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PPAR- γ agonists inhibit profibrotic phenotypes in human lung fibroblasts and bleomycin-induced pulmonary fibrosis

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Abstract

Pulmonary fibrosis is characterized by alterations in fibroblast phenotypes resulting in excessive extracellular matrix accumulation and anatomic remodeling. Current therapies for this condition are largely ineffective. Peroxisome proliferator-activated receptor- γ (PPAR- γ) is a member of the nuclear hormone receptor superfamily, the activation of which produces a number of biological effects, including alterations in metabolic and inflammatory responses. The role of PPAR- γ as a potential therapeutic target for fibrotic lung diseases remains undefined. In the present study, we show expression of PPAR- γ in fibroblasts obtained from normal human lungs and lungs of patients with idiopathic interstitial pneumonias. Treatment of lung fibroblasts and myofibroblasts with PPAR- γ agonists results in inhibition of proliferative responses and induces cell cycle arrest. In addition, PPAR- γ agonists, including a constitutively active PPAR- γ construct (VP16-PPAR- γ), inhibit the ability of transforming growth factor- β 1 to induce myofibroblast differentiation and collagen secretion. PPAR- γ agonists also inhibit fibrosis in a murine model, even when administration is delayed until after the initial inflammation has largely resolved. These observations indicate that PPAR- γ is an important regulator of fibroblast/myofibroblast activation and suggest a role for PPAR- γ ligands as novel therapeutic agents for fibrotic lung diseases.

Keywords

troglitazone; ciglitazone; transforming growth factor

Pulmonary fibrosis occurs in a wide variety of illnesses, including systemic disorders as well as primary lung disease (25, 29,30, 34, 45). Fibrotic changes can also appear as sequelae to insults by a diverse group of infectious, environmental, and therapeutic exposures (20, 38, 40, 43). Some familial and sporadic forms are associated with genetic polymorphisms (58). In other cases, as in idiopathic pulmonary fibrosis (IPF), causation remains unknown (17, 62).

In general, pulmonary fibrosis is characterized by alveolar epithelial cell injury with failure of alveolar reepithelialization, followed by recruitment and persistence of fibroblasts that differentiate into myofibroblasts. These in turn lay down excessive collagen and extracellular matrix, resulting in distortions of the lung architecture that cause decreased gas exchange and reduced pulmonary compliance (19, 31, 62, 64, 65). The fibroblasts may derive from resident populations, a circulating fibroblast population, or hematopoietic or stromal and progenitor cells of the bone marrow. Cells exhibiting a myofibroblastic phenotype may also derive from epithelial or endothelial cells in the injured tissues. It is the myofibroblast that secretes collagen and extracellular matrix components characteristic of clinical fibrosis (13, 14, 48, 51, 71).

Since anti-inflammatory agents have proven largely ineffective, recent therapeutic attempts have targeted various components of the injury initiation and wound-healing process, including oxidative stress, apoptosis, epithelial replacement, angiogenesis, and chemokine and cytokine production (1, 3). Inasmuch as pulmonary fibroblasts and myofibroblasts appear to play a central role in the pathogenesis of the tissue fibrotic response (48, 57, 71), intervention in fibroblast proliferation and differentiation may be a key to treatment.

Peroxisome proliferator-activated receptor- γ (PPAR- γ) is a member of the nuclear hormone receptor family. PPAR- γ controls gene expression, in part by binding as a heterodimer with the retinoid X receptor to specific response elements [peroxisome proliferator response elements (PPREs)] within promoters. In the absence of ligands, the heterodimer binds corepressors that may limit gene expression. Binding of agonist ligands leads to a conformational change; corepressors dissociate and coactivators are bound, leading to an increased rate of gene transcription (5). PPREs regulate a diverse range of tissue effects, including adipocyte differentiation, glucose homeostasis, and inflammatory, immune, and proliferative responses; new functional clusters of genes under PPRE regulation have recently been described (36). In addition, activation of PPAR- γ may produce transrepression of non-PPRE genes by depleting the supply of coactivators required by other nuclear transcription factors (54).

