway inflammation, remodeling and the development of AHR. PPAR γ ligands may also protect dilator responses since they can preserve β ₂-adrenoceptor expression and function in a guinea pig model of homologous desensitization to albuterol [105]. The potential for direct bronchodilator actions is supported by the demonstration of acute PPAR γ -independent relaxation in mouse trachea [91]. Although clinical trial results are limited, evidence of improved lung function in a difficult-to-treat cohort of smokers with asthma [113] supports further investigation of the potential for PPAR γ agonists to target ASM proliferative, inflammatory and contractile functions and their contributions to impaired dilator responses and the consequences of AHR in asthma.

Abbreviations

15dPGJ₂: 15-Deoxy- $\Delta^{12,14}$ -prostaglandin-J₂
 ACQ: Asthma control questionnaire
 AdPPAR γ : Adenoviral PPAR γ

AF-1:	Activator function-1
AHR:	Airway hyperresponsiveness
AMPK:	Adenosine monophosphate-activated protein kinase
AP-1:	Activator protein-1
AR:	Adrenoceptor
ASM:	Airway smoothmuscle
α SMA:	α smooth muscle actin
BAL:	Bronchoalveolar lavage
C/EBP:	CAAT/enhancerbinding protein
CDDO:	2-Cyano-3,12-dioxolean-1,9-dien-28-oic acid
CGZ:	Ciglitazone
DBD:	DNA binding domain
ECM:	Extracellular matrix
ECP:	Eosinophil cationic protein
FFA:	Free fatty acid
FGF2:	Fibroblast growth factor
GCS:	Glucocorticoid
G-CSF:	Granulocyte-colony stimulating factor
GM-CSF:	Granulocyte/macrophage-colony-stimulating factor
GR:	Glucocorticoid receptor
HETE:	Hydroxyeicosatetraenoic acid
HODE:	Hydroxyoctadecadienoic acid
IFN γ :	Interferon γ
IB κ :	NF κ B inhibitory cofactor
IKK:	I κ B kinase
IL-:	Interleukin
i.p.:	Intraperitoneal
LBD:	Ligand binding domain
MAPK:	Mitogen-associated protein kinase
MCh:	Methacholine
MMP:	Matrix metalloproteinases
NF κ B:	Nuclear factor κ B
NHR:	Nuclear hormone receptor
OVA:	Ovalbumin
Penh:	Enhanced pause
PG:	Prostaglandin
PGZ:	Pioglitazone
PI3K:	Phosphoinositide-3-kinase
PPAR γ :	Peroxisome proliferator activated receptor
PPRE:	PPAR response element
PTEN:	Phosphatase and tensin homologue deleted on chromosome ten
RANTES:	Regulated on activation, normal T cells expressed and secreted
RGZ:	Rosiglitazone
RXR:	Retinoid X receptor
SERCA:	Sarco/endoplasmic reticulum Ca ²⁺ ATPase
STAT:	Signal transducers and activators of transcription
SUMO:	Small Ubiquitin-like Modifier
TGF β :	Transforming growth factor β
TGZ:	Troglitazone
TNF α :	Tumour necrosis factor α
VEGF:	Vascular endothelial growth factor.

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Research Article

The Nitrated Fatty Acid 10-Nitro-oleate Diminishes Severity of LPS-Induced Acute Lung Injury in Mice

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Acute lung injury (ALI) is an inflammatory condition culminating in respiratory failure. There is currently no effective pharmacological treatment. Nitrated fatty acids (NFAs) have been shown to exert anti-inflammatory effects. We therefore hypothesized that delivery of NFAs directly to the site of inflammation would reduce the severity of ALI. Pulmonary delivery of 10-nitro-oleate following endotoxin-induced ALI in mice reduced markers of lung inflammation and injury, including capillary leakage, lung edema, infiltration of neutrophils into the lung, and oxidant stress, as well as plasma levels of proinflammatory cytokines. Nitro-oleate delivery likewise downregulated expression of proinflammatory genes by alveolar macrophages, key cells in regulation of lung inflammation. These effects may be accounted for by the observed increases in the activity of PPAR- γ and the PPAR- γ -induced antioxidant transcription factor Nrf2, together with the decreased activity of NF- κ B. Our results demonstrate that pulmonary delivery of NFAs reduces severity of acute lung injury and suggest potential utility of these molecules in other inflammatory lung diseases.

1. Introduction

A variety of pulmonary and extrapulmonary insults can result in acute lung injury (ALI), which is characterized by capillary leakage and resulting pulmonary edema and hypoxemia [1]. These multiple origins of ALI are reflected in different animal models of the disease, of which pulmonary administration of bacterial endotoxin (lipopolysaccharide; LPS) is among the most common. Regardless of the precipitating cause, the earliest phases of ALI feature severe neutrophil-rich alveolar inflammation [2] and associated oxidant stress [3] that represent the proximate causes of much or all of the subsequent pulmonary injury. ALI morbidity and mortality remain high and there is no effective pharmacotherapy [4], underlining the urgent need for improved treatment modalities.

Peroxisome proliferator-activated receptor γ (PPAR- γ) plays a central role in many of the feedback loops that normally limit inflammation and lead to its resolution [5–9] and is therefore a promising target for ALI pharmacotherapy.

Among the many anti-inflammatory effects attributable to PPAR- γ activation are diminished increases in reactive oxygen species, cytokines, chemokines, and adhesion molecules [10]. A major mechanism underlying these actions is decreased activity of proinflammatory transcription factors such as NF- κ B, AP-1, and STAT [5, 6]. Synthetic PPAR- γ agonists are widely used for treatment of diabetes but have been associated with adverse effects. A wide variety of endogenous molecules are known to activate PPAR- γ , but most either exhibit low potency or are present at low concentrations, leading to uncertainty regarding their physiological role.

Nitrated fatty acids (NFAs) are activating ligands for all three PPARs, exhibiting their greatest potency as PPAR- γ agonists [11, 12]. They have also been shown to exhibit a number of PPAR- γ -dependent effects, including induction of adipogenesis [11] and of CD36 receptor expression by macrophages [12]. NFAs are produced endogenously by nonenzymatic reaction of NO or its inorganic reaction products with naturally present unsaturated fatty acids

[13], with positional isomers of nitro-oleic acid (OA-NO₂) and nitrolinoleic acid (LNO₂) found at the highest concentrations in human plasma [11, 14]. The suggested PPAR- γ -mediated anti-inflammatory effects of NFAs have been supported by *in vitro* studies [15, 16], but *in vivo* and potentially translational studies of such effects have been limited. These considerations led us to investigate the ability of treatment with OA-NO₂, the most potent PPAR- γ -activating NFA [11], to reduce the severity of inflammation and lung injury in a murine model of ALI induced by LPS. In order to maximize pulmonary availability, OA-NO₂ was delivered directly to the lung via the intratracheal route.

2. Materials and Methods

2.1. Animals. Male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were used at 6–8 weeks of age (20–25 g). All studies were performed according to protocols reviewed and approved by the Atlanta Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

2.2. OA-NO₂ and LPS Administration. Mice were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine, administered via intraperitoneal injection, and a tracheotomy was performed. ALI was then induced by intratracheal (i.t.) injection of 50 μ g of endotoxin (LPS) prepared from *Escherichia coli* O111:B6 (Sigma-Aldrich, St. Louis, MO). Thirty min later mice were injected i.t. with 50 μ g OA-NO₂ (Cayman Chemical, Ann Arbor, MI) in 50 μ L of 10% DMSO or with vehicle. After a further 5.5 h the mice were euthanized, plasma was obtained, and lung and BAL fluid samples were collected. Neutrophil infiltration into the lung peaks approximately 6 h after i.t. LPS administration [17].

2.3. Lung Wet: Dry Weight Ratio. As an index of lung edema, the amount of extravascular lung water was calculated. The lower lobe of the right lung was ligated and excised and the wet weight was recorded. The lung was then placed in an incubator at 60°C for 24 h to obtain the dry weight. The wet: dry ratio was calculated by dividing the wet weight by the dry weight.

2.4. Bronchoalveolar Lavage (BAL) Fluid Collection and Cell Count. Following removal of the lung's lower right lobe, BAL fluid was collected by flushing 3 \times 1 mL of phosphate-buffered saline (PBS) containing 0.1 mM EDTA into the lung via a tracheal cannula. The pooled BAL fluid was centrifuged at 500 \times g at 4°C for 5 min. Pelleted cells were then resuspended in 1 mL of PBS. Total cell number was counted by hemocytometer and a differential cell count was performed by cytopspin staining with Diff-Quik (Siemens, Newark, DE).

2.5. BAL Fluid Protein. Increase in BAL fluid protein concentration was taken as a measure of increased permeability of alveolar-capillary barriers. Total protein concentration in the

supernatant following BAL fluid centrifugation was determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

2.6. Lung Histopathology. The lungs were inflated and fixed with 10% neutral formalin overnight at room temperature. Lung tissue was dehydrated with increasing ethanol (EtOH) concentrations and then embedded in paraffin. Five-micrometer-thick paraffin sections were stained with hematoxylin and eosin (H&E).

2.7. Assessment of Capillary Leakage. To further assess lung permeability, 50 mg/kg of Evans Blue Dye (EBD; Sigma-Aldrich, St. Louis, MO) dissolved in 200 μ L of PBS was injected into the tail veins of mice following ALI induction. After 30 min, the animals were euthanized and the lungs perfused with 5 mL PBS, after which the lungs were excised *en bloc* and snap-frozen in liquid nitrogen. The frozen lungs were then homogenized in 2 mL PBS. The homogenate was diluted with 2 vol of formamide and incubated at 60°C for 18 h, followed by centrifugation at 5,000 \times g for 30 min. The supernatant was collected and absorbance was measured at 620 and 740 nm. The EBD concentration was determined from standard absorbance curves evaluated in parallel. Correction for contaminating heme pigments was calculated by the formula $E_{620}(\text{EBD}) = E_{620} - (1.426 \times E_{740} + 0.030)$. The EBD concentration was expressed as μ g per g of lung.

2.8. Measurement of Myeloperoxidase Activity. As an index of neutrophil infiltration, BAL fluid and tissue-associated myeloperoxidase (MPO) activity was determined. Frozen lung tissues were thawed, weighed, homogenized, and sonicated on ice in radioimmunoprecipitation assay buffer (RIPA). After centrifugation at 10,000 \times g at 4°C for 20 min, the supernatant was collected and used for determination of MPO activity by a commercially available fluorometric assay kit (700160; Cayman Chemical) according to the manufacturer's instructions. Results were expressed as nmol/min-ml. Similar measurements were performed on BAL fluid supernatant.

2.9. Measurement of Oxidant Stress. Hydrogen peroxide (H₂O₂) production in lung tissue was determined using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's directions. The concentrations of nitrate and malondialdehyde (MDA) in lung homogenates were measured using commercially available colorimetric assay kits (Cayman Chemical) according to the manufacturer's instructions.

2.10. Measurement of Plasma Cytokine Levels. Plasma levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), keratinocyte chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2) were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

TABLE 1: Oligonucleotide primers employed.

Gene		Primer sequence	Tm °C	Amplicon size (bp)
9s rRNA	F	5'-ATCCGCCAACGTCACATT-3'	57.6	115
	R	5'-CCGCCGCCATAAGGAGAAC-3'	64.5	
CD36	F	5'-CAGTCGGAGACATGCTTATTGAG-3'	60.7	151
	R	5'-TTTGCCACGTCATCTGGGTTT-3'	62.5	
COX-2	F	5'-TGTGACTGTACCCGGACTGG-3'	63.0	233
	R	5'-TGCACATTGTAAGTAGGTGGAC-3'	60.0	
FABP4	F	5'-GGGGCCAGGCTTCTATTCC-3'	61.8	114
	R	5'-GGAGCTGGGTTAGGTATGGG-3'	61.1	
GAPDH	F	5'-GGACGCATTGGTCGTCTGG-3'	63.0	204
	R	5'-TTTGCCTGGTACGTGTTGAT-3'	60.2	
IL-12 p40	F	5'-CAAGGCTGTTACATTATCCCA-3'	60.3	107
	R	5'-CCAGTGTGGTCATGGACTTTC-3'	60.6	
MCP-1	F	5'-TTAAAAACCTGGATCGGAACCAA-3'	60.1	121
	R	5'-GCATTAGCTTCAGATTACGGGT-3'	60.7	
NOS2	F	5'-ACATCGACCCGTCCACAGTAT-3'	62.7	177
	R	5'-CAGAGGGGTAGGCTTGTCTC-3'	61.0	
NOX4	F	5'-TGCCTGCTCATTGGGCTGT-3'	62.2	180
	R	5'-CCGGCACATAGGTAAAAGGATG-3'	60.8	
PPAR- γ	F	5'-CCATTCTGGCCACCAAC-3'	66.5	479
	R	5'-CTGAAACCGACAGTACTG-3'	53.8	
TNF- α	F	5'-CCTGTAGCCCACGTCGTAG-3'	61.5	148
	R	5'-GGGAGTAGACAAGGTACAACCC-3'	61.4	

2.11. RNA Isolation and Quantitative Real-Time RT-PCR.

Cells pelleted from BAL fluid were resuspended in DMEM supplemented with 10% fetal bovine serum, allowed to adhere to tissue culture-treated six-well plates for 1 h, and then washed twice to remove nonadherent cells. Adherent alveolar macrophages were lysed, and RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA), with cDNA being generated from 100 ng of total RNA using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) employing random and oligo dT primers. Real-time quantitative PCR was performed using 100 ng cDNA with 2X SYBR Green Master mix (Applied Biosystems) and specific primers for the genes of interest (Table 1). These experiments were performed on an AB 7500 fast thermal cycler using a three-step protocol employing the melting curve method. The average of each gene cycle threshold (C_t) was determined for each experiment. Relative cDNA levels ($2^{-\Delta\Delta C_t}$) for the genes of interest were determined by using the comparative C_t method, which generates the $\Delta\Delta C_t$ as the difference between the gene of interest and the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 9s rRNA for each sample. Each averaged experimental gene expression sample was compared to the averaged control sample, which was set to 1.

2.12. Transient Transfection Assay. PPAR- γ activity in A549 cells (ATCC, Rockville, MD) was determined as previously described [18]. Briefly, cells were transiently co-transfected either with a plasmid containing the luciferase gene under regulation by four Gal4 DNA-binding elements (UAS $_G$ ×4

TK-luciferase) and a plasmid containing the PPAR- γ ligand-binding domain fused to the Gal4 DNA-binding domain or with the luciferase gene under control of the peroxisome proliferator response element (PPRE) isolated from the fatty acid transport protein. All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following treatment with test compounds, activation was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

2.13. Transcription Factor DNA-Binding Activity Assay.

Nuclear proteins were extracted using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The protein concentration was determined using the BCA Protein Assay kit (Pierce). Nuclear extracts were used to quantify DNA-binding activity of PPAR- γ , nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and the p65 subunit of NF- κ B using ELISA-based TransAM kits (40096, 40696, and 50296; Active Motif) according to the manufacturer's instructions.

2.14. Molecular Modeling and Computer Simulations of Binding of OA-NO $_2$ with PPAR- γ . *In silico* construction of OA-NO $_2$ was carried out using Chem Office (ChemDraw and Chem3D; CambridgeSoft Corp., Cambridge, MA). To avoid steric hindrance and clashes that can appear in the building process, the models obtained were subjected to geometry optimization using GaussView with a protocol of 300 steps of conjugate gradients. Each model then was optimized using

a semi-empirical method such as PM3 as implemented in the Gaussian98 package of programs.

Autodock 4.2 (with Lamarckian Genetic Algorithm) or ArgusLab 4 was used to generate the starting complexes. An elitism value of 1 was used, together with probabilities of mutation and crossing-over of 0.03 and 0.07, respectively. From the best solutions obtained according to these parameters, those defined by the user as exhibiting the best probabilities—in our case, 0.07—were further refined by a local search method. Autodock defines the conformational space implementing grids over the entire space of possible solutions. With the aim of testing the ability of Autodock to converge into solutions that are inside the PPAR- γ ligand-binding domain, a grid of 70 Å per side with 0.4 Å spacing between points was set up in such a way that it covered both the external surface and the internal cavity of the PPAR- γ ligand-binding domain. The following procedure was employed for the OA-NO₂-PPAR- γ ligand-binding domain docking simulations: 150 runs were done for each OA-NO₂-PPAR- γ ligand-binding domain. At the end of each run, the complexes were separated into clusters according to their lowest root mean square deviation (RMSD), and the best score value based on a free empiric energy function was determined. Cluster complexes whose average score was -11.50 kcal/mol/L with respect to the best energy obtained in that run were selected. The selected final complexes were optimized using the semi-empirical PM3 method as a refining procedure with Gaussian98.

Constant-volume, constant-temperature molecular dynamics (MD) simulations of the complexes were performed on the Discover, version 2.7 (Biosym Technologies, Inc., San Diego, CA) and MD simulation programs in Chem Office. Energy minimizations were conducted for two systems, both of which assumed 1 molecule of OA-NO₂, 1 molecule of PPAR- γ , and water molecules. The systems simulated were; (i) OA-NO₂ and PPAR- γ separated from each other by a distance greater than the non-bonded cutoff distance (>8.5 Å); (ii) OA-NO₂ complexed with the PPAR- γ ligand-binding domain. Each molecular system was contained in a box size of 25.0 × 25.0 × 37.0 Å with periodic boundary conditions. The step size was 2 femtoseconds (fsec). To start the simulations, different seed numbers were used for initial Maxwellian velocity distribution for each system. Simulations were continued and the coordinates were saved for analysis every 2 fsec.

2.15. Statistical Analysis. Data are presented as mean ± SD. Differences between groups were analyzed using ANOVA, followed by a Bonferroni multiple comparison test using GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered significant.

3. Results

3.1. In Silico Binding of OA-NO₂ to PPAR- γ . Although the crystal structure of LNO₂ bound to the PPAR- γ ligand binding site has been reported [19], no similar information is available for OA-NO₂. We accordingly used *in silico* methods to determine the likely binding mode for this

compound. As shown in Figures 1(a) and 1(b), OA-NO₂ is well accommodated within the PPAR- γ ligand-binding site, with best pose energy of -11.50 kcal/mol, and exhibits the appropriate interactions and hydrogen bonds with the PPAR- γ amino acid residues (ARG 288, GLN 286, HIS 449, and TYR 473) that are known to be important for PPAR- γ activation.

3.2. OA-NO₂ Activates PPAR- γ In Vitro. To confirm the ability of NFAs to activate PPAR- γ we utilized the GAL4 reporter system in A549 airway epithelial cells. Transfected cells were treated with 0.1, 1, or 5 μ M concentrations of OA-NO₂ or, as a positive control, of the synthetic PPAR- γ agonist rosiglitazone. Each compound demonstrated dose-dependent activation as a result of binding to the construct's PPAR- γ ligand-binding domain, with similar activation for given molar concentrations (Figure 1(c)). To demonstrate activation of endogenous PPAR- γ , A549 cells were similarly transfected with the luciferase gene under control of a PPAR response element. This assay likewise demonstrated dose-dependent activation by both OA-NO₂ and rosiglitazone (Figure 1(d)).

3.3. Pulmonary Administration of OA-NO₂ Diminishes Severity of LPS-Induced Lung Inflammation. Pulmonary inflammation is a crucial feature of ALI. Since the nuclear receptor PPAR- γ is known to exert a variety of anti-inflammatory effects and unsaturated long-chain NFAs are activating ligands for PPAR- γ , we hypothesized that pulmonary delivery of an NFA would diminish the severity of ALI. To test this hypothesis, we utilized a well-established murine model of ALI induced by i.t. administration of 50 μ g of LPS. Thirty min after LPS injection, 50 μ g of OA-NO₂ in 10% DMSO was delivered to the lungs via the i.t. route. Control mice received vehicle without NFA. After a further 5.5 h the mice were euthanized, the lower right lobe excised for assessment of edema by wet:dry weight ratio, BAL fluid collected, and the lungs excised for histopathological examination and measurement of inflammation-association molecular markers. Plasma was obtained at the same time.

A prominent aspect of pulmonary inflammation is infiltration of neutrophils (polymorphonuclear leukocytes; PMNs) into the lungs and thus into BAL fluid. We observed an LPS-induced increase in the total number of cells in BAL fluid (Figure 2(a)), and differential staining indicated that neutrophils accounted for most of the increase (Figure 2(b)). Both increases were significantly attenuated by OA-NO₂ treatment. Similar results were seen for measurements of myeloperoxidase in both lung (Figure 2(c)) and BAL fluid (Figure 2(d)). MPO is found in the pulmonary system in association with PMNs and is thus a marker for their presence. These results were confirmed by direct microscopic examination of BAL fluid (Figure 2(e)).

Inflammation is also characterized by oxidant stress, which directly injures lung tissues. Oxidant stress reflects production of reactive oxygen species such as H₂O₂ and superoxide, predominantly by macrophages and neutrophils, and of the reactive nitrogen species NO by a variety of cell types. As expected, measurement of H₂O₂ demonstrated

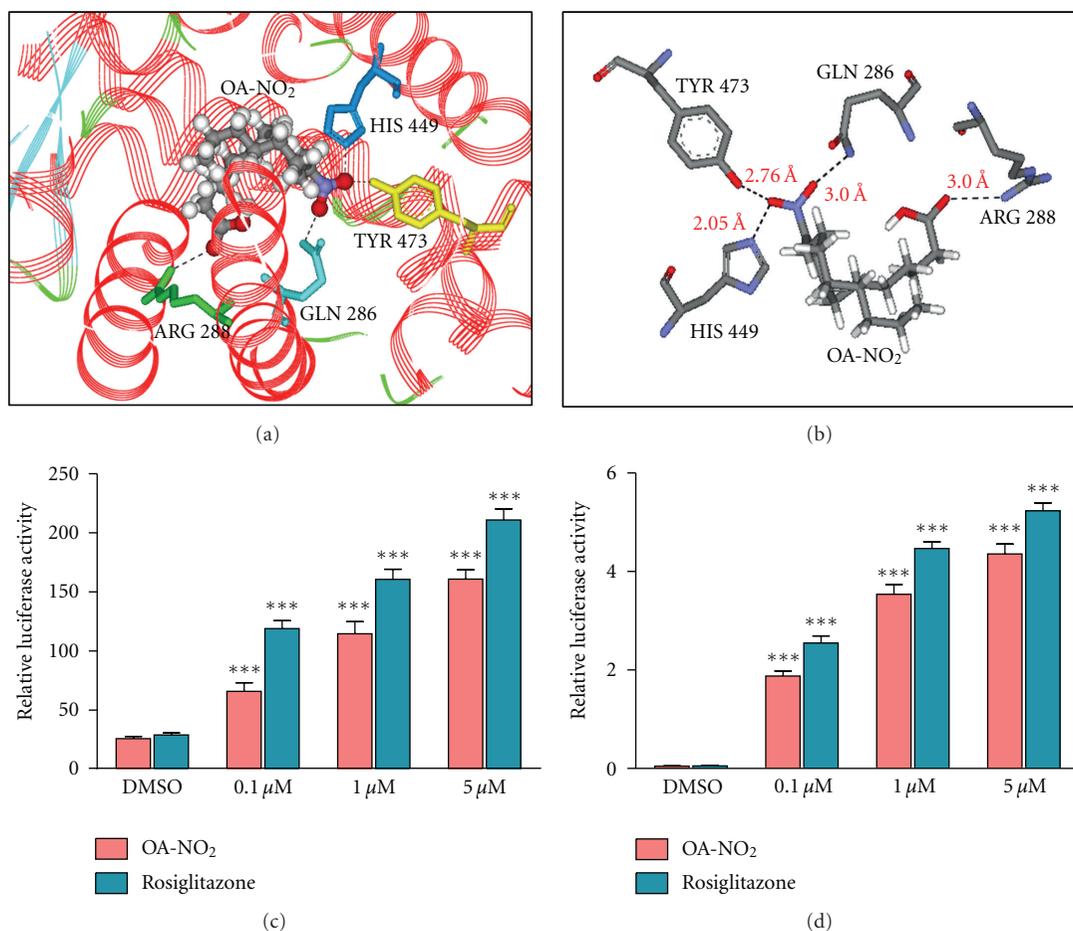


FIGURE 1: OA-NO₂ activates PPAR-γ in airway epithelial cells. Binding of OA-NO₂ to the PPAR-γ ligand-binding domain was modeled *in silico*. Results are presented as (a) space-filling representation; (b) schematic representation showing interactions of PPAR-γ residues with specific groups in the OA-NO₂ ligand. Hydrogen bonds are indicated by dotted lines. A549 cells were transiently transfected with pRLSV40 and with one of the following constructs: (c) PPAR-γ dependent luciferase reporter, pFATPluc; (d) PPAR-γ GAL4 reporter system (UAS_G × 4 TK-luciferase + GAL4-PPAR-γ). Cells were then incubated with vehicle (DMSO), OA-NO₂ (0.1–5 μM), or rosiglitazone (0.1–5 μM). After 24 h, luciferase activity was measured with a dual luciferase activity kit and normalized to that of *Renilla* luciferase. Data are representative of one of two independent experiments; *n* = 6; ****P* < 0.001 versus vehicle.

a large increase following LPS administration that was reduced by OA-NO₂ treatment (Figure 2(f)). Similar results were seen for nitrate, the end product of NO metabolism (Figure 2(g)). Levels of the lipid oxidation product MDA that provides an index of overall oxidative stress, and MDA levels followed the same pattern as the other two markers of oxidant generation (Figure 2(h)).

Inflammation in ALI is likewise associated with release of proinflammatory cytokines and chemokines by neutrophils, macrophages, and other cells. We measured the proinflammatory cytokines TNF-α (Figure 2(i)) and IL-6 (Figure 2(j)), the chemokine KC, which activates and attracts neutrophils (Figure 2(k)), and the related chemokine MIP-2, which has effects similar to KC (Figure 2(l)). Plasma levels of all four markers were greatly increased by LPS administration, but this increase was significantly reduced by OA-NO₂ treatment.

3.4. Pulmonary Administration of OA-NO₂ Diminishes Capillary Permeability and Severity of LPS-Induced Lung Injury. Increased capillary permeability results in lung edema, a driving force for the hypoxemia that is observed in ALI. It also allows escape of plasma proteins into the alveolar space, which can then be detected in BAL fluid. We find that OA-NO₂ treatment attenuates the LPS-induced increase in BAL fluid protein concentration (Figure 3(a)). The increased vascular permeability following LPS administration allowed EBD to extravasate from the vasculature into the lung parenchyma, turning these lungs deep blue (~0.3 μg dye per g lung). This extravasation of EBD was significantly reduced by OA-NO₂ treatment, however, resulting in only a pale blue appearance of the lung (~0.17 μg dye per g lung; Figure 3(c)). The lung edema that results from capillary permeability is reflected in the wet:dry weight ratio, and LPS-induced increases in this parameter were also reduced by

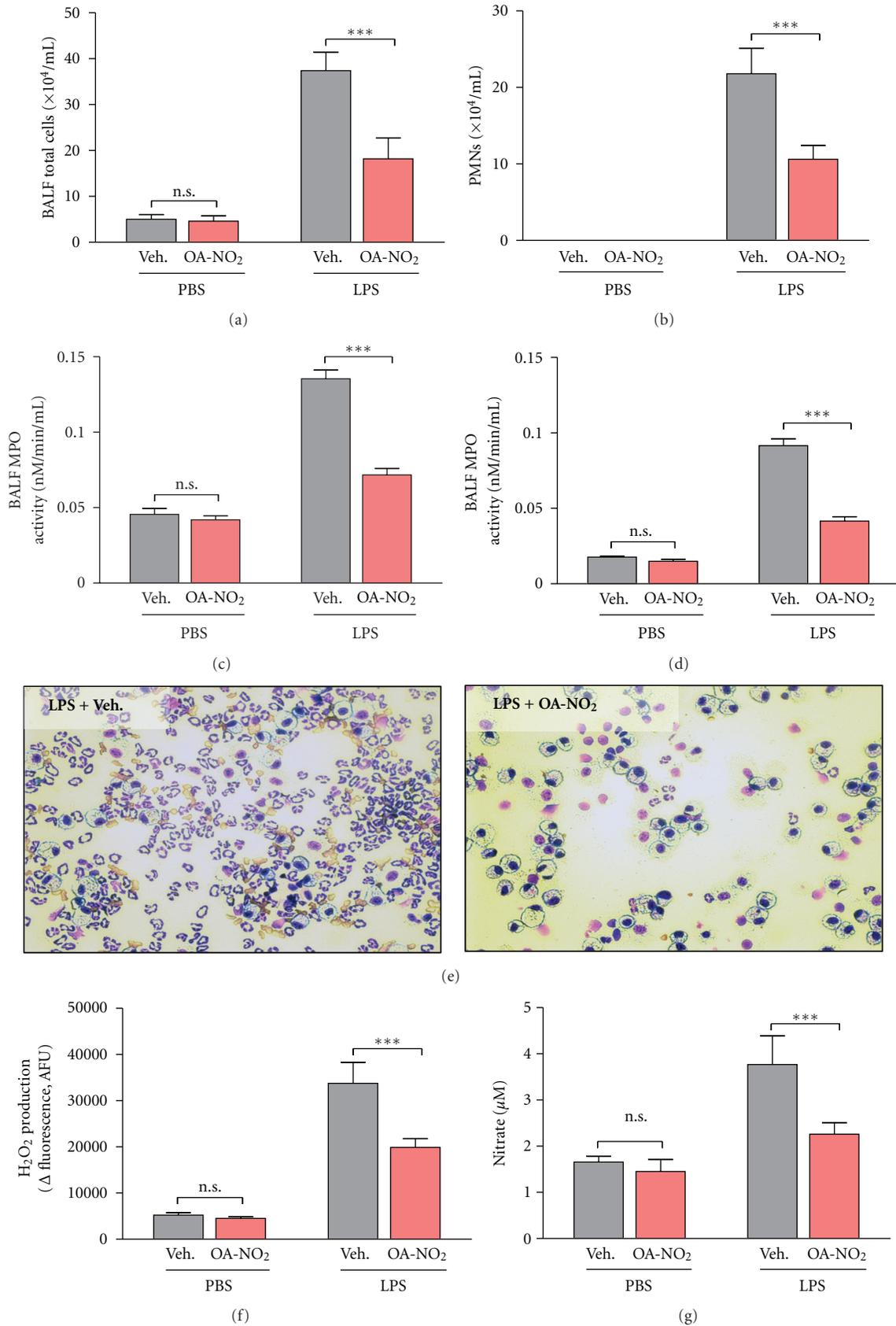


FIGURE 2: Continued.

