PPARγ agonists inhibit TGF-β induced pulmonary myofibroblast differentiation and collagen production: implications for therapy of lung fibrosis

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Pulmonary fibrosis is a general term covering several specific diseases with similar pathology. These diseases are progressive and are characterized by accumulation of myofibroblasts and excessive deposition of extracellular matrix and connective tissue. A variety of different forms of pulmonary fibrosis exist, and many of these result in significant morbidity and mortality. Sadly, there are few if any effective therapies (9, 34). Transdifferentiation of myofibroblasts following exposure to a variety of stimuli, particularly TGF-β (29, 33, 34). Myofibroblasts are fibroblast-like cells with smooth muscle cell characteristics, particularly expression of α-smooth muscle actin (α-SMA). They are one of the major sources of extracellular matrix proteins, especially collagen, as well as fibrogenic cytokines and chemokines. The accumulation of fibroblasts and myofibroblasts and their production of extracellular matrix proteins, e.g., collagen, result in significant damage to the lung architecture and gas exchange abnormalities (29, 30, 34, 38). During normal wound repair, myofibroblasts undergo apoptosis and are removed from the healing area. However, in progressive fibrosis, myofibroblasts fail to apoptose and can persist within the fibrotic scar, perpetuating the scarring process (29, 38). A potential novel and exciting therapy to inhibit scarring is to prevent the conversion of fibroblasts to myofibroblasts and/or to decrease myofibroblast production of collagen and other extracellular matrix proteins. This can be studied in vitro by a model system using TGF-β to drive the differentiation of cultured primary human pulmonary fibroblasts to myofibroblasts (33).

Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor. After ligation with its agonist, PPARγ heterodimerizes with the retinoid X receptor (RXR). This complex recognizes PPAR response elements (PPRE) in promoters on target genes resulting in the regulation of gene transcription (3, 5, 36). PPARγ agonists have novel effects on fibroblast differentiation by promoting their conversion to adipocytes (13, 21). Over the past decade PPARγ agonists have received attention for their ability to regulate adipocyte differentiation and to increase insulin sensitivity in diabetic patients (8, 25, 36). 15-DeoxyΔ12,14-prostaglandin J2 (15d-PGJ2) is a potent naturally occurring PPARγ agonist. It is a spontaneously derived end product from prostaglandin D2 (3, 17). A variety of synthetic PPARγ agonists have now been developed for the treatment of diabetes. These drugs include the thiazolidinediones (TZDs): trioglitazone, rosiglitazone, ciglitazone, and pioglitazone (8, 25, 37). Rosiglitazone is the one of the most specific TZDs for PPARγ (8, 25) and is currently in clinical use as Avandia (GlaxoSmithKline) for therapy of Type II diabetes.
There are important new indications that PPARγ agonists have a role in modulating inflammation (5–7, 36). PPARγ has anti-inflammatory activities, including the repression of the NF-κB and activator protein (AP)-1 pathways (5, 6, 10). We hypothesize that PPARγ agonists have antifibrotic properties by inhibiting pulmonary myofibroblast differentiation and collagen production. In the current study we provide novel data demonstrating the ability of both endogenous and synthetic PPARγ agonists to block key TGF-β-mediated profibrotic effects, including pulmonary myofibroblast differentiation and excess collagen production. These activities support the concept that PPARγ agonists may have exciting potential for therapy of currently untreatable fibrotic lung diseases.