The ligand-binding domain of PPAR- γ is large ($\sim 1,300 \text{ \AA}^3$) compared with other nuclear receptors and can dock a wide variety of compounds of diverse sizes (8, 37, 59). Natural agonists of PPAR- γ include fatty acids and fatty acid derivatives, although the physiologically crucial endogenous ligand or ligands remain unidentified (5, 27). Synthetic PPAR- γ agonists of the thiazolidinedione (TZD) class are used in the treatment of patients with type 2 diabetes mellitus. TZDs exert a number of biological effects that occur through PPAR- γ and independently of this receptor (23, 67).

Ligands of PPAR- γ inhibit activation of a variety of mesenchymal and fibroblast cell types (15, 18, 33, 39, 42, 55, 73). Specifically, they have been shown to block profibrotic actions of transforming growth factor (TGF)- $\beta 1$ by inhibiting binding of the nuclear transcription factor activator protein-1 (21), by interfering with the Smad2 and Smad3 signaling pathways, and by reducing phosphorylation of ERK1/2 (49, 72). Recent studies have demonstrated significant potential roles for PPAR- γ agonists with respect to pulmonary

pathobiology (28, 61). However, investigation of PPAR- γ agonists as in vitro and in vivo regulators of pulmonary fibrotic responses has been limited (6, 16).

In the present study, we examine the potential use of PPAR- γ agonists for treatment of pulmonary fibrosis by studying their effects on normal lung fibroblasts and fibroblasts isolated from patients with idiopathic interstitial pneumonias (IIPs). We then extend our studies to a murine model of fibrosis, demonstrating for the first time the ability of PPAR- γ agonists to inhibit collagen deposition in the lung.

MATERIALS AND METHODS

Reagents

Aliquots of the PPAR- γ agonists ciglitazone (Cig; Cayman, Ann Arbor, MI) and troglitazone (Tro; Cayman) were dissolved in 100% DMSO (Sigma-Aldrich, St. Louis, MO) at 100 mM and stored at -20°C for in vitro experiments. FuGENE 6 was obtained from Roche Applied Science (Indianapolis, IN); TGF- β 1, epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor (PDGF) from R & D Systems (Minneapolis, MN); rabbit polyclonal PPAR- γ antibody (H-100) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal β -actin antibody from Sigma-Aldrich; mouse monoclonal α -smooth muscle actin (α -SMA, clone 1A4) antibody from Dako Automation (Carpentaria, CA); and rabbit polyclonal cyclin D from NeoMarkers (Fremont, CA).

Human fibroblast isolation and culture

IIP fibroblasts were obtained from patients undergoing surgical lung biopsy for diagnosis of IIP. Biopsy-based histopathological diagnoses included usual interstitial pneumonia, nonspecific interstitial pneumonia, and respiratory bronchiolitis-associated interstitial lung disease. Control fibroblasts were obtained from patients undergoing thoracic surgery for nonfibrotic lung diseases. All subjects provided written informed consent in accordance with the University of Michigan Institutional Review Board. Human fetal lung fibroblast (IMR-90) cells were obtained from the Coriell Institute for Medical Research (Camden, NJ). Fibroblasts were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and 10% FBS (Sigma-Aldrich). All experiments were carried out using cells at *passages 5–9*.

Western immunoblotting

Western blot analysis was performed as described previously (53). Briefly, cell lysates were prepared in RIPA buffer containing a 1:100 dilution of protease inhibitor cocktail III (Calbiochem, La Jolla, CA), electrophoresed on SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes by electroblotting. Membranes were probed with antibodies and then with horseradish peroxidase-conjugated secondary antibodies and Super-Signal West Pico chemiluminescence detection reagents (Pierce, Rockford, IL).

Immunocytochemistry of PPAR- γ

IMR-90 fibroblasts were cultured overnight on sterile glass coverslips. The cells were washed and fixed with chilled (-20°C) methanol. After equilibration in a humidified chamber, the cells were blocked in 1% BSA in PBS for 30 min. The cells were then incubated overnight at 4°C with polyclonal anti-PPAR- γ antibody (1:100 dilution). After incubation, the cells were washed three times with PBS and then incubated with Cy3-conjugated sheep anti-rabbit IgG (1:200 dilution; Sigma-Aldrich) for 1 h at room temperature. The cells were washed as described above, and the coverslips were mounted on glass slides and visualized under a fluorescent microscope. Hoechst 33342 vital DNA stain was included with the secondary antibody incubation.