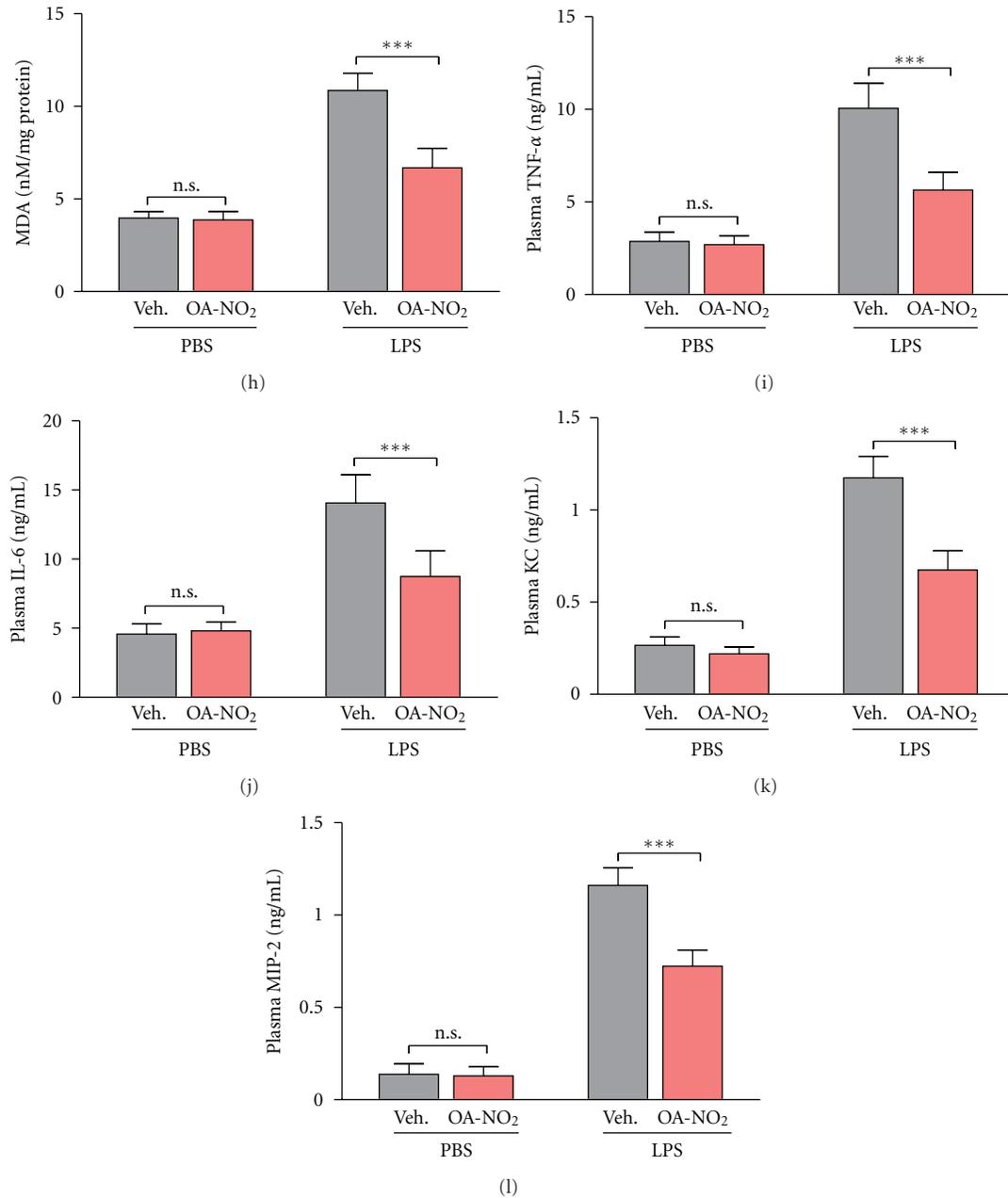


FIGURE 2: Pulmonary delivery of OA-NO₂ reduces LPS-induced lung inflammation. Induction of ALI by i.t. injection of LPS (50 μg) was followed 30 min later by i.t. administration (50 μL) of OA-NO₂ (50 μg) or vehicle (10% DMSO). After a further 5.5 h, BAL fluid, plasma, and lung samples were obtained. (a) Total cell and (b) neutrophil number in BAL fluid. Myeloperoxidase activity in (c) lung tissue and (d) BAL fluid. (e) Microscopic examination following staining of BAL fluid. (f) H₂O₂ production, (g) nitrate concentration, and (h) malonaldehyde/protein ratio in lung. Plasma levels of (i) TNF-α, (j) IL-6, (k) KC, and (l) MIP-2. Data are representative of one of two independent experiments with $n = 6-8$ mice per group; *** $P < 0.001$.

OA-NO₂ treatment (Figure 3(b)). Direct histopathological examination of the lung following H&E staining further confirmed significant lung inflammation and injury in mice treated with vehicle but much less severe abnormalities in those that had received OA-NO₂ (Figure 3(d)).

3.5. OA-NO₂ Produces Anti-Inflammatory Alterations in Activity of PPAR-γ, NF-κB, and Nrf2. We propose that

NFAs exert many of their anti-inflammatory effects by activating PPAR-γ, which is known to decrease activity of the proinflammatory transcription factor NF-κB. PPAR-γ promotes transcription of the antioxidant factor Nrf2, which in turn upregulates PPAR-γ expression in a positive feedback loop [20, 21]. To test our hypothesis, we examined the effects of OA-NO₂ treatment on activity of PPAR-γ, NF-κB, and Nrf2. When followed by vehicle treatment, i.t. LPS

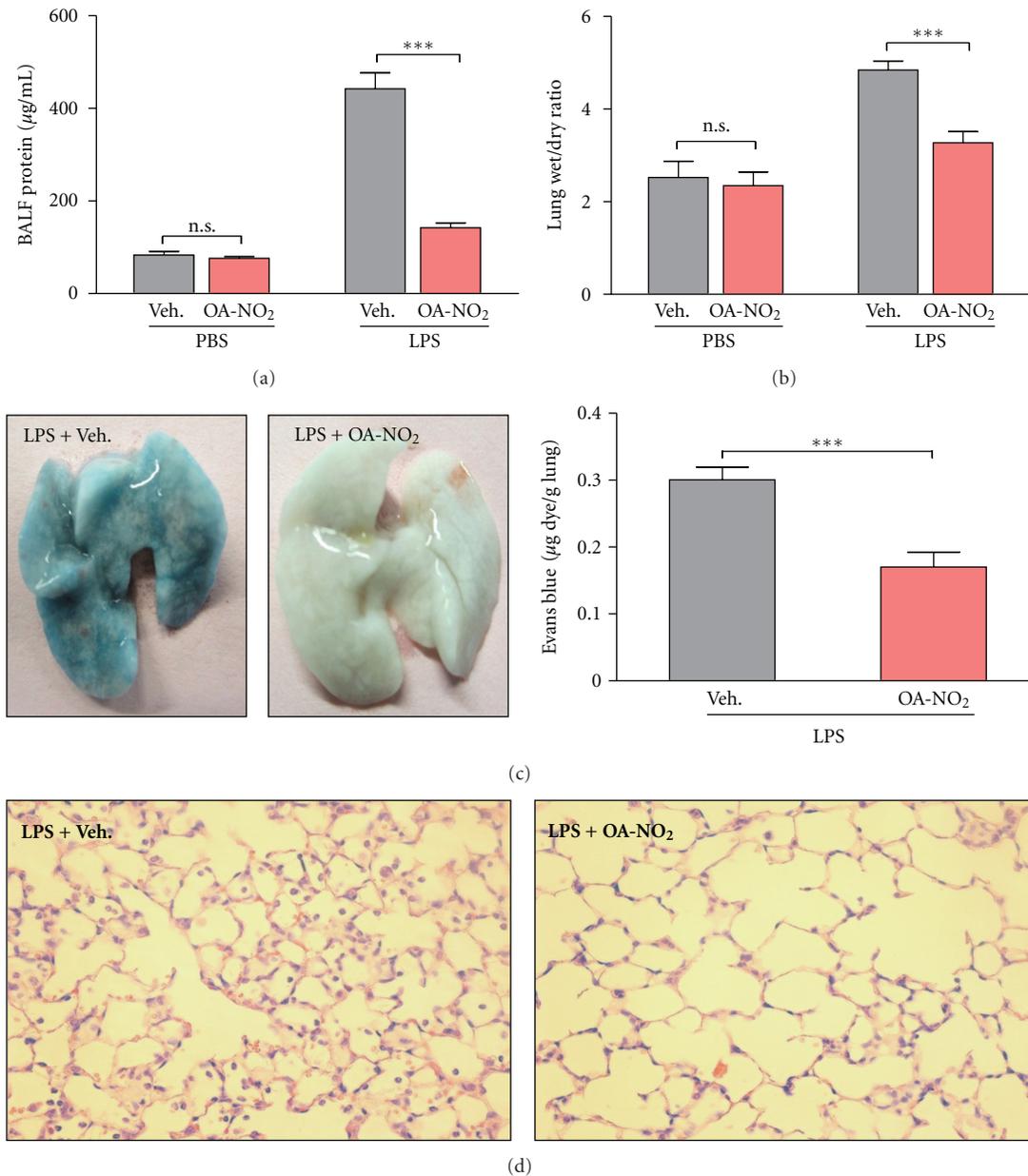


FIGURE 3: Pulmonary delivery of OA-NO₂ reduces LPS-induced lung injury. Induction of ALI by i.t. injection of LPS (50 µg) was followed 30 min later by i.t. administration (50 µL) of OA-NO₂ (50 µg) or vehicle (10% DMSO). After a further 5.5 h, BAL fluid and lung samples were obtained. (a) Protein concentration in BAL fluid. (b) Ratio of lung wet:dry weight. (c) Extravasation of Evans Blue dye into the lung following intravenous injection was photographed and quantitated by spectrophotometry. (d) The lung was examined histologically following H&E staining. Data are representative of one of two independent experiments with $n = 6-8$ mice per group; *** $P < 0.001$.

upregulated the DNA-binding activity of NF-κB, as expected (Figure 4(c)) but decreased activity of PPAR-γ (Figure 4(a)) and Nrf2 (Figure 4(b)). OA-NO₂ treatment, however, not only diminished the increase in NF-κB activity but also increased the DNA-binding activity of PPAR-γ and Nrf2. Under noninflammatory conditions, OA-NO₂ treatment increased PPAR-γ activity but had no significant effect on basal levels of NF-κB or Nrf2 activity. Given the role of NFAs as PPAR-γ agonists and the known anti-inflammatory and antioxidant activities of PPAR-γ, many exerted through

inhibition of NF-κB activity and upregulation of Nrf2, these data support the concept that NFAs act in part via PPAR-γ activation.

3.6. OA-NO₂ Decreases Inflammatory Response of Alveolar Macrophages. Alveolar macrophages play a central role in regulation of the lung's immune system. When activated by stimuli such as LPS, they generate oxidants and secrete molecules that attract neutrophils and other immune cells to the lung. As PPAR-γ is known to play a major role

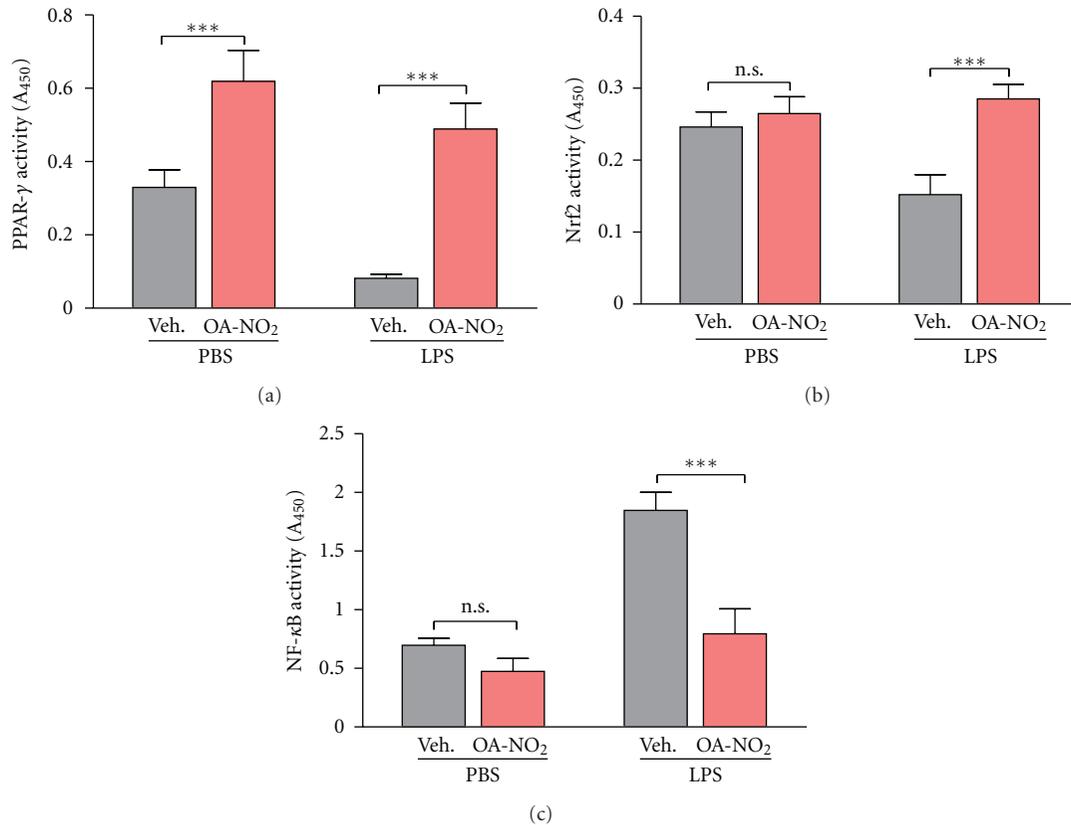


FIGURE 4: OA-NO₂ reverses LPS-induced changes in transcription factor activity. Induction of ALI by i.t. injection of LPS (50 μg) was followed 30 min later by i.t. administration (50 μL) of OA-NO₂ (50 μg) or vehicle (10% DMSO). After another 5.5 h lungs were excised and DNA-binding activity of the transcription factors (a) PPAR-γ, (b) Nrf2, and (c) NF-κB was determined. Data are representative of one of two independent experiments with $n = 6-8$ mice per group; *** $P < 0.001$.

in modulating activation of alveolar macrophages [22], we investigated the ability of NFAs to suppress LPS-induced activation of these cells. Specifically, mice were treated with LPS and either vehicle or OA-NO₂ as previously described. Alveolar macrophages were then isolated and their RNA extracted for determination of relevant gene expression. Results (Figure 5) demonstrate significant suppression of the proinflammatory cytokines TNF-α and interleukin-12 (IL-12) and the chemokine MCP-1. Downregulation was also observed for the inducible form of nitric oxide synthase (iNOS; NOS2) and the superoxide-generating enzyme NADPH oxidase 4 (NOX4), both of which contribute to oxidative stress. OA-NO₂ treatment also reduced alveolar macrophage expression of cyclooxygenase 2 (COX-2), which synthesizes proinflammatory prostaglandins. Conversely, OA-NO₂ upregulated expression of PPAR-γ, and the PPAR-γ target genes fatty acid binding protein 4 (FABP4) and CD36, a receptor that facilitates macrophage phagocytosis of apoptotic and senescent neutrophils and thereby contributes to the resolution of inflammation. All these measurements confirm the ability of NFA treatment *in vivo* to suppress the activated, proinflammatory phenotype of alveolar macrophages.

4. Discussion

Our results establish that delivery of OA-NO₂ directly to the lung significantly reduces the severity of pulmonary inflammation and injury. We also find that treatment with OA-NO₂ suppresses the activated phenotype of alveolar macrophages, key cells in the regulation of pulmonary inflammation. In the lung, LPS-induced increase in activity of the proinflammatory transcription factor NF-κB is largely blocked, as are LPS-induced decreases of PPAR-γ and the antioxidant transcription factor Nrf2. Indeed, PPAR-γ activity is increased over basal levels in both inflammatory and noninflammatory conditions. Upregulation of Nrf2 expression and activity may account for much of the antioxidant activity of PPAR-γ and consequent reduction in inflammation-associated oxidative injury [20, 21].

This appears to be the first study of NFAs' ability to reduce the severity of ALI and one of very few to investigate the protective anti-inflammatory actions of NFAs in an animal model of any disease. Borniquel and colleagues have recently obtained results in a murine model of inflammatory bowel disease that are similar to ours in ALI [23], finding that OA-NO₂ decreased disease severity and the increase in NF-κB expression while increasing expression of PPAR-γ.

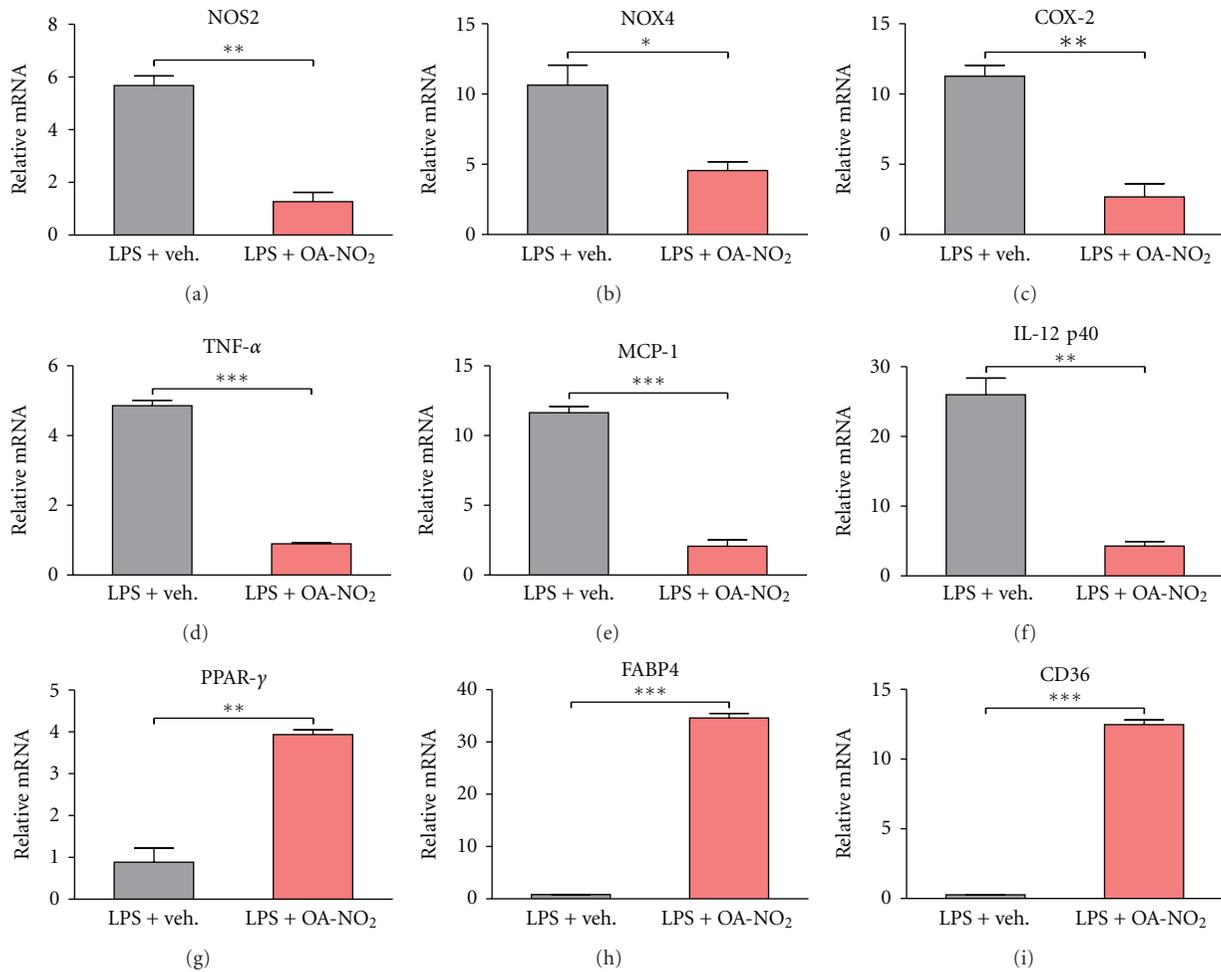


FIGURE 5: OA-NO₂ reduces inflammatory phenotype in LPS-activated alveolar macrophages. Induction of ALI by i.t. injection of LPS (50 μg) was followed 30 min later by i.t. administration (50 μL) of OA-NO₂ (50 μg) or vehicle (10% DMSO). After another 5.5 h BAL fluid was obtained. Alveolar macrophages were isolated from the BAL fluid and plated in DMEM + 10% FBS. After 1 h, RNA was isolated and expression of the indicated genes was determined using real-time PCR; results were normalized to values for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 9s rRNA. Data are representative of one of two independent experiments with $n = 6-8$ mice per group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Significantly, the increase in PPAR-γ expression was abolished by simultaneous administration of a PPAR-γ antagonist. OA-NO₂ was also found to reduce infarct size in cardiac ischemia-reperfusion injury [24]. This was accompanied by a reduction in NF-κB activity but a potential role for PPAR-γ was not investigated. Beneficial effects of OA-NO₂, together with reduced inflammation, have also been seen in renal ischemia/reperfusion injury [25]. It appears likely that a number of other inflammatory conditions might also benefit from NFA treatment.

Activation of the nuclear hormone receptor PPAR-γ has been shown to reduce the severity of LPS-induced ALI [5, 9], and NFAs are known to activate PPAR-γ [11, 12]. That this activation reflects binding to the ligand binding domain is demonstrated both by ability of NFAs to displace the synthetic ligand rosiglitazone [12] and by the elucidated crystal structure of LNO₂ bound to this site [19]. We therefore suggest that the protective effects we

observe are mediated largely by activation of PPAR-γ. Such activation appears adequate to account for most of our observations, including increased activity and expression of both PPAR-γ itself and the antioxidant transcription factor Nrf2, which both upregulates and is upregulated by PPAR-γ [20, 21], as well as decreased activity of the proinflammatory transcription factor NF-κB.

Initial studies suggested that OA-NO₂ and LNO₂ were present in normal human plasma at concentrations exceeding 0.1 μM, which in conjunction with their measured binding affinity would raise the possibility that they were endogenous PPAR-γ agonists [11]. Later studies from another group, however, have suggested concentrations below 1 nM [26]. Notably, both studies were performed with plasma from healthy subjects and do not reflect the increase in NFA concentrations anticipated in inflammatory conditions. This controversy nevertheless has only limited relevance to our studies, which address pharmacological effects of

exogenously delivered OA-NO₂ rather than the role of endogenous NFAs.

Although the ability of NFAs to activate PPAR- γ at readily achievable plasma concentrations is well established, this may not be their only mechanism of action. NFAs are known to alkylate thiol groups via the Michael reaction [27] and the large majority of plasma NFAs are in fact present as protein adducts [28]. These adducts, however, are primarily to serum albumin or other proteins unlikely to be involved in signaling pathways. Nevertheless, alkylation of signaling proteins might well underlie certain NFA effects. It has been shown that NFAs alkylate the p65 subunit of NF- κ B, thus reducing its DNA-binding activity [16]. This mechanism has been proposed to mediate the protective effect of NFAs on cardiac ischemia/reperfusion injury, although involvement of other mechanisms, including PPAR- γ activation, was not ruled out [24]. More recently it has been shown that NFA alkylation of atypical protein kinase C ζ inhibits bradykinin-induced Ca⁺⁺ influx in pulmonary epithelial cells [29]. Other studies have identified NFA effects not readily related to PPAR- γ [30–32], including induced expression of anti-inflammatory and cytoprotective genes under control of the heat shock transcription factor [33], but these studies did not identify a specific mechanism for the observed effects. These data suggest that NFA actions involve both PPAR- γ -dependent and -independent mechanisms, although this question requires further investigation.

Failure of OA-NO₂ to upregulate Nrf2 activity under noninflammatory conditions appears unexpected, given the substantial increase in PPAR- γ activity we observed. PPAR- γ knockdown has been shown to block O₂-induced increases in Nrf2 expression [20] while PPAR- γ agonists upregulate activity of this antioxidant transcription factor [34]. In the latter system, however, induction was weak in the absence of additional stimulation with retinoic acid, an activating ligand for the RXR receptor with which PPAR- γ forms a heterodimer required for transcriptional activity. NFAs have also been reported to upregulate Nrf2 activity and expression by alkylation of the inhibitor protein Keap-1 [35–37], which both maintains a cytosolic location for Nrf2 and marks it for ubiquitination and subsequent degradation. Other evidence suggests that NFAs may activate PPAR- γ and Nrf2 by distinct post-transcriptional pathways, with the latter involving phosphatidylinositol-3-kinase and protein kinase C [38]. Notably, stimulation of reporter gene activity required NFA concentrations at least 10-fold higher for Nrf2 than for PPAR- γ . These different potencies may account for our observation that, under noninflammatory conditions, a single 50 μ g injection of OA-NO₂ upregulated PPAR- γ but not Nrf2 activity. In this context, NFA effects on Nrf2 would appear to be an intriguing area for further investigation.

5. Conclusions

Our results in a murine model of ALI support the anti-inflammatory effects of NFAs that have been demonstrated *in vitro* and in a limited number of other disease models. They also show, for the first time, that direct pulmonary delivery of an NFA can have beneficial effects in lung disease.

Inflammation is an important feature of many lung diseases, including asthma and chronic obstructive pulmonary disease, and synthetic PPAR- γ -activating thiazolidinediones have been proposed as treatments for these diseases. As diabetes therapies, however, these agents are known to be associated with adverse effects. Our results suggest that NFAs, and perhaps OA-NO₂ specifically, might be attractive alternatives for these diseases as well as ALI.

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Review Article

PPAR γ Expression and Function in Mycobacterial Infection: Roles in Lipid Metabolism, Immunity, and Bacterial Killing

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Tuberculosis continues to be a global health threat, with drug resistance and HIV coinfection presenting challenges for its control. *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, is a highly adapted pathogen that has evolved different strategies to subvert the immune and metabolic responses of host cells. Although the significance of peroxisome proliferator-activated receptor gamma (PPAR γ) activation by mycobacteria is not fully understood, recent findings are beginning to uncover a critical role for PPAR γ during mycobacterial infection. Here, we will review the molecular mechanisms that regulate PPAR γ expression and function during mycobacterial infection. Current evidence indicates that mycobacterial infection causes a time-dependent increase in PPAR γ expression through mechanisms that involve pattern recognition receptor activation. Mycobacterial triggered increased PPAR γ expression and activation lead to increased lipid droplet formation and downmodulation of macrophage response, suggesting that PPAR γ expression might aid the mycobacteria in circumventing the host response acting as an escape mechanism. Indeed, inhibition of PPAR γ enhances mycobacterial killing capacity of macrophages, suggesting a role of PPAR γ in favoring the establishment of chronic infection. Collectively, PPAR γ is emerging as a regulator of tuberculosis pathogenesis and an attractive target for the development of adjunctive tuberculosis therapies.