METHODS

Cells and reagents. Normal human lung fibroblast cell strains derived by explant technique were maintained in MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Sigma Aldrich, St. Louis, MO). These explants were derived from anatomically normal lung tissue from patients undergoing surgical resection for benign hamartoma or for small peripheral nodules (20). These cells are morphologically consistent with fibroblasts and express collagen and vimentin. They do not express CD45, factor VIII, or cytoketatin. Cells were used at passages 4–10. The PPARγ agonists ciglitazone and 15d-PGJ2 (Biomol, Plymouth Meeting, PA) and rosiglitazone (20 μM). WY-14643, a PPARγ agonist, and GW-9662 (Cayman Chemical, Ann Arbor, MI) were prepared as 10 mM stocks in DMSO and added to cell cultures to the final concentrations indicated, Wy-14643 (Biomol), a PPARα agonist, and GW-9662 (Cayman Chemical), an irreversible PPARγ antagonist, were prepared in the same manner. DMSO was added to negative control wells at a final concentration of 0.1%. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). A monoclonal α-SMA antibody (Sigma Aldrich) was used for both Western blots and immunocytochemistry (ICC). Western blots were normalized to GAPDH (Abcam, Cambridge, MA). The PPARγ antibody recognizes PPARγ1 and 2 (Calbiochem); the RXR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognizes RXRα, β, and γ and does not cross-react with retinoic acid receptor (RAR).

Detection of PPARγ and RXR protein. Five normal primary human pulmonary fibroblast cultures from different patients were analyzed for PPARγ and RXR protein expression. Lysates containing 10 μg of protein and 500 ng of human adipose tissue as a control were a final concentration of 0.1%. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). A monoclonal α-SMA antibody (Sigma Aldrich) was used for both Western blots and immunocytochemistry (ICC). Western blots were normalized to GAPDH (Abcam, Cambridge, MA). The PPARγ antibody recognizes PPARγ1 and 2 (Calbiochem); the RXR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognizes RXRα, β, and γ and does not cross-react with retinoic acid receptor (RAR).

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agonists block lung myofibroblast differentiation

Response element-luciferase reporter and a fibroblasts. Primary human lung fibroblasts were cotransfected with a PPAR agonists treated with either 15-deoxy-
12,14-prostaglandin J2 (15d-PGJ2, 10 μM). The PPAR agonist 15d-PGJ2 inhibited >95% of the TGF-β stimulated α-SMA induction, whereas rosiglitazone inhibited 40% of the TGF-β stimulated α-SMA induction (Fig. 3B). PPARγ agonists inhibit production of type I collagen in human lung fibroblasts treated with TGF-β. Collagen is one of the major extracellular matrix component in fibrotic tissues (29). To determine whether PPARγ agonists inhibit pulmonary fibroblast production of collagen, cells were treated with TGF-β and PPARγ agonists and collagen protein was assayed (Fig. 3A). The most potent α-SMA suppression was observed with 15d-PGJ2 treatment. The synthetic PPARγ agonist rosiglitazone also significantly decreased expression of α-SMA protein. Again, the PPARα agonist did not reduce α-SMA protein expression (Fig. 3A). Densitometry analysis normalized to GAPDH indicates lung fibroblasts treated with TGF-β exhibited a 25-fold increase in α-SMA expression over untreated control fibroblasts, an indication of myofibroblast differentiation (Fig. 3B). PPARγ agonist 15d-PGJ2 inhibited >95% of the TGF-β stimulated α-SMA induction, whereas rosiglitazone inhibited 40% of the TGF-β stimulated α-SMA induction (Fig. 3B).

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PPARγ agonists block lung myofibroblast differentiation. To determine whether PPARγ agonists block TGF-β-induced differentiation of lung fibroblasts to myofibroblasts in vitro, we selected two different primary human lung fibroblast strains and treated them with TGF-β and the PPARγ agonists 15d-PGJ2, ciglitazone, or rosiglitazone at different concentrations. Representative data from one fibroblast strain are shown. Myofibroblast differentiation was assessed by ICC and Western blot analysis for α-SMA, a marker of myofibroblast differentiation. Cultured fibroblasts treated with media alone had a few constitutively positive α-SMA cells (Fig. 2A), whereas fibroblasts treated with TGF-β demonstrated significant differentiation to myofibroblasts with an increase in α-SMA expression (Fig. 2B). All three PPARγ agonists inhibited myofibroblast differentiation in both fibroblast strains tested. 15d-PGJ2 and rosiglitazone were the most effective, as seen by the decrease in the number of α-SMA positive cells (Fig. 2, E–H). Treatment with a PPARα agonist (WY-14643) did not inhibit TGF-β-driven myofibroblast differentiation (Fig. 2D).