Transient reporter assays

All transfections were performed using the FuGENE 6 reagent according to the manufacturer's instructions. IMR-90 or rat fibroblasts were transfected in the absence of serum with the DNA construct of interest ($2\text{ }\mu\text{g/well}$) and the *Renilla* luciferase plasmid pRL-SV40 ($0.1\text{ }\mu\text{g/well}$; Promega, Madison, WI). DNA constructs included the PPAR-dependent luciferase reporter pFATP-luc, which contains three copies of the mouse FATP gene PPRE upstream of the minimal thymidine kinase promoter, the α -SMA reporter construct pGAL3- α -SMAp-luc (26), or a corresponding empty vector (control). Protein extracts generated from harvested cells were analyzed using a dual luciferase assay system (Promega). Transfections were performed in triplicate.

Cell counts

Cells were detached from six-well plates, and a 0.2-ml aliquot of cell suspension was diluted in Isoton II solution for counting in a Coulter counter (model ZM, Coulter Electronics, Hialeah, FL). The number of cells per dish was calculated on the basis of a dilution factor that was identical for all groups.

Flow cytometric analysis

To determine the effect of Tro and Cig on the cell cycle, synchronization of IMR-90 fibroblasts was accomplished by 48 h of serum starvation. Cells were then returned to medium + 5% FBS and treated with different concentrations of Tro or Cig. After 72 h, cells were harvested, washed, and fixed with 70% ethanol for 30 min at room temperature. After fixation, cells were washed with PBS and suspended in staining buffer consisting of propidium iodide ($50\text{ }\mu\text{g/ml}$) and 0.01% RNase in PBS. Cells were analyzed with an FACS Vantage flow cytometer using CellQuest acquisition and analysis programs (Becton Dickinson, San Jose, CA).

Fibroblast differentiation and transfection

IMR-90 or IIP cells were differentiated into myofibroblasts with TGF- β 1 as previously described (63). For transfection experiments, IMR-90 cells grown in 10% FBS were transfected with a pcDNA3-VP16-PPAR- γ construct ($2\text{ }\mu\text{g/well}$) produced by subcloning of the constitutively active VP16-PPAR- γ fusion cDNA into the mammalian expression vector

pcDNA3 or control vector (pcDNA3). After 6 h of transfection, cells were differentiated as described above.

Rat fibroblast isolation and culture

Fibroblasts were isolated from adult rat (Fischer 344) lungs as described previously (26). Cells were cultured in complete medium composed of DMEM (Sigma-Aldrich) supplemented with 10% plasma-derived serum (Cocalico Biologicals, Reamstown, PA), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Fungizone), 1% insulin-transferrin-selenium (Sigma-Aldrich), 5 ng/ml PDGF, and 10 ng/ml epidermal growth factor.

Measurement of soluble collagen

IMR-90 or IIP cells were differentiated into myofibroblasts, placed in fresh serum-free medium, and then pretreated (1–2 h) in triplicate with Tro (10 or 20 µM) or vehicle followed by TGF-β1 (2 ng/ml). Cells transfected with the pcDNA3-VP16-PPAR-γ construct (see above) were also used. After 24 h, cell culture supernatants were assayed for total soluble collagen (Sircol Collagen Assay Kit, Biocolor, Belfast, N. Ireland). One milliliter of Sirius red reagent was added to 200 µl of test sample, which was then mixed for 30 min at room temperature. The collagen-dye complex was precipitated by centrifugation at 10,000 *g* for 10 min. The supernatants were drained off and dissolved in 1 ml of 0.5 M NaOH. Absorbance was measured at 540 nm. Calibration curves were established using the collagen standard provided by the manufacturer.