1. Introduction

Tuberculosis is a global public health problem, with over 9 million new cases being reported each year that are responsible for almost 2 million deaths annually worldwide [1]. *Mycobacterium tuberculosis* (*M. tuberculosis*), the etiological agent of tuberculosis, is a highly successful pathogen, infecting approximately one-third of the human population, and it has adapted to live within the hostile macrophage environment. Through long-standing coevolution with its mammalian host, *M. tuberculosis* has evolved different strategies to subvert the immune and metabolic responses of the host cells. Pathogenic species of mycobacteria express and regulate numerous genes within the host to evade the host immune responses and suit their intracellular life style. Among the

intracellularly induced genes, several genes have functions in lipid metabolism.

PPAR γ is a member of the lipid-activated nuclear receptor superfamily and plays a recognized role in the transcriptional regulation of cellular proliferation, differentiation, and inflammation in addition to metabolic regulation of lipids and glucose [2, 3]. This receptor is regulated by fatty acid metabolites and acts as a transcription factor, forming heterodimers with the retinoid X receptor (RXR) and binding to specific PPAR response elements (PPREs) in the promoter regions of target genes [4, 5]. PPARs were originally described in adipocytes, monocytes, and macrophages [6, 7]. Since then, they have been described in other immune cell types of hematopoietic origin, including T lymphocytes, B lymphocytes, NK cells, dendritic cells, neutrophils,

eosinophils, and mast cells, where a role for these receptors in inflammation and immunoregulation has been proposed [7–10]. However, the role of PPARs in the host immune responses to intracellular infectious agents is only now being recognized.

Herein, we focus on the role of PPAR γ in intracellular bacterial infection. Specifically, we discuss the host response to *Mycobacterium* infection related to the regulation of PPAR γ expression by mycobacteria and PPAR γ -dependent effects on mycobacterial-induced modulation of host cell lipid metabolism and immune responses. Notably, PPAR γ expression is highly upregulated during mycobacterial infection. Mycobacterial-induced PPAR γ plays roles in host cell metabolism leading to increased lipid droplet formation and downregulates the host immune response to favor pathogen burden, thereby suggesting that pathogens may stimulate PPAR γ activity as an escape mechanism.

2. *Mycobacterium* Infection Triggers Increased PPAR γ Expression

PPAR γ is widely expressed in many cell types in different tissues, including in macrophages and dendritic cells in the lung [2, 11, 12]. Moreover, cytokines and pathogen-derived components may regulate PPAR γ expression in cells of the immune system [13]. Recent studies have demonstrated that mycobacterial infection significantly increases PPAR γ expression in human and mouse macrophages with important consequences for immune and metabolic host responses to infection [14, 15].

Infection of macrophages with either *M. bovis* bacillus Calmette-Guérin (BCG) or *M. tuberculosis* triggers a time-dependent increase in the expression of PPAR in macrophages *in vitro* [14, 15] and *in vivo* in the lung [16]. Increased PPAR γ expression was apparent as early as 2 h after infection and reached maximal levels within 24 h after the infection. Of note, non-pathogenic, fast-growing *M. smegmatis* fails to induce PPAR γ expression in macrophages, suggesting that PPAR γ expression may be related to bacterial pathogenesis [14, 15].

The mechanisms involved in mycobacterial-induced PPAR γ expression have recently been investigated. Interestingly, even infection with dead bacteria triggers PPAR γ expression, as paraformaldehyde-killed *M. tuberculosis* or cell-wall components; mostly mannose-capped lipoarabinomannan (ManLAM) from either BCG or *M. tuberculosis*, are able to induce PPAR γ expression, suggesting the role of pattern recognition receptors in the regulation of PPAR γ [14–16].

During the infection of foam-like-macrophages, pathogenic mycobacteria trigger an innate immune response mediated by pathogen-associated molecular patterns (PAMPs), such as Toll-like receptors (TLR) and NOD-like receptors (NLRs). Recent reports indicate that NLR and TLR pathways are nonredundant in the recognition of *M. tuberculosis* and can synergize to induce a proinflammatory response [17].

TLRs represent some of the most important pattern recognition receptors (PRRs) that recognize mycobacterial

products [18, 19]. Recognition through TLRs results in the rapid activation of signal-dependent transcription factors, including members of the nuclear factor- κ B (NF- κ B), activator protein 1 (AP1), and interferon regulatory factor (IRF) families [20, 21]. Activation of multiple TLRs, including TLR2, TLR4, and TLR9, as well as TLR6 and TLR1 when dimerized with TLR2, contributes to an efficient innate response against mycobacterial infection, resulting in inflammatory responses with cytokine production [18, 19, 22–24]. The NOD proteins are localized in the cytoplasm, and NOD2 has been implicated in the recognition of intracellular pathogens, such as mycobacteria [17, 25, 26]. NOD2 does not play a significant role in controlling *M. tuberculosis* growth during early infection [27], although NOD2 mRNA levels are increased in patients with tuberculosis [28]. In contrast, Brooks et al. [29] reported that NOD2 controls the growth of both *M. tuberculosis* and BCG in human macrophages, whereas it controls only BCG growth in murine macrophages. Collectively, these findings suggest that activation of different pathways is important and leads to different outcomes during mycobacterial infection.

The role of TLR in regulating PPAR γ expression has been investigated. We demonstrated that PPAR γ expression in macrophages infected with BCG or stimulated with ManLAM is requisitely dependent on TLR2 signaling [14]. However, the nonpathogenic *M. smegmatis*, a well-known TLR2 ligand, and the synthetic TLR2 ligand Pam3Cys fail to induce PPAR γ expression in macrophages [14, 15], suggesting that coreceptors of TLR2 are required to induce PPAR γ expression.

The TLR2 coreceptors and the downstream pathways involved in mycobacteria-induced PPAR γ expression are currently unknown. Of note, Rajaram et al. [15] demonstrated that infection with virulent *M. tuberculosis* or the addition of ManLAM upregulates PPAR γ expression independent of NF- κ B in human macrophages.

3. PPAR γ Regulates Host Immune Responses to Mycobacterial Infection

The host immune response to mycobacterial infection requires tightly balanced orchestration of both innate and adaptive immunity. The role of PPAR γ in regulating the immune responses of murine and human macrophages to different species of *Mycobacterium* has been studied. PPAR γ activation was demonstrated during infection by BCG [14, 16] and *M. tuberculosis* [15], as well as its major cell-wall immune-regulatory lipoglycan, namely, ManLAM [14, 15] that culminates with an anti-inflammatory response and downregulation of macrophage functions.

Of major interest during pathogen infection, PPAR γ may repress target inflammatory genes, including proinflammatory cytokines and inducible NO synthase (iNOS) [30–32]. The molecular mechanisms of the negative regulation of inflammatory responses are executed, at least in part, by the ability of PPAR γ to interfere with the activities of other signal-dependent transcription factors by transrepression

[11]. PPAR γ , which binds constitutively to DNA as a heterodimer with RXRs, functions as a transcriptional repressor through ligand-dependent transrepression of NF- κ B target genes and may also function in the absence of ligand by interacting with corepressor complexes containing histone deacetylases (HDACs), nuclear-receptor corepressor (NcoR), or the silencing mediator of retinoic acid and thyroid-hormone receptor (SMRT) [30, 31, 33]. These protein complexes bind to the promoters of inflammatory genes and prevent the acetylation of histones and the aggregation of coactivator complexes. PPAR γ downregulates proinflammatory gene expression by antagonizing the activity of transcription factors, including FOXP3, T-bet, and GATA-3, which are involved, respectively, in the regulation of inflammation and Th1 and Th2 immune responses [2, 3]. PPAR γ serves also as a negative regulator of macrophage activation, altering the expression of many inflammatory genes [7, 9], modulating macrophage differentiation and activation through transrepression of the transcription factors STAT, AP-1, and NF- κ B [32], and attenuating the respiratory burst [34]. The PPAR γ ligands induce an allosteric change in PPAR γ that results in covalent attachment of small ubiquitin-related modifier 1 (SUMO1) to the ligand-binding domain of PPAR γ using the ubiquitin-conjugating enzyme 9 (UBC9) and the protein inhibitor of activated STAT1 (PIAS1) as the SUMO E2 and E3 ligases, respectively, for transcriptional repression [11]. Next, following sumoylation, PPAR γ interacts with the nuclear corepressor (NCoR) complex to prevent signal-dependent recruitment of ubiquitin-conjugating enzymes (such as UBC5) and the 19S proteasome components necessary for NCoR clearance [31]. As a result, the NCoR complex remains bound to the promoter region and exerts repressive activity to the nuclear transcription factors.

The function of PPAR γ activation in the immune response to mycobacterial infection was investigated. PPAR γ was shown to positively regulate prostaglandin (PG) E₂ production in BCG infected macrophages [14], a process potentiated by PPAR γ agonists and inhibited by antagonists. Accordingly, PPAR γ activation led to increased cyclooxygenase (COX) 2 expression [15] and PGE₂ production [35] in *M. tuberculosis* infected macrophages. Of note, PGE₂ is a potent immune modulator that downregulates Th1 responses and bactericidal activity toward intracellular organisms [36, 37].

The production of nitric oxide (NO) and other reactive nitrogen intermediates by innate immune cells is considered an effective host-defense mechanism against microbial pathogens, including mycobacterial infection. During infection, NO is produced by inducible NO synthase (iNOS) in response to bacterial components or a combination of proinflammatory cytokines, such as interferon (IFN)- γ , TNF- α , and IL-1 β [38]. In most cells, iNOS transcription requires activation of NF- κ B by TNF- α and IL-1 β and activation of STAT-1 by IFN- γ [39, 40]. PPAR γ has been implicated in the suppression of iNOS expression in macrophages [7, 32]. Synthetic PPAR γ agonists promote PIAS1-dependent conjugation of SUMO1 to the PPAR γ ligand-binding domain, preventing the signal-dependent ubiquitylation and the clearance of the NCoR complex required for full-gene

activation and preventing the expression of iNOS [31]. Production of NO in macrophages is also regulated by the levels of arginases, which compete with iNOS for the substrate L-arginine, and catalyze the hydrolysis of L-arginine to L-ornithine and urea. Of note, PPAR γ positively regulates arginase I expression in macrophages [41]. A role of PPAR γ in modulating NO production during *M. tuberculosis* infection has been demonstrated. Silencing of PPAR γ in *M. tuberculosis* infected macrophages significantly enhanced iNOS expression and NO production in these cells while inhibited arginase I expression, suggesting an endogenous role for PPAR γ in the downmodulation of NO production during infection [35].

Infection in susceptible hosts are modulated by type 2 immune response with Th2 cells that produce IL-4 and IL-13 while protection is associated with type 1 immune response largely dependent of TNF- α and IFN- γ [42, 43]. IL-4 has been demonstrated as a key activator of PPAR γ by regulating the induction of the 12/15-lipoxygenase-derived PPAR γ ligands and through an interaction between PPAR γ and signal transducer and activators of transcription 6 (STAT6) on promoters of PPAR γ target genes [44, 45]. PPAR γ activation suppresses the production of proinflammatory cytokines, and are critical for the formation, activation, and maintenance of alternatively activated macrophages [45]. Elevated PPAR γ expression in human macrophages is one of the biological markers of IL-4/IL-13-mediated alternative activation. Conversely, deletion of PPAR γ in alternatively activated macrophages leads to a Th1 pulmonary inflammatory response that favor intracellular pathogen killing [46]. PPAR γ expression has been shown to be elevated in human alveolar macrophages, which are characterized as alternatively activated macrophages [15]. Moreover, markers of alternative macrophages are induced in *M. tuberculosis*-infected macrophages through PPAR γ -dependent mechanisms [35]. In addition, the balance between the activities of NF- κ B p65 and PPAR γ has been demonstrated during mycobacterial challenge. Lagranderie et al. [16] showed that in nuclear lung-cell extracts 24 h after challenge with freeze-dried BCG, PPAR γ expression increased and NF- κ B p65 expression decreased, suggesting an association between the regulation of these two factors. Moreover, PPAR γ knock-down in macrophages led to enhanced TNF- α and decreased IL-10 production by *M. tuberculosis*-infected macrophages [15, 35], indicating that PPAR γ activation lead to an increase IL-10/TNF ratio creating an anti-inflammatory environment favorable for pathogen growth. Together, accumulating data on PPAR γ -dependent effects on immune response during mycobacterial infection suggest that PPAR γ induction is advantageous for this host-adapted intracellular pathogen within the lung microenvironment.

4. PPAR γ Regulates Host Metabolism to Mycobacterial Infection

PPAR γ has been shown to function as a key transcriptional regulator of lipid metabolism in macrophages and dendritic cells (DC) (for review, see [3]) through the direct

regulation of genes participating in lipid uptake, transport, and storage [47–49]. Indeed, PPAR γ is robustly expressed in macrophage-derived foam cells within atherosclerotic lesions, where it plays an important role in lipid homeostasis and metabolism [8, 32, 47, 50].

Pathogen-triggered dysregulation of host-cell lipid metabolism is emerging as a key feature in the pathogenesis of mycobacterial infection, as mycobacteria relies largely on host lipids for their survival and growth. Accumulating evidence suggests that modulation of host lipid metabolism through mycobacteria-induced lipid droplet formation is important in tuberculosis and leprosy. Foamy-like macrophages have been shown to play important roles in tuberculosis pathogenesis, both within the initial phases of macrophage infection and in granulomas [37, 51, 52]. In addition, lipid droplets formed in response to BCG and *M. leprae* constitute sites for eicosanoid synthesis, ultimately leading to increased production of PGE₂ by infected macrophages [14, 37, 53].

PPAR γ is regulated and active in lipid droplet-enriched cells, and PPAR γ may regulate processes associated with lipid-droplet formation in leukocytes during intracellular mycobacterial infection. In agreement with these results, the PPAR γ agonist BRL49653 potentiates lipid droplet formation and PGE₂ production induced by a suboptimal dose of BCG. Conversely, pretreatment with an antagonist of PPAR γ (GW9662) significantly inhibits BCG-induced lipid droplet formation and PGE₂ production [14], indicating the requirement for PPAR γ signaling in lipid droplet biogenesis and further prostanoid production during BCG infection. The role of PPAR γ activation in regulating lipid droplet biogenesis and PGE₂ production was subsequently confirmed in *M. tuberculosis* infected macrophages after PPAR γ knockdown by RNAi [35].

The mechanisms involved in PPAR γ -induced lipid droplet biogenesis in mycobacterial infection are still not completely understood. PPAR γ -mediated expression of adipose differentiation-related protein (ADRP) has been described in different cells and conditions [54, 55]. ADRP is a member of the PAT family of proteins that plays an important role in adipocyte differentiation, lipolysis modulation, lipid droplet assembly, and biogenesis (reviewed in [56]). ADRP may act as a nucleation center for the assembly of nascent lipids [57, 58] and is also associated with the surface of lipid droplets in macrophages and Schwann cells during mycobacterial infection [37, 59], which is thought to play a major role in the maintenance of lipid storage and survival of pathogens. Increased expression of scavenger receptors, including MARCO, macrophage scavenger receptor (MRS), and CD36, has been observed in mycobacterial infection and leads to increased uptake and accumulation of host-derived oxidized lipids in infected cells [60]. Conversely, enhancing cholesterol efflux by liver X receptor (LXR) activation with the synthetic agonist GW3965 significantly decreased the cholesterol ester content of cells triggered by TLR pathways, including exposure to *C. pneumonia* and LPS [61]. In addition, treatment with the fatty acid synthase inhibitor C75, a PPAR γ target, has been shown to inhibit significantly lipid droplet formation induced by mycobacterial infection

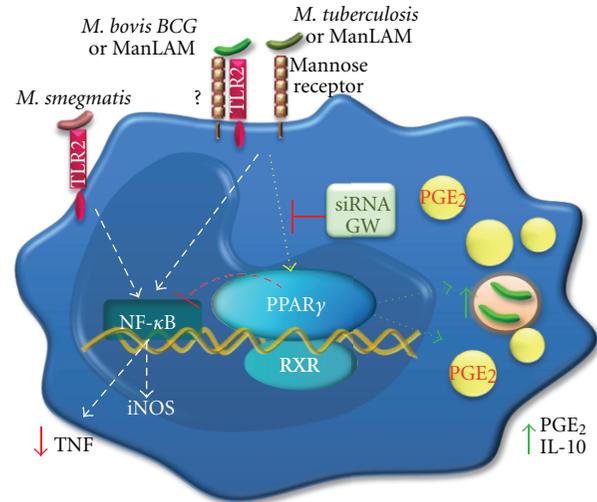


FIGURE 1: Model of PPAR γ functions in mycobacterial infection. The activation of macrophage TLR2 signaling by *M. tuberculosis*, ManLAM, or *M. bovis* BCG results in the activation of PPAR γ and NF- κ B. The activation of PPAR γ by mycobacterial infection induces lipid droplet formation, PGE₂ production and favors mycobacterial survival. Moreover, the PPAR γ activation can down-modulate NF- κ B activity inhibiting the proinflammatory cytokine production. Both inhibition of PPAR γ with the selective antagonist GW9662 or PPAR γ knockdown with siRNA result in a reduction of lipid droplet biogenesis and increased *Mycobacterium* killing by macrophages.

with or without apoptotic cells, confirming the role of new lipid synthesis in lipid droplet biogenesis [62]. Thus, accumulating evidence indicates that mechanisms of increased lipogenesis, decreased lipid degradation, and regulation of lipid influx/efflux act synergistically to form lipid droplets during infection. Based on the different targets of PPAR γ in lipid metabolism, it is conceivable that PPAR γ operates at different levels to regulate lipid droplet biogenesis during infection.

5. Are There Roles for PPAR γ in Mycobacterial Killing and Escape Mechanisms?

PPAR γ has been extensively investigated for its role in many inflammatory diseases; however, its immunoregulatory roles in infectious and parasitic diseases have only recently gained recognition (review [63]).

As discussed above, increased PPAR γ expression during mycobacterial infection is important for lipid metabolism and inflammatory responses of macrophages. Accumulating evidence has suggested that lipid droplet formation may favor intracellular survival and/or replication of *M. tuberculosis*, BCG and *M. leprae* in different models [37, 52, 59, 64]. Moreover, decreased production of proinflammatory cytokines and NO could also contribute to a favorable environment for pathogens, thereby suggesting that mycobacterial-induced PPAR γ expression may act as an escape mechanism for this intracellular parasite.

The impact of PPAR γ expression and activation in mycobacterial survival within macrophages has been investigated. The role of pharmacological inhibition of PPAR γ in macrophage-induced mycobacterial killing was investigated. Pretreatment with the selective PPAR γ antagonist, GW9662, significantly enhanced the capacity of macrophages to kill BCG, as determined by live/dead bacterial staining assessed by flow cytometry [14]. The role of PPAR γ in modulating intracellular bacterial killing was later confirmed by silencing PPAR γ in human macrophages and subsequently infecting the cells with *M. tuberculosis*. Following PPAR γ knockdown, macrophages had significantly better ability to control *M. tuberculosis* growth, as assessed by colony forming assays [15]. The increased control of mycobacterial infection was concomitant with an increase in TNF- α production [15] and a decreased formation of lipid droplets [14], providing evidence that mycobacterial-induced PPAR γ is an important mechanism in favoring mycobacterial growth in macrophages, at least partly through transcriptional regulation of inflammatory cytokines and lipid metabolism. This finding also suggests that lipid droplets may play a role in the pathogenesis of mycobacterial infection via PPAR γ expression and activation dependent mechanisms.

Collectively, these findings indicate that mycobacteria utilize PPAR γ signaling as an escape mechanism that enables survival within the hostile environments of macrophages.

6. Concluding Remarks and Perspectives

Recent studies have begun to shed light on the roles of PPAR γ in mycobacterial infection. Studies on PPAR γ expression and function have revealed that this transcription factor is highly upregulated during intracellular pathogen infection in which PPAR γ plays roles in host cell metabolism and downregulating the host immune response to favor pathogen burden, thereby suggesting that pathogens may stimulate PPAR γ activity as an escape mechanism (Figure 1). Accordingly, inhibition of PPAR γ activity leads to increased mycobacterial killing and infection control, and as such, PPAR γ is emerging as an attractive target candidate for therapeutic intervention strategies.

Although great advances in the understanding of the mechanisms of pathogen-induced PPAR γ expression and its roles in lipid metabolism and inflammatory mediator production have been achieved, critical questions on intracellular pathogen infection remain. Future studies in animal models, as well as clinical studies, will be necessary to characterize the role of PPAR γ in the pathogenesis of tuberculosis and as a target for therapeutic intervention.

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Research Article

Prenatal Rosiglitazone Administration to Neonatal Rat Pups Does Not Alter the Adult Metabolic Phenotype

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Prenatally administered rosiglitazone (RGZ) is effective in enhancing lung maturity; however, its long-term safety remains unknown. This study aimed to determine the effects of prenatally administered RGZ on the metabolic phenotype of adult rats. *Methods.* Pregnant Sprague-Dawley rat dams were administered either placebo or RGZ at embryonic days 18 and 19. Between 12 and 20 weeks of age, the rats underwent glucose and insulin tolerance tests and *de novo* fatty acid synthesis assays. The lungs, liver, skeletal muscle, and fat tissue were processed by Western hybridization for peroxisome proliferator-activated receptor (PPAR) γ , adipose differentiation-related protein (ADRP), and surfactant proteins B (SPB) and C (SPC). Plasma was assayed for triglycerides, cholesterol, insulin, glucagon, and troponin-I levels. Lungs were also morphometrically analyzed. *Results.* Insulin and glucose challenges, *de novo* fatty acid synthesis, and all serum assays revealed no differences among all groups. Western hybridization for PPAR γ , ADRP, SPB, and SPC in lung, liver, muscle, and fat tissue showed equal levels. Histologic analyses showed a similar number of alveoli and septal thickness in all experimental groups. *Conclusions.* When administered prenatally, RGZ does not affect long-term fetal programming and may be safe for enhancing fetal lung maturation.

1. Introduction

Peroxisome proliferator-activated receptor (PPAR) γ is a ligand-activated transcription factor that belongs to the superfamily of nuclear hormone receptors [1]. Several studies have evaluated the role of PPAR γ in lung maturation, demonstrating its critical significance in stimulating the alveolar epithelial-mesenchymal paracrine signaling pathway [2–5]. Recent studies have also shown that PPAR γ agonists such as rosiglitazone (RGZ) significantly enhance lung maturation when administered antenatally. Its efficacy in enhancing pulmonary maturation and neonatal and long-term safety following postnatal administration has also been demonstrated recently [5, 6]. In those studies, lack of any

significant impact on the neonatal and long-term metabolic profile of the exposed offspring was demonstrated [5, 6]. However, data on the long-term effects of RGZ are sparse, and to date no study has examined the effects of RGZ on the metabolic profile of *adult* rats when administered prenatally.

Despite the morbidity and mortality associated with bronchopulmonary dysplasia (BPD), there are no effective pharmacologic preventive or therapeutic options available. Antenatal steroid administration is the standard of care for augmenting pulmonary maturity in the presence of imminent premature labor [7, 8]. However, steroids have both limitations and concerning side effects [9]. Given that antenatal PPAR γ administration enhances lung maturation and may be an alternative to antenatal steroids, it is critically

important to determine its long-term safety before this treatment modality can be considered for human use. Therefore, we wanted to determine the adult metabolic profile and lung structure of adult rats exposed to RGZ antenatally and compare these to the metabolic profile and lung structure of rats exposed to dexamethasone antenatally. To accomplish this, we utilized a previously described animal model to study the effects of prenatally administered RGZ on markers of lung maturation and the metabolic programming [5, 6].

Based on previous studies, we hypothesized that a PPAR γ agonist given prenatally to accelerate lung development would not significantly alter the metabolic profile or phenotype [10]. Given the known effects of PPAR γ agonists on the regulation of insulin and lipid metabolism, we examined the effects of antenatal RGZ on the basic metabolic profile by measuring body weight, glucose and insulin tolerance tests, *de novo* fatty acid synthesis, plasma troponin-I, cholesterol, triglycerides, insulin, and glucagon levels [11–13]. Lung maturation in adult animals was assessed by examining the expression of surfactant proteins B (SPB) and C (SPC), PPAR γ and ADRP, key alveolar epithelial, and mesenchymal molecular markers [5, 14]. Lung morphometry was assessed by determining radial alveolar counts and septal thickness.

2. Methods

Pathogen-free, time-mated, first-time pregnant Sprague-Dawley rats (285–295 g) were obtained at day 16 of gestation (day 21 = term). They were allowed food and water *ad libitum* in a humidity- and temperature-controlled room on a 12-h : 12-h-light : dark cycle. Rats were assigned to each of the 4 treatment groups, receiving either diluent, (cottonseed oil), 0.3 mg/kg of RGZ (Cayman Chemicals, Ann Arbor, MI), 3 mg/kg of RGZ, or 0.25 mg/kg of dexamethasone (Dexa) intraperitoneally (i.p.). The diluent, RGZ or Dexa, was administered using a microsyringe in 100 μ L volumes injected i.p. once daily on gestational days 18 and 19, 24 hours apart, for a total of two doses each. On day 22 of pregnancy, the dams delivered spontaneously. A total of 33 pups from 4 litters (for each study group), with a minimum of 2 males and 3 females in each group were studied. Pups were breast-fed *ad libitum* and then weaned to rat chow on postnatal day 21. Glucose tolerance and insulin tolerance tests were performed at 12 weeks of age. To perform these studies, either glucose or insulin was administered after an overnight fast. At 20 weeks, the left lungs were collected and flash-frozen for later Western hybridization to determine the expression of PPAR γ , ADRP, SPB, and SPC. Right lungs were inflated with saline at a pressure of 20 cm H₂O, after which the trachea was immediately ligated to maintain inflation. Subsequently, the lungs were stored in 30% dextrose for 2 weeks after which they were embedded in paraffin for further sectioning, H&E staining, and light microscopy. Liver, muscle, and perinephric fat were also collected and flash-frozen to determine the effects of prenatal RGZ on the expression of PPAR γ and ADRP, a downstream target of PPAR γ . Peripheral blood was collected

and stored at -80°C for later determination of cholesterol, triglyceride, glucagon, insulin, cardiac troponin, and fatty acids. In a subset of animals ($n = 6$ for each group; 3M : 3F), at 19 weeks *de novo* fatty acid synthesis and incorporation into tissues were analyzed by deuterium (D₂O) labeling and mass spectrometry, as previously described [15]. Briefly, animals received deuterated water (99.9%) prepared in normal saline in an amount equal to $\sim 4\%$ of body weight and administered intraperitoneally and then were given free access to drinking water containing 6% D₂O for 7 days. At the end of the experimental period (~ 20 weeks of age), the animals were sacrificed using 0.1 mL euthasol (= 39 mg pentobarbital sodium, Virbac AH, Ft. Worth, TX) per rat. All animal procedures were performed following the guidelines of the National Institutes of Health for the care and use of laboratory animals and approved by the Los Angeles Biomedical Research Institute, Animal Care and Use Committee.

2.1. Western Blot Analysis. Western analysis was performed as described previously [5]. The primary antibodies used included SPB, SPC (1 : 500, Santa Cruz Biotechnology Inc., Santa Cruz, CA), PPAR γ (1 : 2000, Alexis Biochemicals, San Diego, CA), and ADRP (1 : 500, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blots were subsequently stripped and reprobed with anti-GAPDH antibody (1 : 10,000, Chemicon, Temecula, CA), and the protein values were normalized to the amount of GAPDH as an internal control.

2.2. Glucose Tolerance Test and Insulin Tolerance Test. Either glucose (1 g/kg body wt, intraperitoneal) or insulin (1 unit/kg, subcutaneous) was administered after an overnight fast. Serum glucose levels were assayed at different time points (0, 15, 30, 60, 120, and 180 minutes) using a glucometer (Home Diagnostics, Fort Lauderdale, FL), according to the manufacturer's protocol.

2.3. Cholesterol and Triglyceride Assays. Cholesterol and triglyceride levels were determined using the RAICHEM kit (Cliniqa Corporation, San Marcos, CA, with a dynamic range of 0–600 mg/dl, an intra-assay coefficient of variation of 1.7%), and the Cayman kit (Cayman Chemical Company, Ann Arbor, MI, dynamic range of 0–200 mg/dl, and intra-assay coefficient of variation of 1.34%), respectively, following the manufacturer's protocol.