These results were confirmed by Western blot analysis of α-SMA protein from parallel fibroblast cultures. Cells treated with TGF-β expressed the highest levels of α-SMA protein.

Fig. 1. A: human lung fibroblasts express peroxisome proliferator-activated receptor (PPAR)-γ and retinoid X receptor (RXR) protein. Five normal primary human fibroblast cultures (lanes 1–5) were analyzed by Western blot for expression of PPARγ and RXR proteins. PPARγ and RXR are present in 10 μg of human pulmonary fibroblast lysates at 64 and 65 kDa, respectively. B: the PPARγ transcriptional activation system is active in primary lung fibroblasts. Primary human lung fibroblasts were cotransfected with a PPAR response element-luciferase reporter and a β-galactosidase control plasmid and treated with either 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2, 10 μM), rosiglitazone (Rosi, 20 μM), or DMSO vehicle (Untxt) for 24 h. Luciferase activity was normalized to β-gal activity, and the luciferase activity of mock-transfected cells was subtracted from all values. The results shown are the means ± SD of triplicate wells. Both 15d-PGJ2 and rosiglitazone activate PPARγ-dependent transcription (*P ≤ 0.03).

Fig. 2. Differentiation of primary human lung fibroblasts to myofibroblasts is inhibited by PPARγ agonists. Cultures of primary human lung fibroblasts in chamber slides were treated with 5 ng/ml TGF-β and PPARγ agonists for 72 h, then stained with an α-smooth muscle actin (SMA) antibody and counterstained with hematoxylin. Positively stained cells for α-SMA appear red. A: no treatment; B: transforming growth factor (TGF)-β; C: TGF-β and 0.1% DMSO; D: TGF-β and 25 μM WY-14643; E: TGF-β and 10 μM 15d-PGJ2; F: TGF-β and 25 μM ciglitazone; G: TGF-β and 10 μM rosiglitazone; H: TGF-β and 20 μM rosiglitazone. The panels shown are representative of 3 independent experiments with at least 2 chambers per treatment per experiment.
by ELISA. Uncontracted normal human pulmonary fibroblasts synthesize little collagen. However, treatment with TGF-β significantly increased type I collagen production in human lung fibroblasts (Fig. 4). The PPARα agonist WY-14643 had no effect on the production of type I collagen. In contrast, 15d-PGJ2 and rosiglitazone significantly inhibited TGF-β-induced synthesis of type I collagen by lung fibroblasts compared with the TGF-β-treated cells to P < 0.01 (Fig. 4).

Cell viability is not affected by PPARγ agonists. One simple explanation for the reduction in α-SMA and collagen production is that PPARγ agonists are toxic. To rule out this possibility, viability of human lung fibroblasts treated with PPARγ agonists was measured by MTT assay. Viable cells actively cleave the MTT reagent and form a colored precipitate the appearance of which is proportionate to the number of viable cells (27). Results are shown as a percentage of the negative control (untreated cells). As Fig. 5 demonstrates, there was no evidence of cell toxicity in fibroblasts exposed to PPARγ agonists. Primary human lung fibroblasts were treated in quadruplicate with TGF-β and PPARγ agonists at the previously described concentrations for 72 h, and type I collagen production was measured by ELISA. Assays were performed on quadruplicate wells. *Treatments were statistically significant according to a Student’s unpaired t-test to P < 0.01 compared with TGF-β-treated cells.