Bleomycin-induced model of fibrosis

Eight-week-old female C57BL/6 mice weighing 15–20 g were obtained from Jackson Laboratories (Bar Harbor, ME). Bleomycin sulfate (Sigma, St. Louis, MO) was dissolved in sterile 0.9% saline on the day of intratracheal instillation. A single 50-µl aliquot containing 0.025 U of bleomycin diluted in normal saline was injected intratracheally. The PPAR-γ ligand Tro was administered by oral gavage. Mice were killed and lungs were collected for hydroxyproline and Sircol assay 21 days after bleomycin administration. All experiments were approved by the University of Michigan Committee on Use and Care of Animals.

Hydroxyproline quantification

Whole lungs were homogenized in 2 ml of 0.5 M glacial acetic acid: 1 ml was processed for hydroxyproline assay, and 1 ml was used for Sircol assay. Lung homogenate was dried under vacuum for 16 h, suspended in 1 ml of 6 N HCl, and heated for 8 h at 120°C. The total digest was filtered through a 0.2-µm filter, and 50 µl of clear filtrate were dried for 16 h using a Speed Vac and then suspended in 50 µl of citrate-acetate buffer (pH 6.0) to which 1 ml of chloramine-T solution was added. The resulting mixture was then incubated at room temperature for 20 min before 1 ml of Ehrlich solution (Aldrich, Milwaukee, WI) was added. These samples were incubated for 20 min at 65°C; after they were cooled, the samples were read at 550 nm in a spectrophotometer (model DU 640, Beckman). Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline (0–100 mg/ml).

Sircol assay for collagen in whole lungs

One milliliter of lung homogenate prepared as described for the hydroxyproline assay was mixed overnight at 4°C. After centrifugation, 100 µl of supernatant were mixed with 1 ml of Sircol assay dye reagent and incubated for 30 min at room temperature. After centrifugation, the pellet was suspended in 1 ml of alkali reagent and vortexed to release the dye into solution. Then 100 µl of solution were transferred to a microplate, and absorbance was measured at 540 nm. Values for experimental samples were based on a standard curve of known concentrations of purified rat tail collagen.

Histology

Lungs from euthanized animals were inflated with 10% neutral buffered formalin and fixed overnight. They were then dehydrated in 70% ethanol, processed according to standard procedures, embedded in paraffin, and sectioned. The sections were mounted on slides and stained for collagen with Masson's trichrome.

Cytokine measurement

Lung homogenates were centrifuged at 10,000 rpm for 15 min. Levels of TGF-β1 in the supernatants were measured by ELISA using standardized, specific TGF-β1 antibodies (R & D Systems) that detect protein at concentrations >10 pg/ml and do not cross-react with any other cytokines.

Statistical analysis

Values are means ± SE. Data were analyzed with the Prism 4.0 statistical program (GraphPad Software, San Diego, CA). Comparisons between two experimental groups were performed using a two-tailed Student's *t*-test. Comparisons among three or more experimental groups were performed with ANOVA followed by Dunnett's adjustment for multiple comparisons. Differences were considered significant if $P < 0.05$.

RESULTS

Human lung fibroblasts express different levels of PPAR-γ

In initial studies, we examined the expression of PPAR-γ in fibroblasts isolated from patients with IIPs and from healthy controls. Specifically, fibroblasts were obtained from patients with nonspecific interstitial pneumonia, usual interstitial pneumonia/IPF, respiratory bronchiolitis-associated interstitial lung disease, and normal lung tissue. These cells demonstrated constitutive PPAR-γ mRNA (data not shown) and protein expression (Fig. 1A). The amounts of PPAR-γ protein expression exhibited quantitative variability among individual biopsy samples. When densitometric scans were combined by diagnostic group ($n = 4/\text{group}$), however, no statistically significant differences were seen (Fig. 1B).

To further characterize localization of PPAR-γ, immunofluorescence staining of normal human fibroblast (IMR-90) cells was performed. As shown in Fig. 1C, PPAR-γ is predominantly localized in the nucleus of these cells when cultured in FBS.