2.4. Plasma Insulin and Glucagon. Plasma insulin was measured using an ELISA kit (detection limit of 0.2 ng/mL and 100% specificity) and glucagon was measured via an RIA kit (detection limit of 20 pg/mL and cross-reactivity with oxyntomodulin : $<0.1\%$) purchased from Linco (Linco Research, St. Charles, MO).

2.5. Measurement of Plasma Cardiac Troponin-I Levels. Determination of cardiac troponin-I levels was done based on a rat cardiac Troponin-I ELISA kit as per the manufacturer's protocol (Cat. no. 2010-2-HSP, Life Diagnostics,

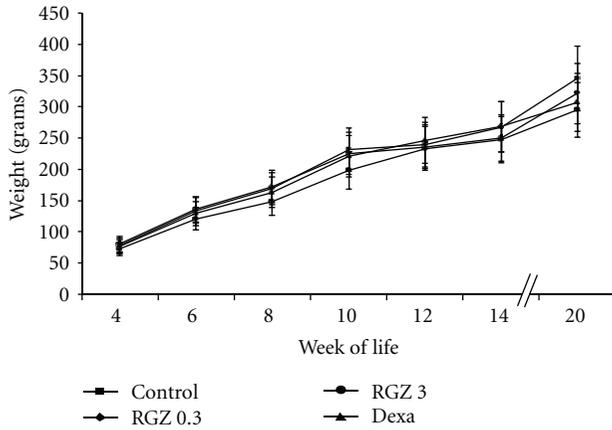


FIGURE 1: Effect of RGZ on postnatal weight gain. RGZ was administered prenatally at doses of 0.3 mg/kg and 3 mg/kg i.p. $\times 2$ doses. Another group was treated prenatally with i.p. dexamethasone (Dexa, 0.25 mg/kg) $\times 2$ doses. Weight was recorded every 2 weeks starting at week 4 until week 14 and on the day of sacrifice. There were no significant differences between study groups throughout the observation period.

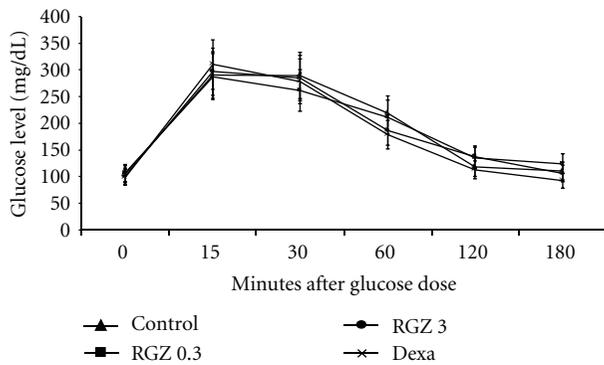


FIGURE 2: Effect of RGZ on glucose tolerance test (GTT). At 12 weeks of age, glucose was administered at 1 g/kg body weight i.p. after an overnight fast. Glucose was assayed at time = 0 (baseline), 15, 30, 60, 120, and 180 minutes following glucose administration. There were no significant differences ($P > 0.05$) in timed serum glucose values in the treated groups compared with controls during the GTT.

West Chester, PA) (detection limit of 0.156 ng/mL and 100% specificity).

2.6. Fatty Acid Analysis. *De novo* fatty acid synthesis was analyzed by deuterium labeling, followed by mass spectrometry, as previously described [5, 15].

2.7. Histologic Analysis. Lung morphometry was performed following previously described methods [10].

2.8. Statistical Analysis. Analysis of variance and two-tailed Student's *t*-test with Bonferroni correction for multiple comparisons were used to analyze the experimental data. *P* values < 0.05 were considered to be statistically significant.

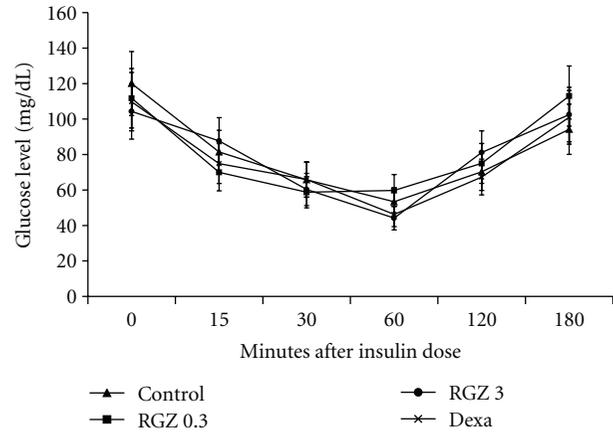


FIGURE 3: Effect of RGZ on insulin tolerance test (ITT). At 12 weeks of age, insulin was administered at 1 unit/kg body weight subcutaneously after an overnight fast. Glucose was assayed at time = 0 (baseline), 15, 30, 60, 120, and 180 minutes following insulin administration. There were no significant differences in serum glucose values between the treated and control groups during any of the time points of the ITT ($P > 0.05$).

3. Results

3.1. Effect of RGZ on Body Weight. A total of 33 pups (8-9/group) were studied in each group. There were no significant differences in birth weight of pups from each experimental group. Body weight was determined every 2 weeks as an overall measure of growth and metabolism starting on day 30 of life until week 14 and at sacrifice. We found no significant differences ($P > 0.05$) in body weight among the treatment groups at all time-points examined (Figure 1).

3.2. Effect of RGZ on Glucose and Insulin Tolerance. Glucose and insulin tolerance tests showed no significant differences in glucose values among the different groups at all time-points examined (Figures 2 and 3).

3.3. Effect of RGZ on Insulin, Glucagon, and Cardiac Troponin Levels. Table 1 shows that there were no significant differences in insulin, glucagon, or cardiac troponin-I levels among any of the groups, ($P > 0.05$ for all).

3.4. Effect of RGZ on Blood Cholesterol and Triglyceride Levels. Table 1 shows no significant differences in plasma cholesterol and triglyceride levels in the control group versus the RGZ or Dexa-treated group, ($P > 0.05$ for all).

3.5. Effect of RGZ on Fatty Acid Synthesis. Analyses of *de novo* fatty acid synthesis and their incorporation into tissues at 19 weeks showed that the fraction of *de novo* synthesized palmitate molecules in the RGZ- and Dexa-treated groups were comparable to the control group (Table 2).

TABLE 1: Insulin, glucagon, lipids, and troponin measurements. Plasma samples taken at 20 weeks for metabolic analyses showed there were no significant differences ($P > 0.05$) in insulin, glucagon, lipids, and troponin measurements in RGZ- and dexamethasone-treated groups compared with controls. Values are mean \pm SD. $N = 24$ (6 in each group).

Treatment group	Insulin (ng/mL)	Glucagon (pg/mL)	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Troponin (ng/mL)
Control	2.3 \pm 1.7	217.3 \pm 12	33.2 \pm 15	94.4 \pm 15	0.18 \pm 0.18
RGZ 0.3 mg/kg	2.6 \pm 1.4	190.6 \pm 6	35.6 \pm 6	105.1 \pm 15	0.16 \pm 0.23
RGZ 3 mg/kg	2.7 \pm 0.9	208.2 \pm 23	32.5 \pm 23	108.9 \pm 14	0.10 \pm 0.08
Dexamethasone 0.25 mg/kg	2.3 \pm 1.4	207.8 \pm 13	30.3 \pm 13	95.8 \pm 21	0.13 \pm 0.13

TABLE 2: Effect of RGZ on fatty acid synthesis. At 19 weeks, the fraction of *de novo* lipogenesis and incorporation into the tissues were analyzed by deuterium labeling and mass spectrometry. There were no significant differences ($P > 0.05$) in the fraction of *de novo* synthesis of palmitate molecules in the RGZ- and dexamethasone-treated groups compared with the controls. m1 = fraction of isotopomer molecules with one deuterium substitution, m2 = fraction of isotopomer molecules with two deuterium atoms. $N = 24$ (6 in each group).

Group	m2/m1	Deuterium enrichment (%)	Fraction of new palmitate molecules
Control	0.36	0.04	0.28
RGZ 0.3 mg/kg	0.41	0.04	0.28
RGZ 3 mg/kg	0.35	0.04	0.25
Dexamethasone 0.25 mg/kg	0.37	0.04	0.30

3.6. *Effect of RGZ on Alveolar Differentiation.* Western blot analysis for SPB, SPC, PPAR γ , and ADRP on protein lysates from whole lung samples from different groups showed that when compared to control, Dexa- and RGZ-treated groups had no significant effect on the expression of all the molecular markers probed ($P > 0.05$ for all, Figure 4).

3.7. *Effect of RGZ on PPAR γ and ADRP Expression in Liver, Muscle, and Perinephric Fat.* Figure 5 shows Western Blot results for the extrapulmonary PPAR γ - and ADRP-expressing tissues (liver, muscle, and perinephric fat) examined. There were no significant differences in PPAR γ and ADRP protein levels among the different treatment groups when compared with controls ($P > 0.05$ for all).

3.8. *Lung Histology.* Morphometric analysis showed no significant differences in septal thickness and alveolar count between the control, Dexa, and RGZ-treated groups ($P > 0.05$, Figure 6).

4. Discussion

In view of the increasing survival of extremely low birth weight infants and the accompanying increased prevalence of BPD, it is imperative that we find optimal preventive and therapeutic interventions to decrease the morbidities and mortality associated with this condition [16]. At present, the standard of care to augment lung maturity during imminent premature delivery is antenatal steroid administration;

however, evidence suggests steroids may increase the risk for significant adverse effects like altered neuronal development [17]. Despite the necessity to find an optimal treatment for lung immaturity, extensive research in the field has not succeeded in finding such an alternative to antenatal steroids. In the last decade, the possibility of using PPAR γ agonists to enhance lung maturation and promote lung injury repair has been explored [1–3]. In addition, our laboratory has shown that in the developing lung PPAR γ agonists can prevent lung injury induced by infection, nicotine, or hyperoxia [18, 19]. Similarly, a recent study by Garg et al. has provided evidence that early postnatal administration of PPAR γ agonists can reverse the effects of growth restriction [20].

Regardless of its evident efficacy, the long-term safety of prenatally administered PPAR γ agonists is unknown. Our present study is the first to examine the long-lasting molecular effects of prenatally administered RGZ, a potent PPAR γ agonist. Our results demonstrate that all of the metabolic parameters examined did not change, and RGZ did not alter the adult phenotype of our experimental groups compared with controls. Given RGZ's known effects on insulin and fat metabolism, we determined the body weight patterns across all study groups and observed no significant differences in growth rate and adult weight at 20 weeks of age [12, 19]. Since PPAR γ activation regulates the transcription of insulin-responsive genes involved in the metabolism of glucose, we also studied the effects of RGZ on glucose and insulin tolerance as well as glucagon and insulin levels in adults following prenatal RGZ administration [21]. We found that RGZ did not affect either the glucose or insulin tolerance tests, nor the serum insulin or glucagon levels in any of the experimental groups.

Given that PPAR γ -related genes are involved in the regulation of lipid metabolism and have effects on the lipid profile, we also determined serum cholesterol and triglyceride levels, as well as *de novo* fatty acid synthesis, among the experimental groups and found no alterations in either serum triglyceride or cholesterol levels when compared to non-treated animals [11]. Results of mass spectrometric analyses did not show alterations in the rate of *de novo* fatty acid synthesis in the experimental groups.

Rosiglitazone is widely used in the adult population for the treatment of hyperglycemia in diabetes [21, 22]. Recent reports have associated RGZ at a dose of 4 mg twice daily for a period of 20 weeks with an elevated risk of cardiovascular events in this population [23]. We measured cardiac troponin due to its well-established validity as a marker for cardiac injury and to allow for comparison with

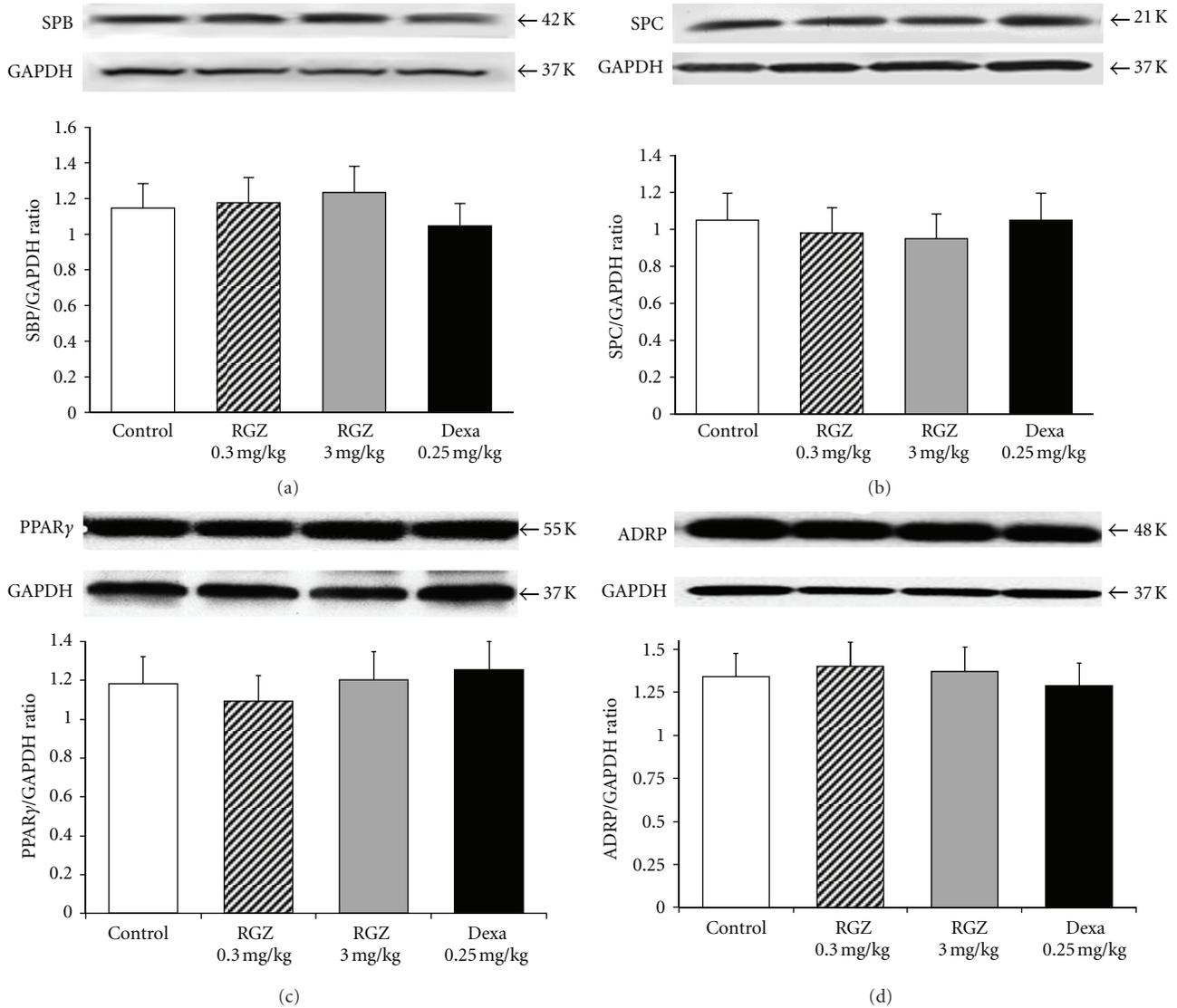


FIGURE 4: Effect of RGZ on SPB, SPC, PPAR γ , and ADRP expression in lung. Utilizing Western blot, SPB, SPC, PPAR γ , and ADRP levels were determined in lung lysates. There were no significant differences ($P > 0.05$) in protein levels of SPB (a), SPC (b), PPAR γ (c), or ADRP (d) in lung, in the RGZ- and dexamethasone- (Dexa-) treated groups compared with the controls. Representative Western blots and the corresponding density histograms are shown ($n = 4$ in each group).

previous data on rat cardiac function studies [24]. Our study did not reflect any differences in troponin-I levels among the study groups. In contrast to the human data, the absence of cardiotoxicity of RGZ is probably due to much shorter and lower doses used in our animal study compared to much greater exposure in adults (2 doses in our study versus 280 doses in the adult studies).

In addition, we measured the effect of antenatally administered RGZ on the expression of SPB, SPC, PPAR γ , and ADRP (the downstream target of PPAR γ) in the lung, and in selected extrapulmonary PPAR γ -expressing tissues such as the liver, adipose tissue, and muscle. Our results show that when compared to controls, there were no significant differences in the expression of SPB, SPC, PPAR γ , or ADRP in either the pulmonary or extra-pulmonary tissues

examined. Lastly, morphologic studies did not show any differences in the septal thickness and number of alveoli between the experimental and control groups.

In summary, long-term followup after prenatal administration of RGZ showed no effects on body weight, insulin and glucagon tolerance tests as well as on insulin, glucagon, triglyceride, cholesterol, or troponin-I levels. In addition, RGZ did not have any effects on fatty acid synthesis or lung morphology, suggesting absence of any long-term metabolic or pulmonary effects following antenatal exposure.

Among the various thiazolidinediones, RGZ was selected for this study based on extensive clinical experience of others and our studies on its role in perinatal lung maturation [5, 25–27]. The results of this study should be interpreted with caution since given the small sample size, the possibility

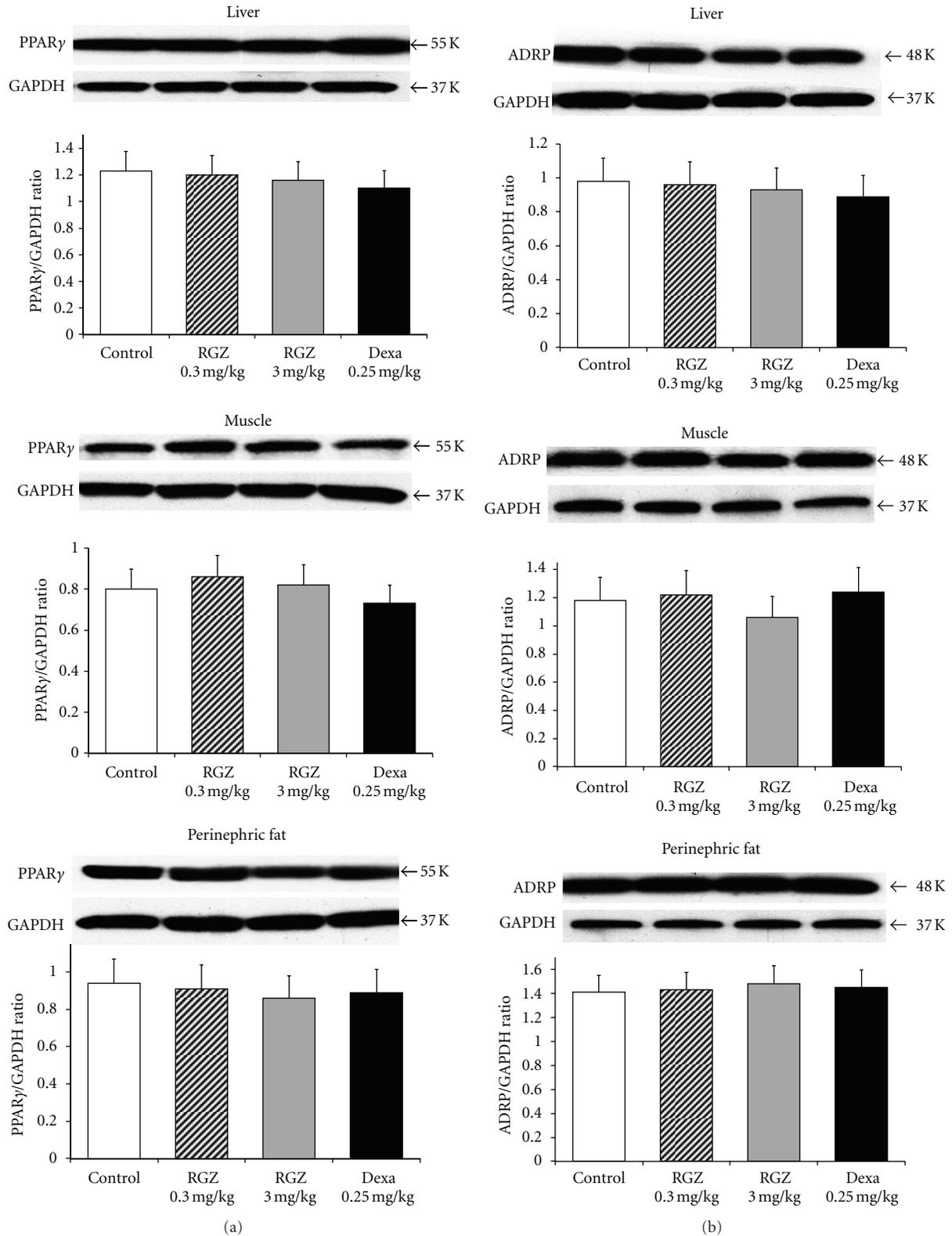


FIGURE 5: Effect of RGZ on PPAR γ and ADRP expression in liver, skeletal muscle, and perinephric fat. Utilizing Western blot assay, PPAR γ (a) and ADRP (b) protein levels were examined in the whole tissue lysates of liver, skeletal muscle, and perinephric fat. There were no significant differences ($P > 0.05$) in the protein levels of PPAR γ and ADRP in the liver, skeletal muscle, or perinephric fat, normalized to GAPDH, in the treated groups compared with the control. Representative Western blots and the corresponding density histograms are shown ($n = 4$ in each group).

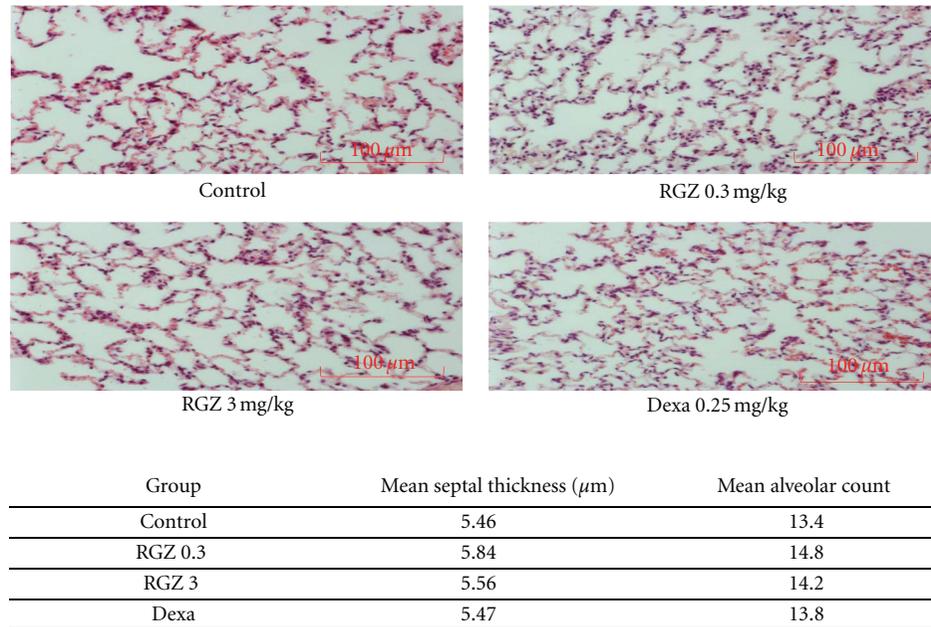


FIGURE 6: H&E staining of lung samples showed no significant differences in the alveolar number and septal thickness at 20 weeks, between control and treated groups. Light microscopy images of the sections at 20x magnification are shown. $P > 0.05$ for all groups and $n = 4$ in each group.

of a type II error cannot be ruled out. However, the promising benefits of thiazolidinediones at the doses used in our studies and the favorable long-term results in the present study strengthen the argument for the use of PPAR γ agonists as an effective and safe alternative for the prevention of BPD.

5. Conclusions

RGZ is an effective intervention in the enhancement of lung maturity and the promotion of lung injury repair. Long-term followup of antenatally treated subjects in our study did not show any changes in their metabolic profile or in their phenotype, suggesting that PPAR γ agonists are a safe alternative for the prevention and treatment of BPD. Though human studies have shown increased cardiovascular risk associated with RGZ, such adverse effects were not seen in this study, probably due to very different dosing regimens [23, 28]. RGZ is a prototype for the thiazolidinedione group of drugs and our results possibly demonstrate a beneficial class effect suggesting the need for pharmacokinetic and pharmacodynamic studies in humans with the goal of developing this class of drugs as an effective and safe alternative to enhance fetal lung maturation.

Acknowledgments

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Research Article

The Effect of PPAR Agonists on the Migration of Mature and Immature Eosinophils

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PPAR γ agonists can either enhance or inhibit eosinophil migration, which is a sum of directional migration (chemotaxis) and random cell movement (chemokinesis). To date, the effects of PPAR agonists on chemokinesis have not been examined. This study investigates the effects of PPAR α , δ , and γ agonists on eosinophil migration and chemokinesis. Eosinophils purified from blood of atopic donors were preincubated with rosiglitazone (PPAR γ agonist), GW9578 (PPAR α agonist), GW501516 (PPAR δ agonist), or diluent. The effects of PPAR agonists were examined on eosinophil chemokinesis, eotaxin-induced migration of eosinophils, and migration of IL-5R α + CD34+ cells. Expressions of CCR3, phospho-p38, phospho-ERK, and calcium release were also measured in eosinophils after rosiglitazone treatment. Low concentrations of rosiglitazone, but not GW9578 or GW501516, increased chemokinesis of eosinophils ($P = 0.0038$), and SDF-1 α -induced migration of immature eosinophils ($P = 0.0538$). Rosiglitazone had an effect on eosinophil calcium flux but had no effect on expression of CCR3 or phosphorylation of p38 or ERK. In contrast, high concentrations of rosiglitazone inhibited eosinophil migration ($P = 0.0042$). The effect of rosiglitazone on eosinophil migration and chemokinesis appears to be through modification of calcium signaling, which alludes to a novel PPAR-mediated mechanism to modulate eosinophil function.

1. Introduction

Eosinophils are effector cells which contribute to the pathology of allergic diseases [1]. They are recruited from the blood into inflamed tissue by local release of chemokines [2, 3]. Eotaxin-1, which is one of the most potent eosinophil chemokines, signals through chemokine receptor 3 (CCR3). Novel approaches toward inhibiting migration of effector cells such as eosinophils are being investigated for treatment of allergic asthma.

The peroxisome proliferator-activated receptors (PPARs) are metabolite-activated transcription factors that have been shown to regulate metabolic and inflammatory responses [4]. There are three identified subtypes of PPARs: PPAR α [5], PPAR γ [6], and PPAR δ [7], which have attracted interest as therapeutic targets in lung disease due to their preferential expression on human airway smooth muscle [8] and inflammatory cells [9] and increased expression

during inflammatory events [10, 11]. In murine models of allergic asthma, PPAR α and PPAR γ agonists (rosiglitazone and GW9578, resp.) inhibit eosinophil influx to the lung following airway antigen challenge [12, 13]. The PPAR γ agonist was more effective than the PPAR α agonist, while the PPAR δ agonist had no effect [13].

At low concentrations, however, PPAR agonists have been shown to enhance eosinophil migration *in vitro* [14]. The observed enhancement in eosinophil migration could be due to an increase in chemotaxis or chemokinesis [15], and the current study will compare the effects of low concentrations of the PPAR agonists GW9578, GW501516, and rosiglitazone on eosinophil migration and chemokinesis.

2. Materials and Methods

2.1. Subjects. Blood was obtained from 10 male and 13 female, nonsmoking, atopic donors aged 19–60 years old.

Atopy was confirmed by skin prick testing. Subjects were not currently using steroidal or nonsteroidal anti-inflammatory medications or antihistamines. Samples of cord blood were obtained from the hospital delivery room from 6 individuals whose atopic status and medication use were not determined. The study was approved by the FHS/HHS Research Ethics Board, and subjects gave informed consent to participate.

2.2. Eosinophil Purification. One hundred mL of peripheral blood was collected from each subject into sodium heparin vacutainers for *in vitro* experiments. Peripheral blood was diluted with an equal volume of McCoy's 5A (Invitrogen Canada Inc., Burlington, ON, Canada), and eosinophils were purified using an AccuPrep density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) followed by a MACS column CD16+ neutrophil depletion (Miltenyi Biotec, Auburn, CA, USA). The purity of each eosinophil sample was determined with a CytoPrep stained for a cell differential count (Diff Quik; Siemens Healthcare Diagnostics, Deerfield, IL, USA). Light microscopy demonstrated that the eosinophil preparations were >90% pure and the majority of contaminating cells were neutrophils.