Overexpression of a dominant-negative PPARγ protein partially reverses PPARγ agonist-induced inhibition of myofibroblast differentiation. Some anti-inflammatory effects of PPARγ agonists may be independent of PPARγ activation (6, 10). To determine whether the effects of PPARγ agonists on myofibroblast differentiation are dependent or independent of PPARγ activation we used a dominant-negative approach. Primary human lung fibroblasts were transfected with a replication-deficient adenovirus expressing a mutated dominant-negative PPARγ gene that binds to the ligand but does not activate transcription at the PPRE (28). An adenovirus with no inserted transgene was used as a control. At an MOI of 30 pfu/cell, >90% of fibroblasts were infected (data not shown). The fibroblasts were then treated with TGF-β and PPARγ agonists to determine whether the dominant-negative PPARγ would prevent the inhibition of TGF-β-driven myofibroblast differentiation by PPARγ agonists. Myofibroblast differentiation was determined by ICC and Western blot analysis. Fibroblasts infected with the control virus and treated with TGF-β differentiated to myofibroblasts as expected (Fig. 6A), and differentiation was inhibited by 15d-PGJ2 (Fig. 6B) or rosiglitazone (Fig. 6C), as previously shown. Fibroblasts infected with the dominant-negative PPARγ adenovirus also differentiate to myofibroblasts when treated with TGF-β (Fig. 6D). However, the dominant-negative PPARγ blocks the ability of 15d-PGJ2 or rosiglitazone to inhibit myofibroblast differentiation (Fig. 6, E and F). This was confirmed by Western blot analysis (Fig. 6).
analysis (Fig. 6, G and H). Fibroblasts treated with TGF-β and 15d-PGJ₂ expressed 11% of the amount of α-SMA expressed by cells treated with TGF-β alone. The dominant-negative virus increased α-SMA expression to 37% of the positive control, blocking 30% of the inhibitory effect of 15d-PGJ₂. Although rosiglitazone is less effective at inhibiting α-SMA expression (52% of the positive control), the dominant-negative virus increased α-SMA levels to 88% of control, blocking 75% of the effects of rosiglitazone.

Inhibition of myofibroblast differentiation by PPARγ agonists is largely via a PPARγ-dependent mechanism. To further investigate the PPARγ dependence of the antifibrotic effects of PPARγ agonists, fibroblasts were treated with TGF-β, 15d-PGJ₂, and GW-9662, a highly specific and irreversible PPARγ antagonist (15). GW-9662 completely inhibits 15d-PGJ₂-driven differentiation of human fibroblasts to adipocytes (C. W. O’Laughlin and R. P. Phipps, personal communication), a PPARγ-dependent process, as well as PPARγ-dependent gene transcription in human chondrosarcoma cells (26). However, GW-9662 had no effect on the ability of 15d-PGJ₂ to inhibit TGF-β driven myofibroblast differentiation and α-SMA expression (Fig. 7).

DISCUSSION

Diseases characterized by pulmonary fibrosis cause significant morbidity and mortality. For example patients with idiopathic pulmonary fibrosis have a median survival from the time of presentation of only 2.9 yr. Sadly, for most of these patients there are few if any effective therapies. By increasing our understanding of the pathogenesis of these diseases we can develop new targets for therapy. The pulmonary myofibroblast is an important cell in the development and progression of fibrosis because it synthesizes excessive extracellular matrix, increases lung contraction, and persists in the lung tissue as it fails to undergo normal apoptosis (29, 38). The myofibroblast is therefore an important potential therapeutic target in scarring of the lung as well as other tissues. Myofibroblasts can be transdifferentiated from fibroblasts in vitro by their exposure to the fibrogenic cytokine TGF-β (12, 33). Furthermore, we and others have shown that TGF-β is a potent stimulus for myofibroblast differentiation and induction of pulmonary fibrosis in vivo (22, 23, 31). This study clearly demonstrates that PPARγ agonists inhibit TGF-β stimulated myofibroblast development and collagen production in primary human pulmonary fibroblasts, highlighting PPARγ agonists as exciting potential antifibrotic therapies.