PPAR- γ ligands activate a PPRE-dependent promoter in human lung fibroblasts

We next assessed whether selective agonists would activate lung fibroblast PPAR- γ . IMR-90 cells were transfected with a PPAR-dependent reporter construct, pFATP-luc, or control vector. Neither Tro nor Cig activated cells transfected with the control vector (data not shown), whereas Tro and Cig activated transcription of the PPAR reporter construct in a dose-dependent manner, with higher levels of activation demonstrated with Tro (Fig. 2). Thus specific agonists can activate PPAR- γ expressed in human lung fibroblasts (IMR-90).

PPAR- γ ligands inhibit proliferation of human lung fibroblasts

Fibroblast proliferation constitutes an important component of the fibrotic response. We therefore assessed whether PPAR- γ activation could alter the proliferation of undifferentiated fibroblasts. IMR-90 cells were treated with 1–20 μ M Tro or Cig for 24–72 h. We found similar dose- and time-dependent inhibitory effects on cell proliferation with Tro and Cig (Fig. 3A). Additionally, we examined the effects of PPAR- γ ligand treatment on fibroblasts isolated from patients with IIPs. The proliferative capacity of these cells, in particular IPF fibroblasts, was less than that of IMR-90 and normal fibroblasts, consistent with previous reports (10, 50). Nevertheless, the antiproliferative effects of PPAR- γ ligands were similar among the various fibroblast types (Fig. 3B). PPAR- γ ligands did not alter cell viability as assessed by trypan blue exclusion and lactate dehydrogenase release (data not shown).

PPAR- γ agonists inhibit proliferative responses of undifferentiated fibroblasts and myofibroblasts to mitogenic growth factors

Proliferation of fibroblasts and myofibroblasts in fibrosing lung diseases is driven by a variety of mitogenic growth factors. We therefore determined whether the antiproliferative effect of PPAR- γ agonists would occur in the presence of a growth factor such as PDGF, a potent simulator of fibroblast proliferation. Treatment of IMR-90 cells with PDGF alone resulted in a significant increase in proliferation at 24, 48, and 72 h compared with controls. By comparison, treatment (1–2 h) of IMR-90 cells with PPAR- γ agonists followed by the addition of PDGF significantly inhibited the stimulation of proliferation (Fig. 4A). Similar results were observed with IIP fibroblasts (data not shown). We then determined the effect of PPAR- γ agonists on myofibroblast proliferative responses. IMR-90 cells were initially serum starved for 48 h and then treated with TGF- β 1 for 24 h to induce myofibroblast differentiation. Cells were then treated (1–2 h) with PPAR- γ agonist before addition of the potent mitogen fibroblast growth factor. Cell counts were obtained 48 h later. Similar to our results with PDGF, Tro exerted significant inhibitory effects on myofibroblast proliferation (Fig. 4B).

PPAR- γ agonists induce cell cycle arrest and decrease expression of cyclin D

To further analyze the antiproliferative effects of PPAR- γ ligands, we examined the cell cycle of treated cells. Flow cytometric analysis after propidium iodide staining of Tro-treated IMR-90 cells revealed a dose-dependent decrease in the number of cells in the S phase after 72 h of treatment (Fig. 5A). Concomitant with this decrease in the number of IMR-90 cells in the S phase, there was also a dose-dependent increase in the number of cells

accumulating in the G₀/G₁ phase of the cell cycle (Fig. 5A), suggesting a block at the G₀/G₁ stage with Tro treatment. The number of cells in the G₂/M phase was not altered by Tro. Since we observed accumulation of cells in the G₀/G₁ phase, we determined the effect of PPAR- γ ligands on cyclin D expression in IMR-90 cells. Treatment of cells with Tro (20 μ M) decreased cyclin D expression at 24, 48, and 72 h (Fig. 5B). These data are consistent with the cell cycle analysis and suggest that PPAR- γ ligands induce G₁ arrest by inhibiting the expression of a G₁ cyclin (cyclin D). Cell cycle analysis and cyclin D results were similar in cells treated with Cig (data not shown).