Purified eosinophils were resuspended in RPMI complete (10% Fetal Bovine Serum, 1 M HEPES in RPMI 1640) and incubated for 20 minutes with 0.1 nM–100 μ M of either a PPAR α agonist (GW9578; Cayman Chemical, Ann Arbor, MI, USA), a PPAR β/δ agonist (GW501516; Axxora LLC, San Diego, CA, USA), or a PPAR γ agonist (rosiglitazone; Cayman Chemical, Ann Arbor, MI, USA), all in a final concentration of 0.1% DMSO, or with diluent (RPMI complete in 0.1% DMSO). Treatment with PPAR agonists had no effect on eosinophil viability as determined by trypan blue exclusion, being >98% viable before and after incubation.

2.3. Eotaxin-Induced Eosinophil Migration. The migration assay used a 48-well Boyden chamber, following protocols from previous studies on eosinophil migration [16]. The PPAR agonists were left in the cell suspension, and the upper wells of the Boyden chamber were filled with 50 μ L of eosinophils at a concentration of 3×10^6 cells/mL. Eotaxin at a concentration of 10 nM (R&D Systems, Minneapolis, MN, USA) in the presence of equivalent concentrations of PPAR agonist (0.1 nM–100 μ M) was placed in the lower wells of the microchemotaxis assembly (Neuro Probe Inc., Gaithersburg, MD, USA). The upper and lower wells were separated by a nitrocellulose filter with an 8 μ m pore, and the chamber was incubated in high humidity for 90 minutes at 37°C. The chamber was then disassembled, and the nitrocellulose filter was fixed with mercuric chloride overnight and stained with hematoxylin and chromotrope 2R. The filter were dehydrated through graded alcohols followed by a 30-minute xylene treatment, then mounted on a glass slide and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA). The number of eosinophils was quantified from 10 random fields at the leading edge, counted from the underside of the filter using a light microscope at 400x magnification.

2.4. PPAR Agonist-Induced Eosinophil Chemokinesis. To determine the effects of PPARs on eosinophil chemokinetic responses, eosinophils were incubated with PPAR agonists (0.1 nM–100 μ M) for 20 minutes then loaded into the upper chamber with similar concentrations of the PPAR agonist in the lower chambers.

2.5. Eosinophil CCR3 Expression. Purified eosinophils were incubated with rosiglitazone at 0.1 nM or 10 nM or diluent for 110 minutes. The cells were then stained for CCR3 surface expression using mouse anti-human Pacific Blue-CD45 (eBioscience, San Diego, CA, USA), FITC-CD16 (Becton-Dickinson Biosciences, Mississauga, ON, Canada), and PE-CCR3 (Medical & Biological Laboratories, Naka-ku, Nagoya, Japan), as well as the isotype control antibodies for CCR3. Cells were acquired with an LSR II flow cytometer (Becton Dickinson Instrument Systems; Becton-Dickinson, Mississauga, ON, Canada) using the FACSDiva software program (Becton-Dickinson Biosciences). Fluorometric compensation was set to minimize autofluorescence, a known issue surrounding eosinophil and flow cytometry [17].

2.6. Eosinophil Migration—Signal Transduction Pathways. Purified eosinophils were treated with rosiglitazone at concentrations which was previously reported to induce cell migration (0.1 nM, 10 nM) [14] or with diluent for 110 minutes in the absence or presence of 10 nM eotaxin. After rosiglitazone treatment, eosinophils were lysed and the protein concentration was standardized using a Bradford assay. Phosphorylation of ERK1/2 and p38 was analyzed using signal transduction assay reaction (STAR) ELISA kits (Millipore, Temecula, CA, USA) and quantified by measuring the absorbance at 450 nm using an EL800 plate reader (BioTek Instruments, Winooski, VT, USA).

2.7. Eosinophil Progenitor Cell Transwell Migration. Cord blood was diluted with an equal volume of McCoy's 5A (Invitrogen Canada Inc.), and mononuclear cells were purified using an AccuPrep density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). Non-adherent mononuclear cells (NAMCs) were then resuspended in McCoy's 3+ (McCoy's 5A with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% 2-mercaptoethanol) and incubated for 2 hours in 5% CO₂ at 37°C and high humidity to remove monocytes. CD34+ progenitor cells were isolated using a MACS column CD34+ positive selection (Miltenyi Biotec, Auburn, CA, USA). The CD34+ progenitor cell preparations were >90% viable after isolation.

CD34+ progenitor cells were resuspended at 1×10^6 cells/mL in RPMI complete (10% fetal bovine serum, 1 M HEPES in RPMI 1640) with 100–1000 nM of PPAR α agonist (GW9578), PPAR β/δ agonist (GW501516), or PPAR γ agonist (rosiglitazone) agonist, all in a final concentration of 0.1% DMSO or diluent (RPMI complete in 0.1% DMSO). SDF-1 α (R&D Systems) at a concentration of 100 ng/mL in the presence of equivalent concentrations of PPAR agonists

(100–1000 nM) was placed in the lower wells of the transwell assembly. The chamber was incubated in high humidity for 18 hours at 37°C. The chamber was then disassembled, and the cells from the lower well were stained for CD34 and IL-5R α surface expression using mouse anti-human CD34-APC, CD45-FITC, and PE-IL-5R α (Becton-Dickinson Biosciences), as well as the isotype control antibodies for CCR3. Cells were acquired with an LSR II flow cytometer (Becton Dickinson Instrument Systems; Becton-Dickinson) using the FACSDiva software program (Becton-Dickinson Biosciences). Migration was expressed as % of total CD34+ cells plated.

2.8. Image Acquisition and Measurement of $[Ca^{2+}]_i$. Intracellular changes in calcium were measured using confocal microscopy, as previously described [18]. Briefly, isolated eosinophils (1×10^6 cells/mL) were loaded for 1 hr on ice with 3.5 μ M of a Ca^{2+} -sensitive fluorescent probe, fluo-3 AM (Invitrogen Canada) dissolved in dimethyl sulfoxide with 0.01% Pluronic F-127. The eosinophils were then loaded onto a culture dish and placed on the stage of a custom-built confocal microscope equipped with a 20x objective. The bathing solution for all experiments was RPMI, maintained at 37°C, which was exchanged constantly via superfusion throughout the experiment. Eosinophils were then illuminated using 488 nm light from a 20 mW photodiode laser (Coherent Technologies; CA, USA). During the recordings eosinophils were exposed to control (diluent or eotaxin) or treatment (diluent with 100 nM rosiglitazone or eotaxin with 100 nM rosiglitazone). Images (480 \times 640 pixels) were collected at 30 Hz with the imaging software “Video Savant” (IO Industries; London, ON, Canada); 9 consecutive frames were then averaged at 1.5 second intervals, giving a final image rate of 0.67 Hz. The image analysis software, “Scion” (Scion Corporation, Frederick, MD, USA), was used to determine the pixel intensity of 10 individual cells for measurement of $[Ca^{2+}]_i$. Fluorescence intensities of the regions of interest were saved and plotted against time. An increase in average fluorescence intensity was interpreted as an increase in $[Ca^{2+}]_i$, and an increase in the average frequency of $[Ca^{2+}]_i$ spikes was interpreted as an increase in $[Ca^{2+}]_i$ oscillations.

2.9. Statistical Analysis. All data are expressed as the mean \pm standard error unless otherwise stated. Statistical analyses were performed using Prism version 5 (GraphPad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) was used to compare PPAR agonist treatments versus diluent at the various doses, with post hoc Tukey tests for prespecified comparisons. For data not normally distributed, the statistics were performed on the log-transformed data. Statistically significant differences were accepted at $P < 0.05$.

3. Results and Discussion

3.1. Subjects. Blood donors that were recruited for the study were 31 ± 12 years old with mild blood eosinophilia ($3.9 \pm 2\%$ eosinophils).

3.2. Effects of PPAR Agonists on Eotaxin-Induced Eosinophil Migration. The chemokine eotaxin (10 nM) induced significant eosinophil migration compared to diluent control (242.9 ± 148.6 versus 46.1 ± 68.1 cells/10 HPF, $P < 0.0001$). PPAR agonists were tested at low concentrations ($n = 9$, Figures 1(a), 1(b), and 1(c)) and high concentrations ($n = 6$, Figures 1(a), 1(b), and 1(c)). Preincubation with 100 μ M rosiglitazone significantly inhibited eosinophil migration (Figures 1(a) and 1(e); $P = 0.0042$). By contrast, there was no effect of GW9578 ($P = 0.9$) or GW501516 ($P = 0.3$) on eotaxin-induced migration (Figures 1(b) and 1(c)).

Rosiglitazone treatment alone at a concentration of 100 nM both above and below the nitrocellulose filter in the micro-Boyden chamber assay significantly increased the eosinophil chemokinesis compared to diluent control ($P = 0.0038$; Figures 2(a) and 2(e)). In contrast, no chemokinetic responses were observed when eosinophils were incubated with equivalent concentrations of GW9578 (PPAR α agonist, $P = 0.9$) or GW501516 (PPAR δ agonist, $P = 0.9$) above and below the filter (Figures 2(b) and 2(c)), or with a high concentration of rosiglitazone (Figure 2(a), $P = 0.05$).

Cell migration is a multistep process, which involves both chemotaxis and chemokinesis in response to a chemokine [19]. Chemotaxis is defined as directed migration towards a chemokine, whereas chemokinesis is defined as non-directional migration. Chemokinesis, alone, is not sufficient for cell accumulation but may contribute considerably by priming a cell to respond more vigorously to a chemotactic stimuli [20]. Previously it was unknown whether the increase in eotaxin-induced migration by PPAR γ agonists was due to chemotaxis, chemokinesis, or both. Although PPAR agonists in this *in vitro* study are used at concentrations similar to the reported EC₅₀s those for rosiglitazone, GW9578, and GW501516 are 43 nM, 50 nM, and 1.1 nM, respectively [21–23], this study has determined that the enhanced migration by rosiglitazone is likely due to a chemokinetic effect. Such “priming” of cells may enhance their response to other stimuli, including chemokines such as eotaxin that are found in the microenvironment of allergic tissue, and thus lead to eosinophilia.

3.3. Effect of Rosiglitazone on Eosinophil CCR3 Surface Expression and Signaling. Incubation with rosiglitazone at 0.1 and 10 nM had no effect on eosinophil CCR3 surface expression (Table 1). Furthermore, eosinophil incubation with rosiglitazone in the presence or absence of eotaxin had no effect on the level of phosphorylation of ERK1/2 or p38 MAPK (Table 1).

To improve our understanding of how PPAR agonists regulate eotaxin-induced eosinophil migration, we investigated the effects of rosiglitazone on the surface expression and downstream signalling of the eotaxin receptor, CCR3. Consistent with studies of other PPAR γ agonists, 15d-PGJ₂ and troglitazone [14], which were studied at similar concentrations, we observed no effect of rosiglitazone on the level of cell surface expression of CCR3 on eosinophils or on phosphorylation of the downstream signaling molecules ERK1/2 and p38.

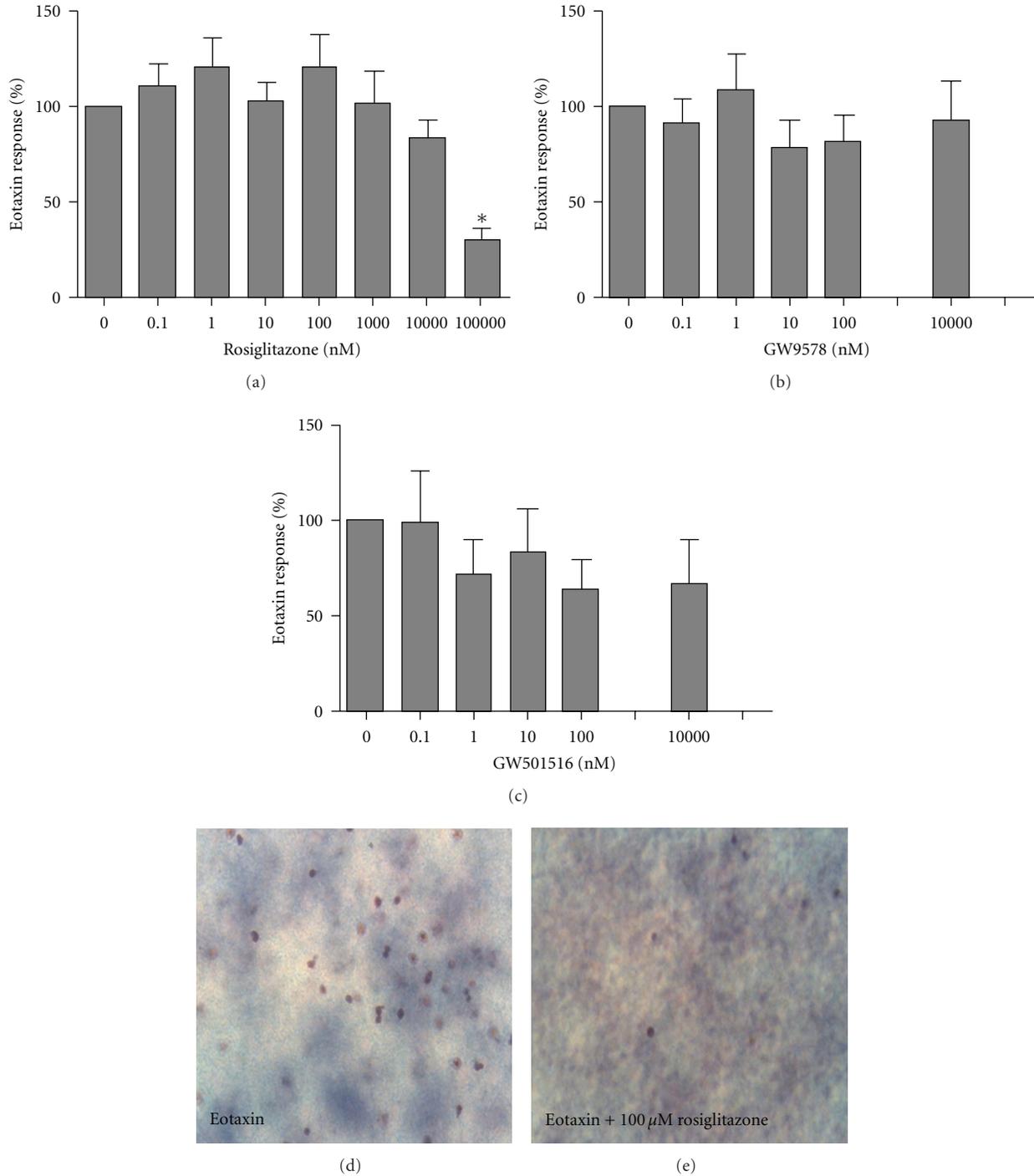


FIGURE 1: The effect on eotaxin-induced eosinophil migration of agonists to PPAR γ ((a) rosiglitazone), PPAR α ((b) GW9578), and PPAR δ ((c) GW501516) at low (0.1–100 nM; 9 subjects) and high concentrations (1000–100,000 nM; 6 subjects). Representative pictures at 200x magnification of the leading edge of the nitrocellulose filter after incubation with eotaxin (d) and eotaxin with 100 μ M rosiglitazone (e). Data are shown as mean \pm SEM and expressed as % of the response to eotaxin.

3.4. Effects of PPAR Agonist on SDF-1 α -Induced Eosinophil Progenitor Cell Migration. Compared to diluent control, stromal cell-derived factor-1 α (100 ng/mL) induced migration of IL-5R α + CD34+ cells isolated from cord blood (8.2 \pm 6.2 versus 33.2 \pm 6.1% of total cells migrated,

$P = 0.0479$). Low concentrations of rosiglitazone increased the migrational response of cord blood-derived IL-5R α + CD34 cells to SDF-1 α compared to diluent control; however, this change did not reach the level of statistical significance (Figure 3; $P = 0.054$). No changes in migration of IL-5R α +

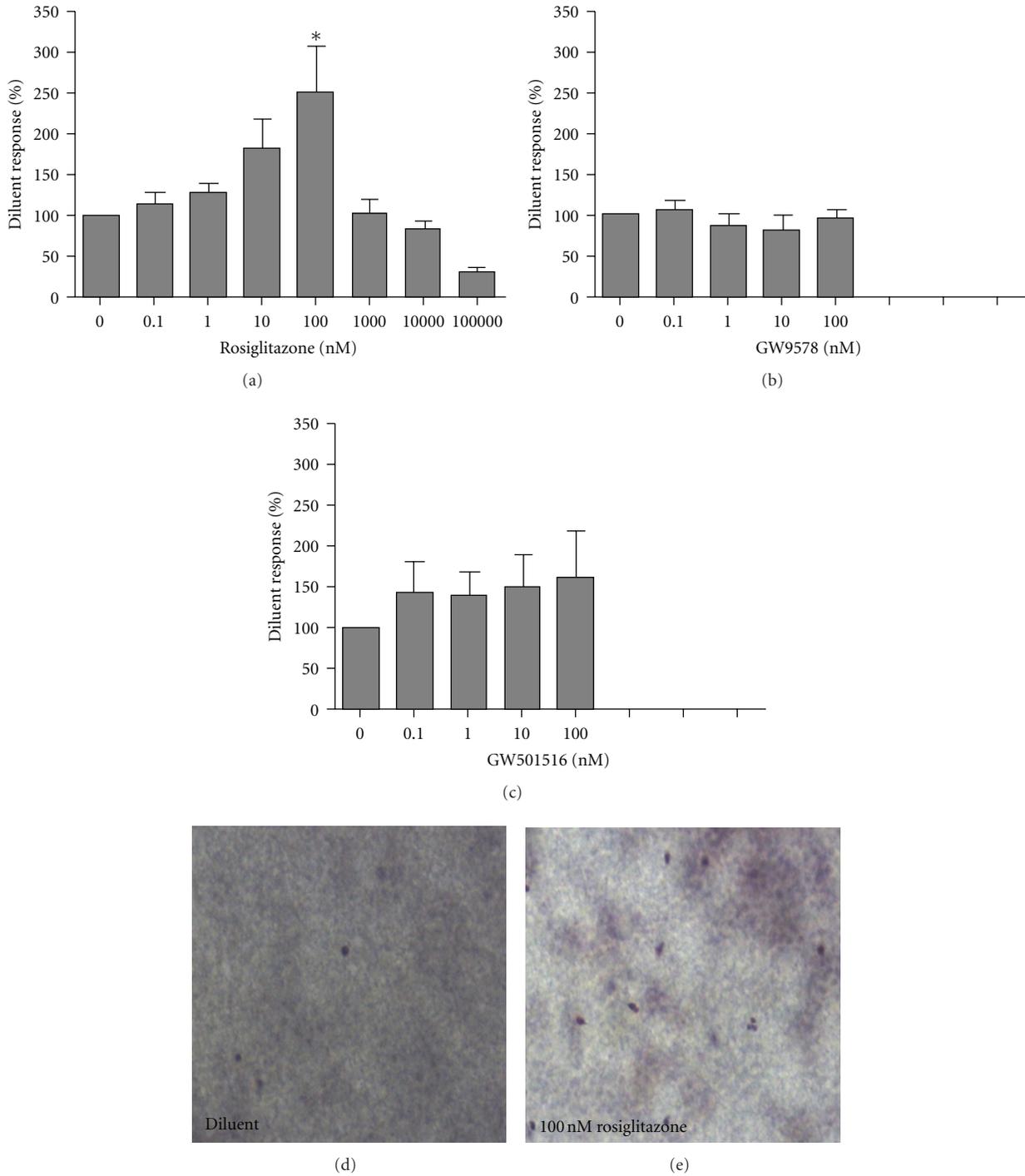


FIGURE 2: The effect on eosinophil chemokinesis of agonists to PPAR γ ((a) rosiglitazone), PPAR α ((b) GW9578), and PPAR δ ((c) GW501516) at low (0.1–100 nM; 9 subjects) and for PPAR γ (a; rosiglitazone) at high concentrations (1000–100,000 nM; 6 subjects). Representative pictures at 200x magnification of the leading edge of the nitrocellulose filter after incubation with diluent (d) and 100 nM rosiglitazone (e). Data are shown as mean \pm SEM and expressed as % of the response to diluent.

CD34+ cells were observed with equivalent concentrations of GW9578 (PPAR α agonist, $P = 0.8$) or GW501516 (PPAR δ agonist, $P = 0.6$) (data not shown).

We also examined the effect of PPAR agonists on the immature eosinophil population of IL-5R α + CD34 cells

purified from cord blood samples. We demonstrated that migration of these immature eosinophils in response to the potent chemoattractant SDF-1 α is likewise enhanced by pretreatment of low concentrations of rosiglitazone. This finding is novel and mirrors our and Koybayashi's [14]

TABLE 1: Eosinophil expression of CCR3 and phosphorylation of ERK1/2 and p38 after treatment with the PPAR γ agonist rosiglitazone.

		Diluent	0.1 nM	10 nM	P value
CCR3 (%)		29.3 \pm 7.5	23.5 \pm 5.7	19.8 \pm 4.7	0.1
CCR3 (MFI)		8.3 \pm 4.4	6.8 \pm 3.3	5.4 \pm 2.8	0.2
Phospho-ERK1/2 (units/mL)	Diluent	3.5 \pm 0.7	3.3 \pm 0.6	3.3 \pm 0.5	0.6
	Eotaxin	3.75 \pm 0.2	3.93 \pm 0.4	3.95 \pm 0.4	0.8
Phospho-p38 (units/mL)	Diluent	3.9 \pm 1	4.8 \pm 1.3	4.5 \pm 1.8	0.9
	Eotaxin	6.7 \pm 1.9	6.2 \pm 1.5	7.0 \pm 1.4	0.8

Data represent mean \pm SEM.

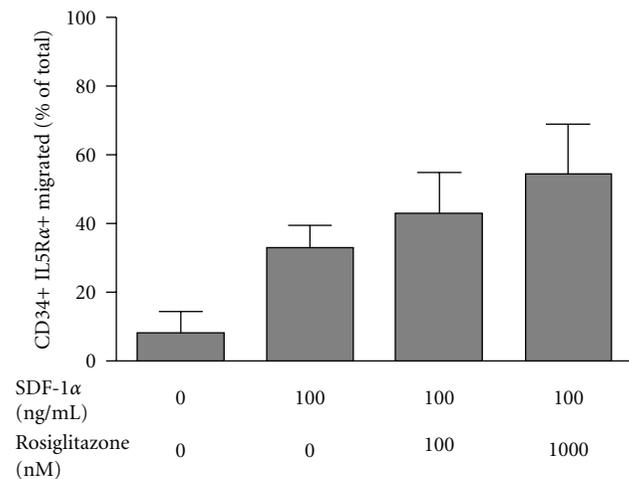


FIGURE 3: The effect of the PPAR γ agonist rosiglitazone on SDF-1 α -induced migration of IL-5R α + CD34+ cells isolated from cord blood (6 subjects). Data are shown as mean \pm SEM and expressed as a % of the total IL-5R α + CD34+ cells.

observations in mature eosinophils, of enhanced migration by low concentrations of PPAR γ agonist, with no effect of PPAR α and δ agonists.

3.5. Image Acquisition and Measurement of $[Ca^{2+}]_i$. Calcium flux was examined in 3 separate eosinophil preparations. Addition of eotaxin to eosinophil preparations caused an increase in maximum fluorescence intensity (Figure 4; diluent 16.6 \pm 6.2 versus eotaxin 66.7 \pm 27.4) and the number of calcium oscillations (diluent 0.007 \pm 0.004 Hz versus eotaxin 0.038 \pm 0.016 Hz). Treatment with 100 nM rosiglitazone consistently reduced the frequency of calcium oscillations observed after the addition of 10 nM eotaxin (control 0.038 \pm 0.016 Hz versus treatment 0.016 \pm 0.007 Hz). However, no consistent effect of rosiglitazone was observed on the maximum fluorescence intensity in the 3 preparations studied (control 66.7 \pm 27.4 versus treatment 58.1 \pm 25.2).

Previous studies have shown that pretreatment with a MEK inhibitor or a p38 MAPK inhibitor had no effect on enhanced migration nor did inhibition of the NF- κ B pathway or inhibition of genomic transcription with actinomycin D [14]. We and others have shown that PPAR agonists have

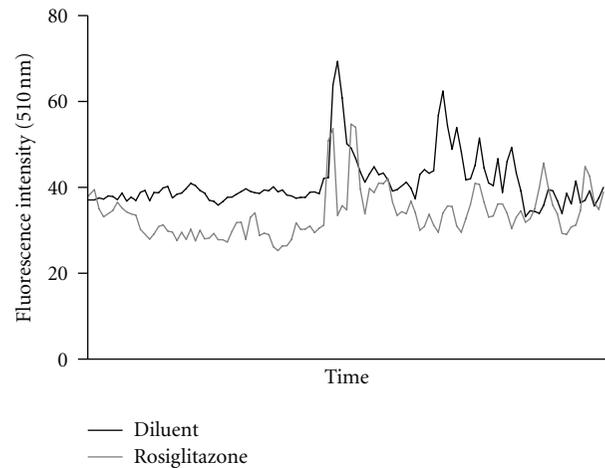


FIGURE 4: The effect of 100 nM rosiglitazone treatment on the eotaxin-induced increase in $[Ca^{2+}]_i$, measured by the frequency of calcium oscillations and maximum fluorescence intensity (representative tracing from 3 experiments).

a modulatory effect on eotaxin-induced calcium mobilization [14], which would suggest that PPAR agonists have downstream targets which have not been identified yet.

4. Conclusion

This is the first study to show increased chemokinesis of eosinophils *in vitro* at low concentrations of rosiglitazone, which has a 100-fold higher binding affinity for PPAR γ than troglitazone [24]. The results of this study also confirm previous observations showing that high concentrations of a PPAR γ agonist inhibit eosinophil migration [24].

We demonstrated that the PPAR γ agonist rosiglitazone, at a concentration of 100 nM significantly increased eotaxin-induced eosinophil chemokinesis *in vitro*. This finding supports the data demonstrating increased eosinophil migration reported by Kobayashi et al. [14]. We also demonstrated that rosiglitazone treatment had no effect on the level of cell surface expression of the eotaxin receptor, CCR3, on eosinophils, or on the downstream signalling events following eotaxin/CCR3 binding, such as phosphorylation of ERK1/2 and p38 MAPK, suggesting that the observed effects

of rosiglitazone are not related to alterations in signalling through the eotaxin receptor. At these same concentrations there was no effect of the PPAR α agonist, GW9578, or the PPAR δ agonist, GW501516.

In summary, at low concentrations the PPAR γ agonist rosiglitazone enhanced chemokinesis of eosinophils isolated from the peripheral blood of atopic subjects and also enhanced the migration of eosinophil progenitors. This chemokinetic effect may at least partially explain the enhancement of eosinophil migration seen at these concentrations. The enhanced chemokinesis was specific for the PPAR γ agonist, as there was no effect of PPAR α or PPAR δ agonists. In light of our findings that a selective PPAR γ agonist can enhance eosinophil chemokinesis at a concentration of 100 nM and decrease eosinophil migration at a concentration of 100 μ M, the therapeutic window for PPAR γ agonists as an anti-inflammatory therapy is narrow and dosage must be titrated carefully.

Acknowledgments

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Review Article

PPAR γ Signaling Mediates the Evolution, Development, Homeostasis, and Repair of the Lung

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Epithelial-mesenchymal interactions mediated by soluble growth factors determine the evolution of vertebrate lung physiology, including development, homeostasis, and repair. The final common pathway for all of these positively adaptive properties of the lung is the expression of epithelial parathyroid-hormone-related protein, and its binding to its receptor on the mesenchyme, inducing PPAR γ expression by lipofibroblasts. Lipofibroblasts then produce leptin, which binds to alveolar type II cells, stimulating their production of surfactant, which is necessary for both evolutionary and physiologic adaptation to atmospheric oxygen from fish to man. A wide variety of molecular insults disrupt such highly evolved physiologic cell-cell interactions, ranging from overdistention to oxidants, infection, and nicotine, all of which predictably cause loss of mesenchymal peroxisome-proliferator-activated receptor gamma (PPAR γ) expression and the transdifferentiation of lipofibroblasts to myofibroblasts, the signature cell type for lung fibrosis. By exploiting such deep cell-molecular functional homologies as targets for leveraging lung homeostasis, we have discovered that we can effectively prevent and/or reverse the deleterious effects of these pathogenic agents, demonstrating the utility of evolutionary biology for the prevention and treatment of chronic lung disease. By understanding mechanisms of health and disease as an evolutionary continuum rather than as dissociated processes, we can evolve predictive medicine.