Data are presented illustrating for the first time that primary human pulmonary fibroblasts express abundant PPARγ and RXR proteins and are capable of PPARγ-dependent transcrip-

Fig. 6. Dominant-negative PPARγ protein reverses the PPARγ agonist inhibition of TGF-β-induced myofibroblast differentiation. A–F: following infection with a dominant-negative PPARγ gene (DN) or a control adenovirus (CV), primary human pulmonary fibroblasts were treated with TGF-β (5 ng/ml) and PPARγ agonists. After 72 h the cells were stained with α-SMA antibody and counterstained with hematoxylin. Positively stained cells for α-SMA appear red. A: control adenovirus (AdV) and TGF-β; B: control AdV, TGF-β, and 10 μM 15d-PGJ₂; C: control AdV, TGF-β, and 20 μM Rosi; D: dominant-negative adenovirus (DN AdV) and TGF-β; E: DN AdV, TGF-β, and 10 μM 15d-PGJ₂; F: DN AdV, TGF-β, and 20 μM Rosi. The photographs shown are representative of 3 independent experiments with at least 2 chambers per treatment per experiment. G: Western blot analysis of fibroblasts infected with either the control (CV) or the dominant-negative PPARγ adenovirus (DN) and treated with TGF-β and PPARγ agonists as above. Cell lysates were prepared for protein, and 2 μg of protein was separated on a 12% SDS gel and examined for expression of α-SMA and GAPDH. H: Western blots (3–4 independent cultures from 2 independent experiments per treatment group) were analyzed by densitometry normalized to GAPDH. Results are expressed as percentage of maximum α-SMA expression (in control AdV + TGF-β-treated fibroblasts). Means ± SE are indicated.
The inhibitory effect of 15d-PGJ2 on myofibroblast phenotype. ICC and Western blot analysis for the expression of α-SMA were used to confirm the inhibition of myofibroblast differentiation by 10 μM 15d-PGJ2 and 1 μM GW-9662. The inhibitory effect of 15d-PGJ2 on α-SMA expression is not reversed by GW-9662. 

Fig. 7. The irreversible PPARγ antagonist GW-9662 does not inhibit the antifibrotic effects of PPARγ agonists. Primary human lung fibroblasts were treated for 3 days with 5 ng/ml TGF-β to induce myofibroblast differentiation, with the addition of 15d-PGJ2 (10 μM) and GW-9662 (1 μM) as indicated. Myofibroblast differentiation was assessed by Western blotting for α-SMA. The inhibitory effect of 15d-PGJ2 on α-SMA expression is not reversed by GW-9662. 

The irreversible PPARγ antagonist GW-9662 does not inhibit the antifibrotic effects of PPARγ agonists. Primary human lung fibroblasts were treated for 3 days with 5 ng/ml TGF-β to induce myofibroblast differentiation, with the addition of 15d-PGJ2 (10 μM) and GW-9662 (1 μM) as indicated. Myofibroblast differentiation was assessed by Western blotting for α-SMA. The inhibitory effect of 15d-PGJ2 on α-SMA expression is not reversed by GW-9662.
TGF-β-stimulated increases in fibronectin mRNA. Understanding similar potential interactions of PPARγ and TGF-β in pulmonary fibroblasts will increase our understanding of the mechanisms by which PPARγ agonists have antifibrotic activities.

Overall, our results convincingly demonstrate that PPARγ agonists potently interrupt two of the most important profibrotic effects of TGF-β on normal human primary pulmonary fibroblasts, the induction of myofibroblasts, and stimulation of excess collagen production. These data are exciting and suggest that the PPARγ pathway is likely a very important future target for therapy of fibrosis of the lungs and other tissues. An added advantage of using PPARγ agonists as novel antifibrotics is that these drugs are currently available for use in patients with diabetes. This will potentially facilitate their rapid translation to use in patients with lung fibrosis, for whom few effective therapies currently exist.

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