PPAR- γ activation inhibits TGF- β 1-induced myofibroblast differentiation

Fibroblast-to-myofibroblast differentiation is a critical process in the pathogenesis of fibrosing lung diseases. To determine whether PPAR- γ ligands inhibit this process, cells from IPF patients and healthy controls were treated with Tro or Cig (1–2 h) and then induced to differentiate with TGF- β 1 (2 ng/ml). Western immunoblot analysis was performed to assess expression of α -SMA as a marker of myofibroblast differentiation. As observed in Fig. 6A and confirmed densitometrically (Fig. 6B), both ligands inhibited TGF- β 1-induced α -SMA expression, although the effect was more pronounced with Tro than with Cig. IMR-90 cells were also transfected with constitutively active PPAR- γ , pcDNA3-VP16-PPAR- γ . As with PPAR- γ ligand treatment, cells transfected with constitutively active PPAR- γ failed to differentiate into myofibroblasts after TGF- β 1 stimulation, suggesting a direct inhibitory role for PPAR- γ in the myofibroblast differentiation process (Fig. 6C). As described above, PPAR- γ ligands did not alter cell viability as assessed by trypan blue exclusion and lactate dehydrogenase release (data not shown).

To determine whether the effect of PPAR- γ on α -SMA expression is mediated, at least in part, at the level of gene transcription, we transfected fibroblasts isolated from normal rat lung with an α -SMA reporter construct (pGAL3- α -SMAp-luc), treated them with Tro or Cig, and then induced differentiation by exposure to TGF- β 1. Cells treated with Tro demonstrated significantly decreased activation of α -SMA compared with controls, whereas those treated with Cig did not demonstrate significant changes in α -SMA activation (Fig. 6D). This suggests a difference between the two ligands in their inhibitory effect on TGF- β 1-induced differentiation.

PPAR- γ activation inhibits myofibroblast collagen secretion

Myofibroblasts are the major collagen-producing cells in fibrosing lung diseases. Therefore, the ability of such ligands to inhibit collagen secretion from these cells was assessed by treatment of myofibroblasts with PPAR- γ ligands. Because fibroblasts constitutively produce collagen, baseline collagen secretion was set to 100%. TGF- β 1-induced differentiation results in significant induction of collagen secretion by myofibroblasts from IPF patients or healthy controls that was inhibited by Tro (Fig. 7A). Similar experiments were carried out using IMR-90 cells, except in some experiments the cells were transiently transfected with the constitutively active PPAR- γ construct pcDNA3-VP16-PPAR- γ . Treatment with Tro or transient transfection with pcDNA3-VP16-PPAR- γ resulted in significant inhibition of collagen secretion from TGF- β 1-activated cells, nearly to baseline unstimulated levels (Fig. 7B).

Tro inhibits bleomycin-induced lung fibrosis

Intratracheal administration of bleomycin results in a murine model of fibrosis. To investigate whether PPAR- γ ligands inhibit fibrosis in vivo, we administered bleomycin intratracheally to mice that were treated with Tro or vehicle beginning 3 days before bleomycin administration ($n = 10/\text{group}$). Lungs were harvested 21 days after bleomycin administration, and fibrosis was assessed by measurement of the amounts of hydroxyproline and collagen. Tro at 200 or 400 mg·kg body wt⁻¹·day⁻¹ beginning 3 days before bleomycin administration significantly inhibited induction of hydroxyproline and collagen accumulation (Fig. 8, *A* and *B*). Administration of Tro at 400 mg·kg body wt⁻¹·day⁻¹ beginning 10 days after bleomycin administration also significantly inhibited hydroxyproline and collagen accumulation, although to a lesser extent (Fig. 8, *C* and *D*). Effects were further confirmed by histological studies using trichrome staining (Fig. 9). Lastly, in support of its antifibrotic actions, Tro administration also altered whole lung levels of the profibrotic cytokine TGF- β 1 (Fig. 10).