“Those who cannot remember the past are condemned to repeat it.” George Santayana

1. Background

Normal lung development is the result of a functionally interconnected series of cell-molecular steps. This sequence of biologic events has been positively selected for evolutionarily over biologic time and space [1], resulting in optimal gas exchange mediated by alveolar homeostasis [2]. Elsewhere we have suggested that chronic lung disease (CLD) causes simplification of the lung in a manner consistent with the reversal of the evolutionary process [3, 4]. Therefore, by identifying those mechanisms that have evolved under selection pressure for optimal gas exchange [5], we have theorized that we can effectively reverse the deleterious effects of CLD by promoting the evolutionarily adaptive mechanism [6], rather than by just treating the symptoms [7]. By

determining the cell-molecular sequence of spatiotemporal signals that have evolved the lung over phylogeny and ontogeny, we can identify physiologically rational targets for effectively preventing and reversing the deleterious effects of endogenous and exogenous factors known to irreversibly damage normal lung development and function.

The ground-breaking tissue culture experiments conducted by Grobstein in 1967 demonstrating that lung development was dependent on endodermal-mesenchymal interactions [8] led to decades of research to determine the underlying cell-molecular mechanisms. The seemingly simple epithelial-mesenchymal interactions during well-defined (embryonic, pseudoglandular, canalicular, saccular, and alveolar), but overlapping stages of lung development result in more than 40 different cell types [9]. Much of what

we currently know about the mechanisms involved in lung development is derived from such studies of cultured lung cells signaling through growth factor-mediated pathways for proliferation and differentiation [10–12]. The discovery that epithelial-mesenchymal signaling induced the lipofibroblast via peroxisome proliferator-activated receptor gamma (PPAR γ) [13] gave rise to the hypothesis that normal lung development could be reconstituted [14] and recapitulated [15, 16]. The following recounts the essential role of PPAR γ in lipofibroblast differentiation and its exploitation for the effective treatment of the preterm lung.

2. Epithelial-Mesenchymal Interactions Generate Alveolar Lung Development

The paracrine growth factor model used to study the maturation of the pulmonary surfactant system and the etiology of CLD is shown in the accompanying schematic (see Figure 1, *steps 1–11*). Briefly, we have observed coordinating effects of stretch on alveolar type II (ATII) cell expression of parathyroid-hormone-related protein (PTHrP) and PGE₂ (Prostaglandin E₂) (*step 1*), the lipofibroblast PTHrP receptor (*step 2*), PPAR γ upregulation (*step 4*) via Protein Kinase A activation (*step 3*), its downstream effect on lipofibroblast ADRP (Adipocyte-Differentiation-Related Protein) expression (*step 5*) and triglyceride (TG) uptake by both the lipofibroblast and the ATII cell (*steps 6a and 6b*), and on the interaction between lipofibroblast-produced leptin (*step 7*) and the ATII cell leptin receptor (*step 8*), stimulating de novo surfactant phospholipid synthesis by ATII cells (*step 9*). The schematic depicts lipofibroblast-to-myofibroblast trans-differentiation (*step 10*) due to decreased PTHrP following exposure to hyperoxia, volutrauma, or infection. All of these effects are shown to be prevented by PPAR γ agonists (*step 11*).

These studies were originally fostered by Barry Smith's seminal observation [10] that glucocorticoids accelerate ATII cell surfactant synthesis by stimulating fibroblast synthesis of an oligopeptide that he termed Fibroblast-Pneumonocyte Factor (FPF). It was known at that time that lung, prostate, and mammary mesodermal development were under endocrine control. Importantly, it was shown that early signals emanated from the epithelium to differentiate the immature mesenchyme in the neighboring epithelium of the developing mammary gland [17]. Moreover, Brody's laboratory had shown that the developing lung fibroblast acquired an adipocyte-like phenotype [18–20], termed the lipid-laden fibroblast, leaving open the question as to whether these cells might be a source of lipid substrate for surfactant synthesis by the ATII cell. The Torday laboratory later discovered the physiologic significance of these lipid-laden fibroblasts by coculturing them with type II cells, which resulted in the rapid trafficking of the lipid from the fibroblast to the type II cell, and its highly enriched incorporation into specific surfactant phospholipids. These data indicated the existence of a specific mechanism for the recruitment of lipid substrate from the vasculature to the type II cell for de novo surfactant synthesis. This trafficking was even more robust when the cocultured cells were treated with glucocorticoids, which

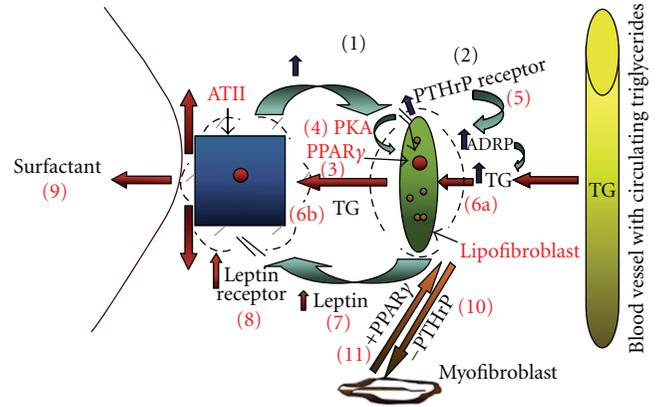


FIGURE 1: Schematic for paracrine determinants of alveolar homeostasis and disease.

are known to stimulate cell-cell interactions in the alveolus in association with increased surfactant synthesis, further reinforcing the notion of a putative mechanism for neutral lipid trafficking for surfactant synthesis since it appeared to be a regulated process [21].

Interestingly, the fibroblasts took up the neutral lipid, but did not release it unless they were in the presence of type II cells; conversely, the type II cells were unable to take up neutral lipid. These observations led to the discovery that type II cell secretion of prostaglandin E₂ (Figure 1, *step 1*), a stretch- and glucocorticoid-regulated mechanism, caused the active release of neutral lipid from the fibroblasts [22]. This effect was further stimulated by glucocorticoid treatment of the lung fibroblasts [22], but the nature of the lipid uptake mechanism by the type II cells remained unknown. Yet we were well aware that the synthesis of pulmonary surfactant was a so-called “on demand” system [23–25], in which increased alveolar distension resulted in increased surfactant production, suggesting the existence of a stretch-sensitive signal emanating from the type II cell. With this in mind, we began studying the role of PTHrP in lung development because (a) it was expressed in the embryonic endoderm [26], (b) its receptor was present on the adepithelial mesoderm [27], (c) it had been shown to be stretch regulated in the urinary bladder [28] and uterus [29], and distension of the lung was known to be of physiologic importance in normal lung development [30], (d) knockout of PTHrP caused stage-specific inhibition of fetal lung alveolarization in the transition from the pseudoglandular to the canalicular stage [31].

Early functional studies of PTHrP had shown that it was a paracrine factor that stimulated surfactant phospholipid synthesis [32], and that it was stretch regulated [11] (Figure 1, *step 1*). We subsequently discovered that PTHrP stimulated neutral lipid uptake by developing lung fibroblasts (Figure 1, *steps 1 and 2*), which we chose to call lipofibroblasts [33], by upregulating ADRP (Figure 1, *step 2*), a molecule previously shown to be necessary for lipid uptake and storage [34] (Figure 1, *step 5*). We subsequently found that ADRP was the factor necessary for the uptake of neutral lipid by the lipofibroblast (Figure 1, *step 6a*) and transit of neutral lipid from

the lipofibroblast to the ATII cell for surfactant phospholipid synthesis (Figure 1, *step 6b*) [35, 36]. The missing component for the PTHrP regulation of lung surfactant was the putative lipofibroblast paracrine factor that empirically stimulated ATII cell surfactant synthesis (32). Reasoning that lipofibroblasts were homologs of adipocytes, we hypothesized that lipofibroblasts, like fat cells, expressed leptin, which would bind to the type II cell and stimulate surfactant synthesis—we found that lipofibroblasts did indeed express leptin during rat lung development, plateauing immediately prior to the onset of surfactant synthesis by the type II cell, and that leptin stimulates ATII cell surfactant synthesis [37] (Figure 1, *step 7*). Importantly, from a mechanistic standpoint, we discovered that type II cells express the leptin receptor [38] (Figure 1, *step 8*), thus providing a ligand-receptor signaling pathway between the lipofibroblast and type II cell. Moreover, PTHrP was discovered to stimulate leptin expression by fetal lung fibroblasts [37] (Figure 1, *steps 1, 2 and 7*), thus providing an integrated, growth factor-mediated homeostatic paracrine loop for the synthesis of pulmonary surfactant, as predicted by the PTHrP-based model of lung development.

Since the major inducers of bronchopulmonary dysplasia (BPD)—barotrauma [39], oxotrauma [40] and infection [41]—all cause ATII cell injury and damage, we investigated the effects of PTHrP deprivation on the lipofibroblast phenotype, only to discover that in the absence of PTHrP, the lipofibroblast transdifferentiates to a myofibroblast, the cell-type that characterizes lung fibrosis. Furthermore, myofibroblasts cannot support type II cell growth or differentiation, whereas lipofibroblasts can [13], demonstrating the functional significance of these two fibroblast phenotypes for lung development; importantly, when myofibroblasts are treated with a PPAR γ agonist, they revert back to the lipofibroblast phenotype, including their ability to promote type II cell growth and differentiation. As a result of these seminal observations, we have found that all of the above-mentioned BPD inducers cause downregulation of alveolar lipofibroblast PPAR γ expression [38, 42, 43], inhibiting normal lung development. Moreover, in all of these conditions, PPAR γ agonists have been found to prevent delayed lung development, and in the case of nicotine inhibition of lung development, to even reverse this process [42–51].

3. The Evolution of Peroxisome Biology

Peroxisomes were first observed by Rhodin in 1954 [52] and were characterized as a novel cellular organelle by de Duve and Baudhin, whose laboratory first isolated peroxisomes from rat liver and determined their biochemical properties [53]. Since the core mechanisms involved in peroxisome biology are shared by a wide variety of organisms, it suggests a common evolutionary origin. Speculations about the evolution of peroxisomes began shortly after their discovery. Early photomicrographs suggested interactions between the peroxisome and endoplasmic reticulum (ER), leading some to speculate that peroxisomes were derived from the endomembrane system [54]. Subsequently, an alternative

view that peroxisomes are independent organelles originating by endosymbiosis was proposed after it was observed that the peroxisomes formed from the division of existing peroxisomes, and that they import proteins [55], both features resembling those of bacterially derived organelles such as mitochondria and chloroplasts. But the most elaborate hypothesis regarding the evolutionary origins of the peroxisome was that of de Duve [56], who proposed a metabolic scenario for the establishment of an endosymbiosis mechanism that entailed the role of peroxisome enzymes in the detoxification of highly reactive oxygen species. In this scenario, the protoperoxisome was acquired at a time when the level of atmospheric oxygen was increasing and represented a toxic compound for the majority of living organisms. This concept is consistent with the evolution of the lung lipofibroblast [15] as an example of how vertebrates have entrained otherwise toxic substances in the environment as physiologic mechanisms [57]. Csete et al. [58] have observed that skeletal muscle satellite cells in culture will spontaneously become adipocytes in 21% oxygen, but not in 6% oxygen, suggesting that the episodic increases and falls in atmospheric oxygen over the last 500 million years may have caused the evolution of fat cells in the lung (lipofibroblasts) and periphery (adipocytes) [3]. Such a mechanism is a selection advantage since the lipofibroblast protects the alveolus against oxidant injury [59], and its production of leptin [37, 38] may have fostered modern-day stretch-regulation of alveolar surfactant [60–63], facilitating the increase in lung surface area [1, 4, 15] and mediating ventilation-perfusion matching [64]. The concomitant production of oxygen free radicals, lipid peroxides and other oxidative products likely generated eicosanoids (22) as a balancing selection for endogenous PPAR ligands. Bolstered by the popularity of the serial endosymbiotic theory [65], this view has been the most widely accepted among biologists.

More recently, the endosymbiosis theory for the origin of the peroxisome has been challenged. Experimental evidence shows a close relationship between the ER and peroxisome formation—certain peroxisomal membrane proteins must first be targeted to the ER before they reach the peroxisome [66], and peroxisome-less mutant yeast can form new peroxisomes from the ER upon introduction of the wild-type peroxisome gene [67]. And independent evidence for an evolutionary link between peroxisomes and the ER was provided by phylogenetic studies showing that homologous relationships between components of the peroxisomal import machinery and those of the ER-decay (ERAD) pathway [68, 69]. These data have led the research community to conclude that the peroxisome originates in the ER [70, 71], but have not excluded the possibility of an endosymbiont [71].

In the early 1990s, based on sequence homology with previously identified members of nuclear hormone receptor superfamily, three PPAR isotypes (PPAR α , β/δ , and γ) were identified, initially in *Xenopus laevis* and the mouse, and later in human, rat, fish, hamster, and chicken [72, 73]. These isotypes were initially shown to be activated by peroxisome proliferators, a group of substances able to induce peroxisome proliferation. Subsequently, various endogenous and exogenous PPAR ligands were identified, including fatty

acids, eicosanoids, synthetic hypolipidemic, and antidiabetic agents [74]. Though PPARs are involved in several aspects of rodent development, they are most importantly involved in various aspects of lipid metabolism and energy homeostasis, with PPAR γ 's role in adipogenesis and lipid storage and PPAR α 's role in fatty acid catabolism in the liver being the best characterized [74, 75].

4. PPAR γ Mediates the Evolutionary History of the Adipocyte: Homologies Run Deep

Over the course of vertebrate evolution, during the Phanerozoic Period (the last 500 million years) the amount of oxygen in the atmosphere has increased to its current level of 21%. However, it did not increase linearly; instead, it increased and decreased several times, reaching concentrations as high as 35% and falling to as low as 15% over this time-period [76]. As pointed out above, the increased oxygen tension may have caused the differentiation of muscle satellite cells into lipofibroblasts, or lung adipocytes, in the lung, as the first directly affected anatomic site where the increased atmospheric oxygen would have generated selection pressure for evolutionary change. Consistent with this hypothesized adaptive response to the rising oxygen tension in the atmosphere, we have previously shown that the lipids stored in alveolar lipofibroblasts protect the lung against oxidant injury [59]. Like adipocytes, lipofibroblast differentiation requires upregulation of PPAR γ [13, 42, 44], which stimulates differentiation of myofibroblasts to lipofibroblasts [45]. In turn, the leptin secreted by the lipofibroblasts binds to its receptor on the alveolar epithelial cells lining the alveoli, stimulating surfactant synthesis [37, 38], and reducing alveolar surface tension. This results in a more deformable and efficient gas-exchange surface. Such positive selection pressure could have led to the stretch-regulated coregulation of surfactant and microvascular perfusion [77] by PTHrP, recognized physiologically as the mechanism of ventilation-perfusion matching. The evolution of these molecular mechanisms could ultimately have given rise to the definitive mammalian lung alveolus, with maximal gas exchange resulting from coordinate stretch-regulated surfactant production and alveolar capillary perfusion, thinner alveolar walls due to PTHrP's apoptotic or "programmed cell death" effect on fibroblasts [78], and a blood-gas barrier buttressed by type IV collagen [79]. We speculate that this last feature may have contributed generally to the molecular blueprint for the peripheral microvasculature of evolving vertebrates, given its effect on angiogenesis [80]. One physiologic consequence of the increased oxygenation may have been the concomitant induction of fat cells in the peripheral circulation, which led to endothermy or warm bloodedness- Mezentseva et al. [81] have shown that thermogenic fat cells differentiate from embryonic limb bud mesenchymal cells in association with the expression of PPAR γ . The resulting increase in body temperature synergized increased lung oxygenation because lung surfactant is 300% more active at 37°C than at ambient atmospheric temperature (i.e., the body temperature for

cold-blooded organisms). For example, map turtles (*Graptemys geographica*) show different surfactant compositions depending on the ambient temperature [82]. Therefore, the advent of thermogenesis would have facilitated the physical increase in lung surfactant surface-tension-lowering activity. Moreover, it has been shown that treatment of cold blooded lizards with leptin, a product of adipocytes, increases their body temperature [83]. These synergistic selection pressures for adipogenesis would have been further functionally enhanced by the coordinate physiologic effects of epinephrine on the heart [84], lung [85], and fat depots [86], underpinned structurally by the increased production of leptin by fat cells, which is known to promote the formation of blood vessels [80] and bone [87], accommodating the infrastructural changes necessitated by the evolution of complex physiologic traits.

5. Everything Put Together Falls Apart in Bronchopulmonary Dysplasia

Since BPD can be induced by all of the varied factors cited above, disrupting epithelial-mesenchymal interactions, we designed experiments to determine the spatiotemporal effects of these disruptors on PTHrP-PPAR γ signaling. The effective distension of the newborn lung has a profound physiologic effect on pulmonary homeostasis [60, 61], and stretching of the ATII cell increases the expression and production of PTHrP [11]. In contrast, overdistension of the type II cell [88] results in downregulation of PTHrP expression, and hence PPAR γ , simulating the consequences of volutrauma [43]. Since hyperoxia also augments the transdifferentiation of lipofibroblasts to myofibroblasts *in vitro* [44], we determined the occurrence of hyperoxia-induced alveolar lipo-to-myofibroblast transdifferentiation *in vivo*. Either 24 hour or 7d *in vivo* exposure to hyperoxia significantly decreased the expression of lipogenic markers, and significantly increased the myogenic markers in association with arrested alveolarization; the lungs demonstrated relatively larger air spaces, thinned interstitia, decreased secondary septal crest formation, and a significant reduction in radial alveolar counts. Moreover, since lung inflammation is a key factor predisposing preterm infants to BPD, we determined the effects of lipopolysaccharide (LPS) on key alveolar epithelial-mesenchymal paracrine interactions [46]. There were acute (24 hour), significant increases in the expression of PTHrP, PPAR γ , ADRP, and surfactant protein-B (SP-B), without any significant effects on the expression of α -smooth muscle actin (α SMA). This was followed (72 h) by significant decreases in the expression of PTHrP, PPAR γ , ADRP, and SP-B, accompanied by a significant increase in the expression of α SMA, the key molecular and functional marker for BPD. And since nicotine affects lung growth and development [47], we determined the effect of *in utero* nicotine exposure on epithelial-mesenchymal interactions as well. Nicotine indirectly inhibited ATII cell proliferation and metabolism via its paracrine effects on the alveolar epithelial lipofibroblasts [48], causing lipo-to-myofibroblast transdifferentiation [49, 89]. In all of the above-cited studies, a PPAR γ agonist blocked

the disruptive effects, even reversing them in the case of nicotine.

6. PPAR γ Agonists Turn on a “Master Switch” for Normal Lung Development That Universally Prevents BPD

It is clear from the work outlined above that lipofibroblast PPAR γ signaling plays a central role in epithelial-mesenchymal interactions by maintaining alveolar homeostasis in volutrauma, oxotrauma, infection, and nicotine-mediated lung injury. The lipofibroblast expresses PPAR γ in response to PTHrP signaling from the ATII cell, resulting in both the direct protection of the mesoderm against oxidant injury [59], and protection against atelectasis by augmenting surfactant protein [37] and phospholipid [38] synthesis. Molecular injury to either the ATII cell or the lipofibroblast downregulates this molecular signaling pathway, causing myofibroblast transdifferentiation. And as indicated above, myofibroblasts cannot promote ATII cell proliferation and differentiation [13], leading to the failed alveolarization characteristic of BPD [50]. In contrast, lipofibroblasts support ATII cell proliferation and differentiation under the influence of factors implicated in the pathogenesis of BPD. This scenario is validated by a plethora of *in vitro* [13, 44–46, 51, 89, 90] and *in vivo* [42, 43, 48, 89] studies. Importantly, these studies show that PPAR γ agonists such as Prostaglandin J₂ and rosiglitazone can prevent or reverse myofibroblast transdifferentiation, potentially preventing the inhibition of alveolarization in the developing lung, the hallmark of CLD of the newborn [13, 42, 45, 47–49, 51, 89, 90].

7. Conclusion

Using a basic cell biologic approach to elucidate the pathophysiology of BPD based on evolved cell-physiologic principles, we have determined the paracrine cell/molecular mechanism by which stretch coordinates epithelial-mesenchymal signaling, upregulating key genes for the induction of the prohomeostatic lipofibroblast phenotype—including PPAR γ , ADRP, and leptin—and the retrograde stimulation of ATII cell surfactant phospholipid and protein synthesis by the lipofibroblast product leptin. Each of these paracrine interactions requires cell-specific receptors on adjacent cells derived from the endoderm or mesoderm, respectively, that is, PTHrP receptors on the mesoderm and leptin receptors on the endoderm, to specifically mediate the signaling pathways within each cell type. More importantly, we have exploited the cell-specific molecular nature of this mechanism in order to effectively and comprehensively prevent and treat lung injuries that affect this signaling pathway. By identifying deep homologous mechanisms that have determined both the phylogeny and ontogeny of the lung, by using exogenous PPAR γ agonists we have been able to prevent and even reverse the effects of a wide variety of injurious agents affecting the epithelial-mesenchymal interactions that have evolved to determine the gas-exchange surface of the lung [1–5].

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Review Article

Emerging PPAR γ -Independent Role of PPAR γ Ligands in Lung Diseases

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Peroxisome proliferator activated receptor (PPAR)- γ is a nuclear hormone receptor that is activated by multiple agonists including thiazolidinediones, prostaglandins, and synthetic oleanolic acids. Many PPAR γ ligands are under investigation as potential therapies for human diseases. These ligands modulate multiple cellular pathways via both PPAR γ -dependent and PPAR γ -independent mechanisms. Here, we review the role of PPAR γ and PPAR γ ligands in lung disease, with emphasis on PPAR γ -independent effects. PPAR γ ligands show great promise in moderating lung inflammation, as antiproliferative agents in combination to enhance standard chemotherapy in lung cancer and as treatments for pulmonary fibrosis, a progressive fatal disease with no effective therapy. Some of these effects occur when PPAR γ is pharmaceutically antagonized or genetically PPAR γ and are thus independent of classical PPAR γ -dependent transcriptional control. Many PPAR γ ligands demonstrate direct binding to transcription factors and other proteins, altering their function and contributing to PPAR γ -independent inhibition of disease phenotypes. These PPAR γ -independent mechanisms are of significant interest because they suggest new therapeutic uses for currently approved drugs and because they can be used as probes to identify novel proteins and pathways involved in the pathogenesis or treatment of disease, which can then be targeted for further investigation and drug development.

1. PPARs and PPAR Ligands

Peroxisome proliferator activated receptors (PPARs) are a family of ligand-binding nuclear hormone receptors that are involved in multiple important regulatory pathways including fat metabolism, cell differentiation, proliferation, apoptosis, and inflammation. The first to be identified was PPAR α , the transcription factor responsible for the upregulation of peroxisome proliferation by fatty acids and certain hypolipidemic fibrate drugs. Three PPARs have now been identified, α , β/δ , and γ , encoded by three separate

genes. PPAR γ is expressed as two different isoforms, PPAR γ 1 and PPAR γ 2, with different transcription start sites. Their detailed domain structure, characteristics, and mode of action have been discussed in detail elsewhere [1, 2].

PPARs have two functional domains, a ligand-binding domain (LBD) and a DNA Binding domain (DBD). Both of these domains are highly conserved in all three receptors. The ligand-binding pocket of the LBD is relatively large and is assumed to bind to a wide range of ligands [2, 3]. Endogenous ligands of the PPARs include fatty acids and fatty acid metabolites; as a result, PPARs are believed to act

in part as lipid sensors. PPARs also bind leukotrienes and prostaglandins such as 15d-PGJ₂, which can have powerful regulatory effects on differentiation and immune responses [4]. At high enough concentration, the PPARs exhibit relatively nonselective binding for fatty acids. However, the significance of this is unclear because the concentration of any ligand in the cellular microenvironment has not been determined [2–5].

Synthetic ligands for all three receptors have been identified and developed. The thiazolidinediones (TZDs) (rosiglitazone, pioglitazone, troglitazone, and ciglitazone) were developed as hypoglycemic agents in the 1980s [6], and the first clinical trial of a TZD as an insulin sensitizer was reported in 1991 [7]. Only after the TZDs were in clinical use were they recognized as ligands of PPAR γ , activating gene transcription to upregulate expression of adipocyte genes and induce adipocyte differentiation [8]. Although TZDs bind mainly to PPAR γ , select TZDs activate other PPARs as well [2, 9, 10].

The fibrate drugs (clofibrate, fenofibrate, gemfibrozil, ciprofibrate) have been in clinical use since the 1930s as cholesterol-lowering agents, but it was not until 1995 that it was discovered that they acted via PPARs [11]. The fibrates act most strongly on PPAR α but have activity against PPAR β/δ and γ as well. For example, benzafibrate is a pan-agonist with similar activity against all three PPAR isoforms. Other dual or pan-PPAR ligands have been developed including KRP-297, a dual PPAR α/γ agonist. Some nonsteroidal anti-inflammatory drugs (NSAIDs) (ibuprofen, indomethacin, and fenoprofen) weakly activate both PPAR γ and α [12].

The triterpenoids are a newly recognized class of natural exogenous PPAR γ ligands initially identified in herbal medicines. These compounds are being studied in their native forms and as synthetic derivatives with increased stability, potency, and selectivity. Ursolic acid, a plant triterpenoid, activates PPAR α and has been shown to regulate hepatic lipid metabolism [13]. Oleanolic acid and its naturally occurring derivative 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate (CDDO) are potent PPAR γ agonists [14, 15].

2. PPAR γ and Lung Disease

Beyond its classical role in regulating fat metabolism, PPAR γ plays a role in regulating cell differentiation and inflammation. It is thus of high interest as potential target for therapies for diseases involving dysregulated inflammation or differentiation. Chronic inflammation due to cigarette smoking and exposure to other environmental toxicants is a contributing factor to numerous lung diseases including COPD (emphysema and chronic bronchitis), asthma, lung cancer, and fibrosis. PPAR γ expression is dysregulated in patients with cystic fibrosis, sarcoidosis [16], COPD [17, 18], and acute lung injury [19]. Often, and particularly with respect to COPD and fibrosis, current therapies only treat the symptoms and do not modify the course of the underlying disease [17, 20]. Thus, there is great interest in understanding the molecular pathways of chronic lung disease and of using

PPAR γ ligands both as tools to probe these pathways and as potential therapies.

The lung represents a particularly useful target for this research as (a) many lung diseases involve acute or chronic inflammation or cell proliferation, which are processes targeted by PPAR γ ligands, (b) drugs can be delivered by inhalation, possibly sparing systemic side effects like heart disease and weight gain, and (c) PPAR γ ligands appear particularly effective against lung fibrosis, a disease for which effective therapy can be challenging. There are numerous studies demonstrating the potential role of PPAR γ and PPAR γ agonists in regulating or treating lung diseases including cancer, fibrosis, and diseases of chronic inflammation. Surprisingly, many of the most interesting effects appear not to require PPAR γ itself as a transcription factor.

3. PPAR γ -Independent Effects of PPAR γ Ligands

Because they play important roles in multiple critical cellular pathways including differentiation, fat metabolism, proliferation, and control of inflammation, PPAR γ and its ligands have come under increasing scrutiny. However, some effects of PPAR γ ligands appear to be PPAR γ -independent—that is, they do not require PPAR γ -dependent transcriptional activation and can occur when PPAR γ is absent or functionally inactivated (Figure 1). The independent effects of PPAR γ were first recognized in 2000 by Thieringer et al. [21] who reported that 15d-PGJ₂ had anti-inflammatory activity on human peripheral blood monocytes *in vitro* that was mediated by a PPAR γ -independent mechanism. More recently, it was reported that 15d-PGJ₂ and ciglitazone induce apoptosis in both normal and malignant human B lymphocytes independent of PPAR γ activation [22]. It should be noted that PPAR γ -independent effects can be determined *in vitro* with relative confidence by using PPAR γ -antagonists, gene deletions, overexpression of dominant-negative mutants, or siRNA. However, it is more difficult to discriminate between PPAR γ -dependent and -independent effects *in vivo* because complete PPAR γ deletion is embryonically lethal [14].

Emerging reports demonstrating PPAR γ -independent effects of PPAR γ ligands are igniting interest in understanding the mechanisms responsible for the PPAR γ -independent effects of these compounds. Although they were initially identified as ligands of PPAR γ , their off-target or direct effects are important as these ligands regulate numerous signaling components that are independent of the classical PPAR γ pathway. These effects are important for two reasons. First, as PPAR γ ligand-based therapies enter into clinical testing, it will be critical to understand both their PPAR γ -dependent and -independent effects, so that their mechanism of action and potential side effects can be evaluated [23]. Second, PPAR γ ligands with PPAR γ -independent effects can be used to identify novel metabolic and regulatory pathways that play a role in human disease and may lead directly to novel therapeutic uses of existing drugs, or to development

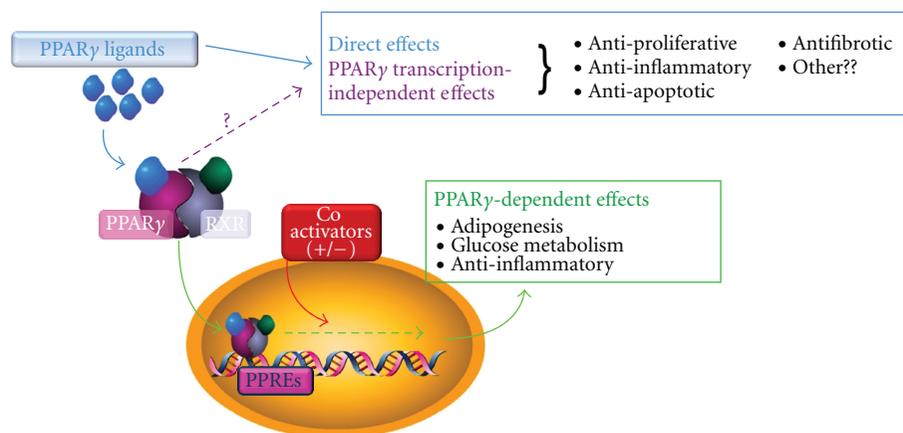


FIGURE 1: PPAR γ ligands have multiple PPAR γ -independent effects. In the classical PPAR γ -dependent pathway, ligand-bound PPAR γ forms a heterodimer with RXR and binds to PPAR γ -response elements (PPREs) which leads to modulation of transcription. However, PPAR γ ligands also exhibit direct effects that do not involve transcriptional activation by PPAR γ /RXR. These direct effects may involve PPAR γ protein interacting with PPAR γ ligands in a “non-classical manner” (not involving RXR or PPRE) or may be completely independent of PPAR γ (functioning even in the complete absence of PPAR γ protein, i.e., direct effects). PPAR γ -independent effects can alter multiple cellular programs including regulation of differentiation, inflammation, apoptosis and may be of significant therapeutic interest.

of new drugs that target these novel pathways uncovered by investigating PPAR-independent effects of these ligands.

4. Mechanism of PPAR γ -Independent Action of PPAR γ Ligands

A key question that must be answered as new PPAR γ -ligand-based therapies are developed is what the mechanisms and targets of the PPAR γ -independent effects of these compounds are. Current evidence suggests that the triterpenoids CDDO (and its derivatives) and the prostaglandin 15d-PGJ₂ contain electrophilic carbon atoms that mediate their effects. These carbons can form covalent bonds with free sulfhydryl groups in cellular proteins via the Michael addition reaction, which can then alter the function of the protein. For example, 15d-PGJ₂ can bind p50, which changes its DNA-binding activity [24]. CDDO is a strong electrophile that binds promiscuously to cellular proteins thus making its mechanism of action complex. A recent study in human embryonic kidney cells identified 577 cellular proteins that could bind to CDDO. The analysis was unable to discriminate between high abundance-low affinity targets and low abundance-high affinity targets, and many of these proteins may interact with CDDO without changing their function [25]. Nevertheless, direct protein binding by CDDO and other electrophilic PPAR γ ligands likely plays a key role in their function, and efforts are underway to identify specific binding partners in specific cell types and disease models.

One target of CDDO and 15d-PGJ₂ is cellular antioxidant defenses. Both CDDO and 15d-PGJ₂ bind glutathione, a key cellular antioxidant. By binding glutathione, CDDO and 15d-PGJ₂ appear to deplete cellular stores of free glutathione, leading to increased oxidative stress and upregulation of antioxidant genes [26–28]. In some but not all cell types,

CDDO also binds Keap-1, which then undocks from its binding partner, the transcription factor Nrf2. Nrf2 is then free to translocate to the nucleus where it is a master regulator of the oxidative stress response. Oral dosing with a CDDO Imidazole derivative prevented emphysema-like changes in lung structure in mice exposed to chronic cigarette smoke, and this effect was abrogated in Nrf2-knockout mice [29]. Activation of antioxidant defense systems may be one mechanism by which PPAR γ ligands can be used to prevent or treat diseases, especially cancer.

Glucocorticoid receptors (GRs) are also being explored as possible targets of PPAR γ -independent signaling mechanism. Initial work done by Ialenti et al. showed that rosiglitazone, and ciglitazone decrease IL-6 production in E8.2/GR3 and J774 cells, which is blocked by the GR inhibitor RU486 [30]. Rosiglitazone, pioglitazone and ciglitazone increased GR translocation to the nucleus in HeLa, A549, and PPAR γ -deficient fibroblasts [31]. In addition, evidence was found for rosiglitazone-induced GR recruitment of the transcriptional coactivators SRC-1 and NCoR in HeLa cells [31]. It was also reported in human bronchial cells that the antiproliferative effects of rosiglitazone and troglitazone were mediated by calcium channel signaling through the GPR40 receptor [32].

It should also be noted that it is not always possible to determine whether an effect that is independent of PPAR γ -regulated transcriptional control is also independent of the presence of PPAR γ protein (Figure 1). PPAR γ ligands may have “direct” effects that bypass classical RXR/PPRE interactions but which nevertheless require a functional PPAR γ protein acting in a nonclassical fashion (possibly as a result of covalent modification). Some studies make use of a small molecule inhibitor of PPAR γ , GW9662, that irreversibly binds to and inactivates the ligand-binding pocket of PPAR γ , while other studies have been performed in cells that are genetically deficient in PPAR γ . In these cases, it seems reasonable to conclude that the effects of the PPAR γ ligands

are truly PPAR γ -independent. However, other studies utilize a dominant-negative PPAR γ construct that can bind ligands and the PPRE but cannot recruit transcriptional co-factors, or ligand derivatives that do not activate PPRE reporter constructs. In these cases, it can not be ruled out that PPAR γ ligands bind PPAR γ protein and carry out their function by a nonclassical pathway that bypasses RXR and the PPRE. Ligands may also act via different pathways in different cell types. More study is needed to understand these PPAR γ -independent mechanisms.

5. Inflammation

In the lung, PPAR γ is expressed in endothelial cells, fibroblasts, smooth muscle cells, airway epithelial cells, and resident alveolar macrophages, as well as infiltrating leukocytes including neutrophils, eosinophils, and mast cells [33, 34]. PPAR γ expression has been identified in multiple lung cell lines including A549, BEAS-2B, and NCI-H292 [35].

Clinical evidence suggests that PPAR γ and PPAR γ ligands are involved in regulating inflammatory responses in the lung [36, 37], and chronic or dysregulated inflammation is an important pathologic feature in many lung diseases including asthma, COPD, sarcoidosis, and pulmonary fibrosis. Expression of PPAR γ is increased in lung tissue from asthmatic patients, specifically localized in mucosal, epithelial, and smooth muscle cells and is associated with the severity of inflammation [34]. PPAR γ expression was decreased in patients with severe pulmonary hypertension, particularly around plexiform lesions [38].

Troglitazone, ciglitazone, and 15d-PGJ₂ strongly inhibited LPS-induced secretion of TNF α and IL-6 and downregulated expression of Cox-2 and iNos in mouse macrophages. PPAR γ -deficient macrophages responded similarly to wild-type control, suggesting a PPAR γ -independent signaling mechanism [39]. Troglitazone and 15d-PGJ₂ also activated PI3K signaling independently of PPAR γ in A549 cells (a human lung epithelial carcinoma line) via increased ERK phosphorylation and ERK/MAPK signaling, leading to increased production of Cox-2 and PGE₂ [40]. Troglitazone and rosiglitazone promoted proliferation in nonmalignant human bronchial epithelial cells by a PPAR γ -independent mechanism, while 15d-PGJ₂ blocked proliferation by a PPAR γ -dependent mechanism [32]. Recently, we reported that 15d-PGJ₂ and CDDO inhibited the silica-induced inflammatory response of primary human lung fibroblasts via a largely PPAR-independent pathway attributed to the electrophilic properties of non-TZD ligands of PPAR γ [41].

In light of their anti-inflammatory properties *in vitro*, animal models have been used to explore the potential of PPAR γ ligands as therapeutic agents in inflammatory lung diseases. Oral administration of ciglitazone decreased cell infiltration, epithelial dysplasia, and IL-2, IL-4, and IFN γ production in a mouse model of allergic airway inflammation [42]. A similar effect was observed with intranasal administration of ciglitazone, with decreased cell infiltration in the lung, particularly that of eosinophils, and reduced airway remodeling [43]. Other TZDs such as rosiglitazone

also decreased neutrophil and eosinophil infiltration and reduced airway hyperresponsiveness to methacholine [44]. Ciglitazone also decreased local inflammation in the lung in a model of *S. pneumoniae*-induced pneumonia, with concurrent reductions in TNF α , IL-6, IL-12p70, and IFN γ secretion [45]. However, it is difficult to attribute the effects of the ligands in *in vivo* studies to PPAR γ -dependent or -independent mechanisms, and further study is needed.

6. Lung Cancer and PPAR γ Ligands

Lung cancer accounts for more cancer-related deaths than any other type of cancer worldwide [46]. In 2010, there were 157,300 lung cancer-related fatalities in the United States alone [47]. As a disease, lung cancer can be divided into two main categories, nonsmall cell lung cancer (NSCLC), which comprises around 85% of all lung malignancies, and small cell lung cancer (SCLC), which comprises the other 15% [48]. NSCLC can be further classified into three subgroups, large cell cancer, adenocarcinoma, and squamous cell carcinoma, depending upon cell type and histological classification. As lung cancer is a substantial cause of human suffering and death, a multitude of therapeutic approaches have been employed. Many therapeutic options are actively being pursued including several recent investigations that utilize PPAR γ ligands as treatments [49, 50]. While recent work demonstrates PPAR γ ligands have beneficial PPAR γ -dependent and -independent properties in treating malignancies, emerging insights into PPAR γ -independent functions in lung cancer are highlighted here.

6.1. In Vitro Models of Lung Cancer and PPAR γ Ligands. PPAR γ ligands including the TZDs, 15d-PGJ₂, and triterpenoids (CDDO and its derivatives) have been investigated in lung cancer cells *in vitro*. Troglitazone dramatically reduces cell proliferation of A549 cells, a human lung adenocarcinoma cell line, at concentrations of 10 to 40 μ M. Because PPAR γ activity, determined by reporter assays, reached a maximal level at 15 μ M while the beneficial reductions in cell proliferation continued up to 40 μ M, the authors suggested that troglitazone had desirable PPAR γ -independent effects at elevated doses [51]. Troglitazone decreased expression of the cell cycle regulatory protein cyclin D1 and increased the number of cells that were growth-arrested in the G₁/G₀ phase of the cell cycle [51]. Recently, a troglitazone analog, delta2-Troglitazone, that does not stimulate PPAR γ -dependent transcription, was shown to increase proteasomal degradation of cyclin D1 in breast cancer cells [52]. This suggests that troglitazone, at least in part, affects cyclin D1 expression independent of PPAR γ . Cyclin D1 is highly expressed in many types of tumors, including the lung [53, 54]. Troglitazone also modifies ERK-1/2 signaling in NSCLC cells [51, 55–57] and triggers apoptosis in two lung cancer cell lines, but not in a control, nonmalignant line, underscoring the potential of TZDs to specifically target tumor cells [56]. In A549 cells, troglitazone directly interacts with the epidermal growth factor receptor (EGFR), binding the EGFR and augmenting

its internalization into the lysosome where it is subsequently degraded [58]. The reduction in EGFR protein levels leads to a loss of EGFR signaling activity, which consequently halts cell proliferation. Interestingly, EGFR mutations are some of the most common mutations found in lung cancer patients [48].

Pioglitazone and rosiglitazone also show a number of beneficial effects in attenuating growth of lung cancer cells *in vitro*. In 2006, Han and Roman determined that rosiglitazone had significant PPAR γ -dependent and -independent effects on blunting NSCLC cell growth [59]. In this study, rosiglitazone impaired proliferation of NSCLC cells by increasing expression of the tumor suppressor phosphatase PTEN (phosphatase and tensin homolog), reducing Akt and p70S6K phosphorylation, and increasing phosphorylation of AMPK. The irreversible PPAR γ inhibitor GW9662 reversed the effects on PTEN expression and Akt phosphorylation but not AMPK and p70S6K phosphorylation, suggesting a mixture of PPAR γ -dependent and -independent effects. AMPK is a master regulator of energy metabolism and regulates mammalian target of rapamycin (mTOR) mediated cell growth, proliferation, and protein synthesis pathways. Phosphorylation of AMPK inhibits mTOR signaling to reduce proliferation and protein synthesis through a reduction of p70S6K phosphorylation. Interestingly, rosiglitazone enhanced the effects of rapamycin on halting NSCLC cell growth, accentuating the potential for combination therapies. The mechanism whereby rosiglitazone activates AMPK phosphorylation is still unclear.

Pioglitazone and rosiglitazone also decreased production of the prostaglandin PGE₂ in both A549 and A427 NSCLC cell lines. Increased levels of PGE₂ stimulate proliferation and pro-survival pathways in numerous malignancies, including NSCLC, suggesting that decreasing PGE₂ production may reduce cancer cell growth. The effects of the agonists were not reversed by either GW9662 or by expression of a dominant-negative PPAR γ , demonstrating that the effects were PPAR γ -independent. Investigation into the mechanism determined a corresponding increase in the expression of 15-hydroxy prostaglandin dehydrogenase (15-PGDH), which converts PGE₂ into a biologically inactive 15-keto prostaglandin derivative [60]. Rosiglitazone also inhibited expression of the α 4 nicotinic acetylcholine receptor (α 4-nAChR) in a PPAR γ -independent manner in three different NSCLC cell lines [61]. Nicotine stimulates proliferation of NSCLC cells through activation of α 4-nAChR. Rosiglitazone activated Erk-1/2 signaling to prompt the tumor suppressor protein p53 to inhibit α 4-nAChR expression. The effects of rosiglitazone treatment on α 4-nAChR expression were independent of PPAR γ as demonstrated by depletion of PPAR γ protein by PPAR γ siRNA as well as through the use of GW9662 [62]. Rosiglitazone and GW1929, another TZD, also inhibit fibronectin expression by tumor cells *in vitro* [63]. Fibronectin expression and extracellular deposition promote tumor cell interactions.

An interesting non-lung study demonstrated that TZDs reduce expression of the oncogenic transcription factor FoxM1 in hepatoma cells [64]. Regulation of FoxM1 expression by TZDs was independent of PPAR γ expression, as

expression of FoxM1 was reduced by TZDs even in the presence of PPAR γ siRNA. Because FoxM1 is often elevated in NSCLC, further investigation to see if TZDs can also reduce FoxM1 expression in lung cancer may be worthwhile.

The endogenous PPAR γ ligand, 15d-PGJ₂, has also been shown to have beneficial effects in treating lung cancer cells. 15d-PGJ₂ promotes apoptosis of A549 cells by activating caspase 3 and attenuating expression of cyclin D1 in a PPAR γ -independent manner [65]. 15d-PGJ₂ also increased production of reactive oxygen species (ROS) in lung cancer cells while reducing intracellular glutathione levels [66]. The effects could not be reversed by a PPAR γ siRNA or the antagonist GW9662 but was reversible with quercetin, a powerful antioxidant, suggesting that 15d-PGJ₂ modulated the redox state of the cell. Interestingly, 15d-PGJ₂ has been shown to covalently attach to glutathione and lead to its oxidation [67], which may account for the loss of glutathione seen in the studies.

The novel triterpenoid CDDO and its synthetic derivatives CDDO-Methyl ester (CDDO-Me) and CDDO-Imidazole (CDDO-Im) have also been investigated as chemotherapeutic agents in treating lung cancer. CDDO-Im promoted apoptosis in A549 and H358 human lung cancer cell lines in a dose-dependent manner with a rapid decrease in STAT5 phosphorylation [68]. STAT5, a phosphorylation-dependent transcription factor in the JAK/STAT pathway, is involved in the transcription of proliferation and pro-survival genes. CDDO-Im induced expression of SOCS-1 (a STAT inhibitor) and SHP-1 (a phosphatase that targets STAT5) in as little as 30 minutes. Because SOCS-1 and SHP-1 are both regulated by the antioxidant response transcription factor, Nrf2, the authors speculate that CDDO-Im activates Nrf2 by binding to its regulatory partner Keap-1 [68].

6.2. Animal Models of Lung Cancer and PPAR γ Ligands. Although it has been difficult to dissect out the specific PPAR γ -independent functions of the PPAR γ ligands from their PPAR γ -dependent functions *in vivo*, the ability of PPAR γ ligands to inhibit tumor cell growth *in vitro* has led to a number of significant studies in animal models. Ciglitazone, which inhibits proliferation of A549 cells *in vitro* by a PPAR γ -independent mechanism as discussed above, also significantly reduced A549 tumor weights in the nude mouse xenograft model, with concomitant reduction in cyclin D1 and increased expression of the cell cycle inhibitor p21 [51]. The xenograft model was also used to demonstrate that daily treatment with 15d-PGJ₂ had potent antitumor effects against subcutaneous tumors comprised of A549 or H460 cells. Of note, 15d-PGJ₂ significantly enhanced the effects of the chemotherapeutic drug docetaxel [65].

In the lung, Girnun et al. utilized a transgenic mouse model containing inducible activating mutations in either KRAS or epidermal growth factor (EGFR), two genes commonly mutated in human lung cancer [69]. Rosiglitazone, in combination with the standard chemotherapeutic agent carboplatin, significantly reduced tumor size by increasing apoptosis and decreasing cell proliferation. Importantly, neither drug alone had significant effects, and rosiglitazone

did not enhance the myelosuppressive effects of carboplatin, suggesting that combination therapy would be safe and effective. Addition of rosiglitazone to a chemotherapeutic cocktail of hydrazine, selenium, and phenylbutyrate significantly reduced lung hyperplasia and adenoma in a mouse carcinogen model compared to the cocktail alone [70]. In another carcinogen-induced lung cancer model in mice, pioglitazone treatment had a clear preventative effect [71]. Mice given oral doses of pioglitazone 8 weeks after initial injection of carcinogen had no change in number of adenocarcinomas but a 64% decrease in tumor volume compared to control, along with a 35% reduction in the number of squamous cell carcinoma.

The CDDO derivatives CDDO-Me and CDDO-ethyl amide also inhibit lung cancer in preclinical mouse studies. In one study, mice were fed the CDDO derivatives starting one week after treatment with vinyl carbamate, a carcinogen that induces adenocarcinoma of the lung. The CDDO-treated mice had a dramatically reduced tumor burden [72]. The authors suggest that CDDO prevented tumor formation through upregulation of the antioxidant heme-oxygenase-1 (HO-1) and through inhibition of STAT phosphorylation leading to increased apoptosis, as demonstrated *in vitro*. In another recent study using the transplantable tumor model, Lewis lung carcinoma (LLC), CDDO-Me drastically reduced tumor size and inhibited myeloid-derived suppressor cells that are involved in the immune suppressive response against tumors [73]. These data are very intriguing as they suggest CDDO-Me may augment cancer vaccine therapies.

7. PPAR-Independent Effects of PPAR Ligands in Pulmonary Fibrosis

Fibrotic remodeling following injury and repair occurs in many tissues including the kidney, heart, skin, and liver [74]. Pulmonary fibrosis is characterized by pathologic remodeling of the lung, with thickening of interstitial spaces, deposition of collagen and other matrix proteins, contraction and stiffening of lung tissue, loss of alveolar architecture, reduced gas exchange and ultimately respiratory failure. Pulmonary fibrosis can be caused by a variety of insults including chronic inflammation and inhalation of particulates like asbestos and silica, radiation, drugs, and as a sequelae of connective tissue diseases. Idiopathic pulmonary fibrosis (IPF) is a severe form of pulmonary fibrosis with no current therapy. The only “cure” is a lung transplant. The disease is progressive and the median time from diagnosis to death at 2.9 years is shorter than that for lung cancer [15].

One of the key pathogenic processes in lung fibrosis is the differentiation and activation of fibroblasts and myofibroblasts to produce the components of scar tissue (hypercellularity, collagen, and other extracellular matrix proteins). The key pro fibrotic cytokine appears to be TGF β . Because of the central role of TGF β and myofibroblasts, there is intense interest in developing novel therapies that target these key players. Pirfenidone, a small orally active molecule that inhibits synthesis of TGF β and TNF α and attenuates growth of fibroblasts *in vitro*, was recently approved for

clinical use in IPF in Europe, but not yet in the United States. The clinical benefits of pirfenidone appear to be small but significant in some studies [75]; no other therapies have been approved that specifically target the underlying cellular pathology of lung fibrosis.

7.1. *In Vitro* Models of Pulmonary Fibrosis and PPAR Ligands.

There is a growing body of evidence that both natural and synthetic PPAR γ agonists have powerful antifibrotic effects *in vitro* [76], and these results are beginning to be translated to preclinical animal models. Typical experiments examine differentiation of human lung fibroblasts to myofibroblasts *in vitro* and associated changes in expression of profibrotic cytokines and matrix proteins. A variety of nontransformed human lung fibroblast (HLF) cell lines are used including fetal, neonatal, adult nonfibrotic, and adult fibrotic (derived from patient biopsies).

We and others reported that rosiglitazone and 15d-PGJ₂ inhibited TGF β -driven myofibroblast differentiation of primary HLFs [77–80]. Expression of a dominant PPAR γ was able to reverse the inhibitory effect of rosiglitazone more effectively than 15d-PGJ₂, suggesting that rosiglitazone can act via both PPAR γ -dependent and -independent mechanisms while 15d-PGJ₂ acts predominantly via an independent mechanism [77]. Since primary HLFs express abundant PPAR γ and RXR proteins and are capable of PPAR γ -dependent transcriptional regulation [77], this suggested that the antifibrotic effects of the PPAR γ agonists were mediated through both PPAR γ -dependent and -independent mechanisms. We also investigated CDDO, a triterpenoid originally identified in herbal preparations with anti-inflammatory properties. We determined that CDDO has an EC₅₀ for inhibition of myofibroblast differentiation that is 20-fold lower than 15d-PGJ₂ and 400-fold lower than rosiglitazone, and it acts independently of PPAR γ as confirmed by pharmacological and genetic approaches [78]. Recently, we reported that 15d-PGJ₂ and CDDO inhibit TGF β -induced phosphorylation of phosphatidylinositol 3-kinase-protein kinase B (PI3K-Akt) and focal adhesion kinase (FAK), but not TGF β -induced p38-MAPK phosphorylation, and that the mechanism was independent of PPAR γ [79]. We also noted that there is a strong correlation between the ability of a PPAR γ ligand to inhibit Akt phosphorylation with its ability to suppress myofibroblast differentiation (Figure 2). We find that rosiglitazone is a weak inhibitor of TGF β -induced Akt phosphorylation (Figure 2) and may be therefore a poor choice as an antifibrotic treatment [79].

It is essential to note that the three main mechanisms of activation of myofibroblast differentiation—TGF β , mechanical stress, or adhesion and integrin activation—act via a common pathway in which FAK is autophosphorylated, leading to increased levels of the active kinase (phospho-FAK). It is conceivable that once TGF β activates myofibroblast differentiation, the increased deposition of extracellular matrix proteins would cause additional mechanical stress at the cell surface leading to sustained and continual activation of FAK. Since FAK itself upregulates myofibroblast differentiation, once TGF β initiates this process, sustained activation of FAK

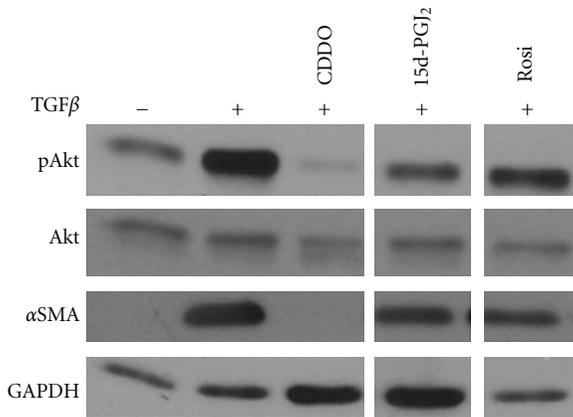


FIGURE 2: PPAR γ ligands inhibit TGF β -induced Akt phosphorylation and myofibroblast differentiation with varied potency. Primary human lung fibroblasts were treated with TGF β (5 ng/mL), alone or in combination with three PPAR γ ligands (CDDO (1 μ M), 15d-PG₂ (5 μ M) and rosiglitazone (20 μ M)). Protein lysates were electrophoretically separated on the same gel, and representative lanes from a single experiment are shown here. The potency of a PPAR γ ligand to inhibit Akt phosphorylation corresponds to its ability to inhibit myofibroblast differentiation. While CDDO inhibits both Akt phosphorylation and α SMA potently, rosiglitazone is a weak inhibitor of both.

would be able to perpetuate the fibrotic response even in the absence of additional external profibrotic signals.

Several other laboratories have also investigated PPAR γ ligands in lung fibroblast differentiation and activation. Lin et al. reported that rosiglitazone inhibited migration, proliferation, and phenotypic differentiation of cultured fetal human lung fibroblasts induced by fetal bovine serum albumin. The inhibitory effect of rosiglitazone on myofibroblast differentiation, determined by expression of α -smooth muscle actin, was also PPAR γ -independent [81]. Troglitazone and ciglitazone were evaluated against normal human fetal lung fibroblasts and fibroblasts isolated from patients with idiopathic interstitial pneumonias. Both ligands inhibited proliferative responses of normal and fibrotic lung fibroblasts to platelet-derived growth factor (PDGF) and TGF β in a dose- and time-dependent fashion and arrested cells at the G₁/G₀ stage. Troglitazone and ciglitazone inhibited expression of cyclin D1, similar to their activity in the A549 adenocarcinoma cell line (see above). Interestingly, they also found that both troglitazone and ciglitazone efficiently inhibited collagen expression by myofibroblasts and that collagen synthesis was also inhibited by overexpression of a constitutively active form of PPAR γ [80]. Since cyclin D1 is a known downstream target of PI3K-Akt pathway, it is conceivable that these and other ligands of PPAR γ (15d-PG₂ and CDDO) inhibit cyclin D1 and arrest fibroblast proliferation via a PPAR γ -independent mechanism involving Akt but inhibit collagen synthesis and other profibrotic functions by a PPAR γ -dependent mechanism.

Not all myofibroblasts in lung fibrosis are derived from preexisting lung resident fibroblasts. Some myofibroblasts may originate from lung epithelial cells via epithelial to

mesenchymal transition (EMT). EMT is a process during which terminally differentiated epithelial cells transform into a mesenchymal phenotype and give rise to fibroblasts and subsequently to myofibroblasts. This process is believed to play an important role in normal repair following injury to alveolar epithelial cells (AECs) [74]. The *in vivo* significance of EMT is controversial, but it is well-described *in vitro* and is characterized by decreased expression of E-cadherin and increased expression of N-cadherin [82]. TGF β potently stimulates EMT and alters the morphology of human AECs. Tan et al. investigated whether PPAR γ ligands could inhibit TGF β -induced EMT in the A549 cell line, an adenocarcinoma of AEC origin. Rosiglitazone and ciglitazone rescued TGF β -mediated repression of E-cadherin via a PPAR γ -dependent mechanism but, at the same time, inhibited TGF β -induced increased N-cadherin expression independent of PPAR γ [82].

Myofibroblasts in lung fibrosis can also originate from circulating adult progenitor cells called fibrocytes. Fibrocytes are distinct from epithelial and endothelial cells, monocyte/macrophages, lymphocytes, dendritic cells, or tissue fibroblasts [83]. Work in the Strieter laboratory demonstrated that TGF β induces myofibroblast differentiation of fibrocytes harvested from human peripheral blood mononuclear cells as measured by α SMA expression [84]. Troglitazone inhibits TGF β -induced α SMA expression and myofibroblast differentiation, and this effect is not reversed by the PPAR γ inhibitor GW9662, indicating that the effects of troglitazone are PPAR γ -independent. TGF β signaling activates the Smad and MAP kinases pathways in fibrocytes, and PPAR γ agonists negatively regulate this process through a PPAR γ -independent pathway. Of special note, troglitazone inhibited JNK activation, which both inhibited myofibroblast differentiation and stimulated differentiation of fibrocytes to adipocytes (fat storage cells) [84]. Thus, pharmacological intervention with PPAR γ ligands may alter the fate of circulating myofibroblast precursors before they ever reach the lung.

7.2. PPAR γ Ligands in Animal Models of Lung Fibrosis. There are currently only a few published reports investigating PPAR γ ligands in animal models of fibrosis, although additional studies are in progress. Troglitazone given orally (200 or 400 mg/kg/day) inhibited bleomycin-induced lung fibrosis in mice, with reductions in lung collagen content and TGF β levels [80]. Rosiglitazone and 15d-PG₂ were also tested in the mouse bleomycin model and reduced mortality, inflammation, cellular influx, and fibrosis [85]. To investigate the PPAR γ dependence of the effect, mice were cotreated with bisphenol A diglycidyl ether (BADGE), a PPAR γ antagonist. BADGE reversed the antifibrotic effects of rosiglitazone and 15d-PG₂, suggesting that at least some of the antifibrotic activity of these compounds is mediated by a PPAR γ -dependent mechanism *in vivo* [86]. However, while BADGE has been reported to be a PPAR γ antagonist in some studies [85], it activates PPAR γ in others [87]. Interestingly, the stimulation of heme-oxygenase (HO-1) by 15d-PG₂ in rat primary cortical neuron cultures was blocked

by BADGE [88], but, in human lung fibroblasts, HO-1 induction was not blocked by another PPAR γ antagonist GW9662 [41]. This suggests that the same PPAR γ ligand may have dependent effects in some cell types and independent effects in others.

A different result was observed in a neonatal hyperoxia model. Premature infants requiring hyperoxia (100% O₂) during the first weeks of life suffer from a high rate of bronchopulmonary dysplasia (BPD), characterized by mild inflammation and fibrosis, and failed alveolarization. In neonatal mice, hyperoxia leads to upregulation of TGF β and Wnt signaling and downregulation of PPAR γ signaling owing to reduction in both PPAR γ mRNA and protein [89, 90]. While rosiglitazone markedly prevented lung injury in hyperoxic neonates, the effect was reversed by administration of GW9662, suggesting a PPAR γ -dependent mechanism [89]. It should be noted that BPD is thought to represent a failure of the developmental program, and therefore the signals and mechanisms involved in neonatal hyperoxic fibrosis may be different than the signals involved in fibrosis of fully differentiated adult lungs, and it would be interesting to study PPAR γ -independent effects of electrophilic ligands of PPAR γ in the neonatal animal models [91].

Additional study is needed to characterize the *in vivo* mechanism of action of PPAR γ ligands. Because the bleomycin model is characterized by acute early inflammation, it is possible that the majority of the antifibrotic effects seen in these early studies are a result of PPAR γ -mediated inhibition of inflammation that precedes fibrosis. Therefore, it will be important to investigate the effects of PPAR γ ligands in fibrosis models that do not also have overt inflammation. CDDO and its derivatives have strong antifibrotic effects *in vitro* at much lower concentrations than TZDs but have not yet been investigated *in vivo* for lung fibrosis. However, it is clear that PPAR γ ligands have strong therapeutic potential in these studies regardless of whether the effects are dependent or independent of PPAR γ -mediated transcriptional regulation.

8. Other Pulmonary Diseases

Rosiglitazone attenuates pulmonary arterial hypertension in an ApoE null mouse model fed a high-fat diet [92]. This appears to be PPAR γ -dependent as mice with targeted deletion of the PPAR γ gene in smooth muscle cell tissue spontaneously developed pulmonary arterial hypertension [92]. Furthermore, under hypoxia-induced pulmonary hypertension conditions, rosiglitazone prevents and reverses pulmonary hypertension by downregulating Nox4 [93].

Acute promyelocytic leukemia (APL) is a subtype of acute myelocytic leukemia in which myeloid precursor cells grow aberrantly and can infiltrate into the pulmonary tract causing severe disease. APL is characterized by a genomic translocation fusing the PML tumor suppressor and the retinoic acid receptor α gene. While APL is normally treated with all-trans retinoic acid (ATRA), cells can become resistant to this therapy, thus novel drug cocktails are needed. CDDO has been shown to increase proapoptotic

and differentiating effects of ATRA in APL-derived NB4 cells and partially reverse ATRA resistance in ATRA-resistant NB4-derived cells [94]. In a mouse model of APL initiated by a PML-RAR α transgene, CDDO and ATRA cotreatment significantly increased animal survival rates by compared to ATRA treatment alone. The PPAR γ selective antagonist T0070907, or PPAR γ siRNA only partially impaired the effects of CDDO treatment, suggesting both PPAR γ -dependent and -independent effects of CDDO in APL cells. Another report suggested beneficial, proapoptotic effects of ciglitazone in APL [95]. Ciglitazone treatment caused a significant increase in APL cell apoptosis demonstrated by DNA fragmentation analysis, increased active caspase-3, cleaved PARP, and decreased expression of the X-linked inhibitor of apoptosis protein. Notably, the effects of ciglitazone were reversed by treatment with GW9662, suggesting a PPAR γ -dependent mechanism.

9. Potential Therapies

Animal models clearly show extraordinary translational potential for PPAR γ ligands to serve as either preventative therapies or cotreatments for lung malignancies. Recent clinical studies also suggest favorable outcomes in lung cancer patients treated with PPAR γ ligands. In a clinical study encompassing patients with diabetes, lung cancer occurrence in patients receiving TZD therapy was decreased by 33% compared to patients not taking a TZD [96]. Notably, the decrease in lung carcinomas was still evident after confounding factors (i.e., age and race/ethnicity) were accounted for. At the present time, clinical trials are underway to investigate the effectiveness of TZDs in the prevention and/or treatment of lung cancer, either alone, or in combination with other chemotherapies. It is important to note that TZDs show both PPAR γ -dependent and -independent modes of action and further exploration of their mechanism of action is needed to elucidate their specific targets. CDDO and its derivatives have proapoptotic and antiproliferative effects on cancer cells *in vitro* and are currently being investigated in phase 2 clinical trials for treatment of cancer [97]. Further studies are required to determine if CDDO-Me will also be useful as a myelosuppressive agent in combination with chemotherapies.

Currently, there are no PPAR γ ligands in clinical trial for fibrotic lung diseases. However, there is evidence in other organ systems suggesting that PPAR γ ligands may have antifibrotic potential in human lung fibrosis. Intriguingly, kidney remodeling is a frequent complication of diabetes, and improvements in renal function have been noted in patients with type II diabetes treated with TZDs [98, 99]. The use of TZDs in clinical therapy may be limited by undesirable side effects. However, unlike diabetes, which can often be managed with alternate therapies, there are no current treatments for IPF and the median survival time after diagnosis is only 2-3 years, which may shift the risk-benefit assessment in favor of use.

In addition to lung cancer and fibrosis, pioglitazone and rosiglitazone are in ongoing clinical trials for

asthma (e.g., NCT01134835 and NCT00614874), cystic fibrosis (e.g., NCT00322868 and NCT01060566), pulmonary hypertension (e.g., NCT00825266 and NCT00006071), and pulmonary arterial disease (e.g., NCT00153166 and NCT00064727). These studies will evaluate whether the *in vitro* anti-inflammatory effects of PPAR γ ligands can be translated to improved patient outcomes.

Current PPAR γ ligands act via both PPAR γ -dependent and -independent mechanisms, and the impact of both pathways must be considered in evaluating their efficacy in animal models and clinical trials. However, the existence of PPAR γ -independent effects creates the potential for development of novel non-PPAR-based therapies that access the independent pathways uncovered by PPAR γ ligands. In this event, currently available PPAR γ ligands with strong PPAR-independent effects, such as 15d-PGJ₂ and CDDO, may play a more important role as probes for novel disease modifying pathways than as direct clinical treatments, paving the way for new molecules that target critical pathways (such as Akt, FAK, ERK, and JNK) without also activating PPAR γ .

It is interesting to note that some of the PPAR γ -independent effects of PPAR γ ligands are mediated by modulating the phosphorylation status of key regulatory enzymes such as Akt, FAK, ERK, and JNK. Imatinib mesylate (Gleevec), a broad-spectrum tyrosine kinase inhibitor currently approved to treat some cancers, has been tested in several small open-label trials in systemic sclerosis, a fibrosing disease that often involves the lung. In some studies, imatinib improved lung function and reduced skin contraction, although the number of adverse effects was high [100, 101]. However, in a randomized, placebo-controlled trial of patients with mild to moderate IPF, imatinib did not significantly reduce disease progression [29]. These studies demonstrate the clinical potential as well as limitation of antikinase treatments in lung fibrosis and encourage further exploration of the non-PPAR molecular targets of PPAR γ ligands.

10. Conclusion

Discovered serendipitously, the off-target, or PPAR γ -independent effects of PPAR γ ligands may prove as interesting and therapeutically useful as their PPAR γ -dependent effects. PPAR γ agonists have potent PPAR γ -independent effects *in vitro* and *in vivo*, regulating proinflammatory responses and acting to promote apoptosis and inhibit differentiation, which may be beneficial in treating cancer and fibrosing diseases. Clinical trials are underway, investigating the currently approved TZDs in novel diseases, and investigating novel agonists such as CDDO and its derivatives. Significant clinical progress can be expected in the next few years.

The most complex problem associated with the use of PPAR γ agonists as disease therapies is indeed the combination of potent PPAR γ -dependent and -independent activities they exhibit. This presents both multiple challenges and multiple opportunities for translational investigation. The PPAR γ -dependent and independent effects can be decoded with specificity using *in vitro* models, but the results are

difficult to confirm *in vivo* because homozygous PPAR γ deletion is lethal. And, of course, patients will be subjected to both the dependent and independent effects of any new drugs, regardless of their primary mode of action *in vitro*. Thus, it will be critically important to carefully evaluate both the PPAR γ -dependent and -independent therapeutic effects and side effects of any new therapies.

Some PPAR γ ligands exhibit PPAR γ -dependent and -independent effects that act in the same direction, such as the antifibrotic activities of the TZDs. Unfortunately, the TZDs carry the risk of significant side effects including edema, weight gain, and cardiovascular effects. Troglitazone (Rezulin) has been withdrawn from the market due to hepatotoxicity [102], while rosiglitazone (Avandia) has been withdrawn in Europe and is under restriction in the US due to cardiovascular effects [103]. In the context of pulmonary disease, while there are alternative therapies for diabetes, there are no effective current therapies for lung fibrosis, and despite advances in chemotherapy, the five-year survival rate for NSCLC is only 15%. Thus, it is possible that these drugs will be revived for use in these deadly disease, either alone or in combination with other therapies. Alternatively, direct lung administration by inhalation may increase the effectiveness of the drugs while sparing the patient from systemic side effects, as is the case with inhaled steroids for asthma.

Also promising is the ability to use PPAR γ ligands as probes to uncover novel disease regulatory pathways which can then be targeted by new, specific therapies. Cyclin D1, Keap-1, Akt, and FAK are examples of disease targets that have been identified through the PPAR γ -independent effects of PPAR γ ligands. It is now possible, through mass spectrometry and other techniques, to determine exactly how these compounds bind to their targets and alter their function. This should allow the development of new compounds that have specific targeting activity against their new targets, while eliminating or reducing their affinity for PPAR γ and thus reducing or eliminating PPAR-dependent activity and its associated side effects. As these ligands enter clinical trials, there is an urgent need to understand their PPAR-dependent and -independent mechanisms of action for the future of targeted and personalized medicines.

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Review Article

PPAR γ as a Potential Target to Treat Airway Mucus Hypersecretion in Chronic Airway Inflammatory Diseases

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Airway mucus hypersecretion (AMH) is a key pathophysiological feature of chronic airway inflammatory diseases such as bronchial asthma, cystic fibrosis, and chronic obstructive pulmonary disease. AMH contributes to the pathogenesis of chronic airway inflammatory diseases, and it is associated with reduced lung function and high rates of hospitalization and mortality. It has been suggested that AMH should be a target in the treatment of chronic airway inflammatory diseases. Recent evidence suggests that a key regulator of airway inflammation, hyperresponsiveness, and remodeling is peroxisome proliferator-activated receptor gamma (PPAR γ), a ligand-activated transcription factor that regulates adipocyte differentiation and lipid metabolism. PPAR γ is expressed in structural, immune, and inflammatory cells in the lung. PPAR γ is involved in mucin production, and PPAR γ agonists can inhibit mucin synthesis both *in vitro* and *in vivo*. These findings suggest that PPAR γ is a novel target in the treatment of AMH and that further work on this transcription factor may lead to new therapies for chronic airway inflammatory diseases.

1. Introduction

Airway mucus hypersecretion (AMH) is a common pathological feature in chronic airway inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF). Growing studies have suggested that AMH is associated with the progression of chronic airway inflammatory diseases and it is a significant contributor to morbidity and mortality. To control, AMH plays an important role in the treatment of chronic airway inflammatory diseases; however, effective therapies that target AMH are lacking [1, 2].

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors belonging to the nuclear hormone receptor family. PPARs are related to retinoid, glucocorticoid, and thyroid hormone receptors [3]. They regulate diverse physiological processes, such as lipid biosynthesis and glucose metabolism, by binding to sequence-specific PPAR response elements in the promoter region of target genes. Recently, PPARs and their ligands have been found to inhibit the expression of proinflammatory

genes, implicating them as regulators of immune and inflammatory responses. Several studies have demonstrated that PPAR ligands possess anti-inflammatory properties that may prove useful in the treatment of inflammatory lung diseases [4–6].

PPAR γ is the most extensively studied PPAR subtype. It is involved in a series of lung diseases, including lung fibrosis, pulmonary vascular diseases, acute lung injury, and lung cancer [7–10]. Besides its regulatory role in adipocyte differentiation, glucose, and lipid metabolism, PPAR γ activation reduces the synthesis and release of immunomodulatory cytokines from various cell types that participate in the regulation of inflammatory and immune processes. Specifically, mounting evidence suggests that PPAR γ plays important roles in regulating processes related to airway inflammation, airway remodeling, and airway hyperresponsiveness, indicating that PPAR γ and its ligand show potential as targets to develop treatments for chronic airway inflammatory diseases [11]. In fact, PPAR γ has been implicated in AMH. Recent studies have shown that chronic airway inflammatory diseases in humans are associated with altered PPAR γ

expression and that PPAR γ ligand can attenuate AMH in both *in vitro* and *in vivo* experimental models. This paper will summarize recent work implicating PPAR γ in AMH.

2. AMH and Chronic Airway Inflammatory Diseases

2.1. Significance of AMH. Mucus secretion is essential for protecting airways. It is vital for air humidification, warming, and cleaning [12]. However, in patients with chronic airway inflammatory diseases, excessive mucus is secreted into the airway, which leads to hospitalization and death in many patients. This hypersecretion, termed AMH, obstructs the airways, limits airflow, impairs gas change, and causes ventilation-perfusion mismatch. Patients with chronic airway inflammatory diseases that also present with AMH generally have poor lung function and high rates of hospitalization and death. Compromised mucociliary function can reduce mucus clearance, which can encourage bacterial colonization, leading to chronic pulmonary infections and exacerbations. In addition, chemicals synthesized by bacteria or released when bacteria are degraded by the immune system can stimulate mucin synthesis and mucus secretion, resulting in a vicious circle [1, 13].

Studies suggest that AMH is not merely a clinical symptom but instead a critical factor in the pathogenesis of chronic airway inflammatory diseases. AMH is associated with greater susceptibility to COPD, a decline in forced expiratory volume in one second, hospitalization, and excess mortality [14–16]. The incidence of COPD in young adults presenting with AMH symptoms is three-fold higher than that in subjects who have never reported AMH symptoms, regardless of their smoking habits [17]. These findings are supported by an analysis of the Framingham offspring cohort studies [18].

AMH has long been recognized as a major cause of death in asthma. One study found luminal occlusions covering 20–100% of the cross-sectional area in patients who died from asthma, leading the authors to conclude that luminal obstruction of airways by an exudate composed of mucus and cells is a major contributor to asthma fatality [19]. In addition, AMH symptoms such as chronic cough or phlegm are independently and significantly related to uncontrolled asthma, and a history of persistent symptoms related to AMH is associated with a more severe asthma phenotype [20]. AMH also contributes to morbidity due to cystic fibrosis (CF). In CF patients, the reduced ability of epithelial cells to secrete chloride usually leads to reduced mucus accumulation in the airways, which favors chronic infection by *Pseudomonas aeruginosa* and other organisms. AMH contributes to CF morbidity by increasing the frequency and severity of pulmonary infections as well as by impairing lung function [21].

2.2. Inflammation and AMH. Abnormal inflammatory response is the major component of chronic airway inflammatory diseases. Such inflammatory response has been associated with AMH in airway diseases. Numerous studies

have demonstrated that inflammatory stimuli and mediators/cytokines contribute to excess mucin synthesis and mucus secretion in airways. For instance, cigarette smoke, neutrophil elastase, and *P. aeruginosa* proteases are well-known inflammatory stimuli that can cause significant inflammatory responses, which result in goblet cell metaplasia and hyperplasia, leading in turn to mucin overproduction and mucus hypersecretion in airways [22–24]. Inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, IL-8, IL-13, and IL-17 can upregulate the expression of MUC5AC, the marker of goblet cell metaplasia, through different signaling pathways [25–29]. This is the most important event in the pathogenesis of AMH. Thus, the ability to control airway inflammatory responses should reduce AMH and thereby benefit patients with chronic airway inflammatory diseases. However, current anti-inflammatory treatments with corticosteroids are not effective in all patients with chronic airway inflammatory diseases. Thus, the search for novel drug targets for these diseases continues.

In summary, AMH arising as a result of inflammatory processes can lead to physiologically and clinically measurable mechanical airway obstruction in patients with chronic airway inflammatory diseases. In this way, AMH significantly affects the pathogenesis, progression, and prognosis of these diseases. Therapies that aim to control the inflammation that triggers AMH may be effective at treating chronic airway inflammatory diseases [30–32].

3. Expression and Anti-Inflammatory Role of PPAR γ in the Lung

3.1. Expression of PPAR γ in Lung Cells. PPAR γ is expressed in many types of structural cells in the lung, including fibroblasts, ciliated airway epithelial cells, alveolar type II pneumocytes, and airway smooth muscle cells [3, 4, 32]. A study in PPAR γ -knockout mice has highlighted the role of this transcription factor in animal models. A specific PPAR γ deletion in airway epithelium produced persistent enlargement of the airspaces in adult mice, which showed more severe emphysema symptoms and higher macrophage numbers after exposure to cigarette smoke than did mice carrying the PPAR γ gene [33]. These results suggest that epithelial PPAR γ is required for proper lung maturation and response to injury.

PPAR γ is also present in several types of inflammatory, immune cells in the lung, where it exerts anti-inflammatory and immuno-modulatory effects. Specially, PPAR γ has been found in macrophages, eosinophils, T cells, B cells, and dendritic cells [34–38]. It plays an anti-inflammatory role in lung cells mainly by inhibiting cytokine production and enhancing phagocytosis of apoptotic neutrophils, a process essential to resolve inflammation.

Increasing evidence suggests that altered PPAR γ expression and function may be associated with chronic airway inflammatory diseases and that stimulation of this receptor may attenuate inflammatory responses. A study examining the expression of PPAR γ in lung tissues of patients with COPD found the protein to be present mainly in alveolar

epithelial cells and smooth muscle cells in the bronchial walls. The intensity of PPAR γ was inversely proportional to the severity of the COPD, with the signal stronger in patients with milder COPD [39]. Another study found that PPAR γ is expressed in CF- and non-CF-type human airway epithelial cells, but appears to be either less abundant, less functional in binding its target DNA sequence, or both, in CF [40]. In asthmatic individuals, but not healthy objects, segmental allergen challenge led to downregulation of PPAR γ mRNA and protein expression in alveolar macrophages [41]. These findings are suggestive of a role for PPAR γ agonists in the treatment of chronic lung inflammatory diseases.

3.2. The Anti-Inflammatory Role of PPAR γ . Building on the evidence linking PPAR γ and airway inflammatory diseases, several studies have investigated the therapeutic role of the receptor and its ligand in cellular and animal models of these diseases. Administration of PPAR γ ligand to BALB/c mice sensitized and challenged with ovalbumin (OVA) reduced the levels of proinflammatory mediators in bronchoalveolar lavage fluid (BALF) and attenuated the inflammatory response in the lung [35, 42, 43]. Treating human airway epithelial cell line A549 with the PPAR γ agonist troglitazone blocked the ability of phorbol 12-myristate 13-acetate to stimulate an increase in TNF- α levels. However, this effect of troglitazone required the presence of MUC1/Muc1 [44]. The authors concluded that PPAR γ exerts an anti-inflammatory effect by stimulating MUC1/Muc1 expression, which then blocks the production of TNF- α /IL-8 induced by phorbol 12-myristate 13-acetate in airway epithelial cells [44]. In addition, PPAR γ plays an essential role in the pathway through which monocyte/macrophage-derived microparticles activate NF- κ B and ultimately induce upregulation of proinflammatory mediators in human lung epithelial cells [45].

The anti-inflammatory effect of PPAR γ is thought to be due mainly to its ability to downregulate proinflammatory gene expression. It does so by sequestering shared coactivators, reducing the ability of inflammatory transcription factors to bind their target DNA. The second way is through ligand-dependent transrepression, preventing other transcription factors' association with DNA sequences. At the same time, PPAR γ can increase the transcription of anti-inflammatory genes. Agonist binding to PPAR γ induces a conformational change in the receptor, allowing the dissociation of corepressors and the association of coactivator molecules. This allows the formation of PPAR γ dimers or heterodimers and subsequent interaction with peroxisome proliferator response elements, which increases anti-inflammatory gene transcription [46].

Taken together, these findings suggest that PPAR γ participates in both physiological and pathological processes in chronic airway inflammatory diseases. These studies make a strong case for PPAR γ activation as a potential treatment for chronic airway inflammatory diseases.

4. PPAR γ and AMH

Since the inflammatory response is the major contributor to AMH, and PPAR γ and its ligand help regulate airway

inflammation, several studies have investigated the potential involvement of PPAR γ and its ligand in AMH.

Lee and colleagues carried out the first study to examine the role of PPAR γ ligand on mucin production in human airway epithelial NCI-H292 cells exposed to cigarette smoke [47]. Exposure caused a significant increase in TNF- α and MUC5AC production, which was attenuated by administration of the PPAR γ agonist rosiglitazone. Rosiglitazone increased the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and inhibited the PI3K/Akt pathway. PTEN antagonizes PI3K-mediated signaling, whose role in mucin production is well documented [47, 48]. These results provide the first evidence that a treatment targeting PPAR γ can significantly inhibit cigarette smoke-induced mucin production, raising the possibility that this approach may be useful in the clinic. Indeed, the study authors proposed that using a PPAR γ agonist to activate PTEN and inhibit PI3K/Akt may be a potential therapy for cigarette smoke-induced mucin secretory diseases [47].

Subsequent studies evaluated the effect of rosiglitazone on airway mucin production in animal models of AMH. Exposing rats for two weeks to acrolein, one of the most toxic components in cigarette smoke, induced goblet cell hyperplasia in bronchial epithelium and upregulated MUC5AC mRNA and protein expression in rat lungs. These changes were associated with airway inflammation, as evidenced by the increased numbers of inflammatory cells and levels of inflammatory cytokines (IL-1 β , IL-8, and TNF- α) in BALF. Treating the rats with rosiglitazone before acrolein exposure attenuated these changes in a dose-dependent manner [49]. Rosiglitazone has also been shown to inhibit NF- κ B activation, which is well known to play an important role in regulating not only multiple inflammatory cytokines but also AMH in inflammatory lung diseases [50, 51]. Therefore, inhibition of NF- κ B activation by rosiglitazone may explain the observed downregulation of proinflammatory mediators and the reduction in AMH. From these findings, Liu et al. concluded that ligand-induced activation of PPAR γ may be a novel therapy for AMH in chronic airway inflammatory diseases such as COPD [49].

AMH is also an important pathologic feature of asthma, which is characterized by nonspecific airway inflammation. Several studies have examined the involvement of PPAR γ and its ligand on AMH in a mouse model of asthma. AMH was observed in airways of animals sensitized and challenged with OVA, while it was barely detectable in airways of nonsensitized animals. Administration of the PPAR γ agonist ciglitazone via nebulizer reduced OVA-induced mucus gland hyperplasia and airway occlusion due to mucus hypersecretion by approximately 75% [52]. In a different mouse model, mice were exposed or not to toluene diisocyanate to induce asthma. Exposed mice that received PPAR γ agonists (rosiglitazone or pioglitazone) or adenovirus carrying PPAR γ 2 cDNA showed a lower percentage of airway epithelium staining positive with PAS than did exposed mice that did not receive agonists or adenovirus [53]. In addition, both of these studies found that PPAR γ agonists support protective remodeling of the airway wall by regulating TGF- β or TGF- β 1 and collagen deposition. Results from both of

these mouse models support the possibility of using PPAR γ agonist to treat AMH in humans.

In addition to these observations in cell lines or animal models, several lines of indirect evidence link activation of PPAR γ and AMH. Activation of PPAR γ by rosiglitazone or pioglitazone markedly reduced mRNA expression of matrix metalloproteinase-9 (MMP-9) and inhibited TNF- α induced MMP-9 gelatinolytic activity [54]. Since MMP-9 is an important MMP related to AMH [55], these results suggest that PPAR γ may attenuate AMH through a pathway involving MMP-9. Simvastatin is a statin that modulates inflammatory processes and that can inhibit mucin production and AMH [56]. A recent study indicated that its anti-inflammatory effect may involve PPAR γ activation [57], which means that simvastatin may suppress AMH via a mechanism mediated by PPAR γ . It seems clear from these diverse lines of evidence that the effects of PPAR γ and its ligand on AMH are mediated by multiple mechanisms. Further work is needed to identify new ligands of PPAR γ and investigate the signaling pathways affected by the receptor.

In summary, substantial evidences indicate that PPAR γ is involved in AMH and that PPAR γ ligand suppresses AMH by inhibiting proinflammatory cytokines and inflammatory signaling pathways. Therefore, PPAR γ agonists may have potential as treatments for AMH in chronic airway inflammatory diseases.

5. Considerations for Targeting PPAR γ in the Treatment of AMH

Although several studies have suggested that PPAR γ is involved in AMH and that PPAR γ agonists inhibit AMH, there is still a long road ahead before laboratory studies can be translated to the clinic. For one thing, the pathogenesis of chronic airway inflammatory diseases such as COPD, asthma, and CF is extremely complicated. Only a few studies, most of them descriptive, have directly investigated the role of PPAR γ and PPAR γ ligand in AMH. As a result, the signaling pathways affected by PPAR γ or PPAR γ agonists remain poorly understood. Further investigation of these areas will be required to define the effects of PPAR γ ligands on airway epithelial cells and the mechanisms by which these pro- and/or anti-inflammatory responses occur.

A second consideration for targeting PPAR γ in the clinic is that the route of PPAR γ agonist administration may be important. In Honda's study [52], treating Balb/c mice with nebulized ciglitazone led to a reduction in mucus production in the airways, whereas orally administered ciglitazone had no such effect [58]. Oral gavage of rosiglitazone or pioglitazone reduced toluene diisocyanate-induced AMH in Balb/c mice [53], while oral rosiglitazone treatment had no effect on goblet cell hyperplasia in C57BL/6 mice [59]. Future work should clarify whether the different results observed with different agonists or models of administration reflect differences in the sensitivity of mouse strains to regulation of airway inflammation and AMH, or whether agonist identity and mode of administration are crucial.

Finally, the safety of PPAR γ agonists in patients with chronic airway inflammatory disease should be carefully

examined. Recent studies suggest an association between rosiglitazone treatment and increased risk of cardiovascular events in patients with type 2 diabetes [60, 61]. Thus it remains to be seen whether PPAR γ agonist is safe for patients with chronic airway inflammatory diseases.

6. Summary

The present paper describes the clinical importance of AMH in the pathogenesis of chronic airway inflammatory diseases and makes the case for exploring PPAR γ and PPAR γ ligand as potential target in treating AMH. Corticosteroid is the first-line anti-inflammatory drug used to chronic airway inflammatory diseases, but its therapeutic effects are controversial, and physicians have begun to pay attention to its undesirable side effects [62]. In fact, it is generally accepted that steroids have only limited effects on AMH [63], so there is a demand for novel and effective anti-inflammatory drugs that can reduce AMH. Increasing evidence suggests that PPAR γ is involved in mucin production and that PPAR γ agonists inhibit AMH both *in vitro* and *in vivo*. Indeed, a recent clinical trial found that rosiglitazone improved lung function in steroid-resistant asthma patients [64], providing direct evidence that patients with chronic airway inflammatory diseases can benefit from PPAR γ agonist treatment. Thus, PPAR γ agonists may represent a novel class of pharmacological agents useful in the management of AMH in chronic airway inflammatory diseases.

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