DISCUSSION

In the present study, we demonstrate several significant findings. 1) Fibroblasts isolated from normal subjects and patients with IIP express the nuclear hormone receptor PPAR- γ . 2) Treatment with PPAR- γ agonists resulted in cell cycle arrest and decreased proliferative responses to mitogenic stimuli in fibroblasts and myofibroblasts. Cell cycle arrest of fibroblasts correlated with inhibition of cyclin D expression. 3) PPAR- γ agonists or VP16-PPAR- γ inhibited the ability of TGF- β 1 to induce fibroblast-to-myofibroblast differentiation or collagen secretion. This occurred, in part, through inhibitory effects on the α -SMA promoter. 4) Extending our studies to in vivo models, we found that the PPAR- γ agonist Tro inhibited fibrosis, as assessed by measurement of total lung hydroxyproline and collagen and by trichrome staining in the bleomycin-induced murine model. This inhibition was observed not only when PPAR- γ agonist treatment began before bleomycin administration, but even when it was begun when inflammation was resolving and the postinflammatory fibrosis that most closely resembles human IPF was beginning. Although the reduction in total lung collagen deposition was smaller when PPAR- γ agonist administration was delayed, it is likely that little or no further collagen deposition occurred once treatment began.

This reduction in collagen deposition may reflect on not only inhibition of TGF- β 1 action, but also on the decreased levels of this cytokine observed in the present study. TZDs have been similarly reported to reduce TGF- β 1 synthesis and resulting fibrosis in mouse models of asthma (24) and chronic pancreatitis (66). PPAR- γ ligand-induced reductions of TGF- β 1 expression and fibrosis-related markers have also been observed in several types of cultured cells (46, 47, 69, 70). These dual effects on TGF- β 1 synthesis and activity can be expected to further enhance the beneficial influence of Tro on lung fibrosis.

The results obtained in the present study not only support a role for PPAR- γ agonists in the suppression of mesenchymal/fibroblast profibrotic activities (6, 15, 18, 33, 41, 55, 73), but they also provide the first direct evidence from an animal model that these agents may be useful in human fibrosis. Indeed, only one previous study has examined the effects of PPAR- γ agonists in the murine model of bleomycin-induced lung injury (16). That study, however,

used a relatively high bleomycin dose that was associated with considerable mortality, and the focus was on reductions in inflammation and mortality-related effects of acute lung injury. Inflammation was still in progress when mice were killed 15 days after bleomycin administration, so there was no opportunity to examine postinflammatory fibrosis.

Studies in which potential therapeutic agents were administered during the postinflammatory phase of an animal model of pulmonary fibrosis have been limited (44). Similarly, no prior study has examined the effects of PPAR- γ ligands on fibroblasts isolated from patients with fibrotic lung diseases. In vitro treatment of IIP fibroblasts with PPAR- γ agonists resulted in antiproliferative responses in all conditions tested, including the presence of potent, biologically relevant mitogenic factors. The inhibitory effects on proliferation with the agonists were very similar across a variety of fibroblast types and resulted in cell cycle arrest and decreased cyclin D expression. These findings are consistent with results seen with human lung adenocarcinoma (A549) cells (32) and other cell types (2).

The induction of fibroblast-to-myofibroblast differentiation by TGF- β 1 is a well-characterized event in vitro; excessive activation of these cells promotes progressive fibrosis and tissue compromise. Myofibroblasts are key effector cells with respect to extracellular matrix deposition and contractility. We demonstrate that Tro or a constitutively active form of PPAR- γ (VP16-PPAR- γ) and, to a lesser extent, Cig inhibit the differentiation of fibroblasts to myofibroblasts and result in decreased collagen synthesis by myofibroblasts. These results are similar to a recently published report in which 15d-PGJ₂ and rosiglitazone (Rosi) demonstrated inhibitory effects on TGF- β 1-induced myofibroblast differentiation (6). Interestingly, Rosi, similar to Cig, was a less potent inhibitor of the differentiation response. Additionally, we observed less effective inhibition of fibroblast proliferation by Rosi than by Tro or Cig (data not shown). Another study has similarly shown that introduction of a PPAR- γ expression vector inhibits the ability of TGF- β 1 to stimulate myofibroblast differentiation of ocular fibroblasts (56). In this case, the construct was not constitutively active and, thus, may have been activated by unspecified endogenous ligands.

It is well established that TZDs and other agonists such as 15d-PGJ₂ exert PPAR- γ dependent and -independent effects (9, 22, 35). Similar to the suppressive effects on TGF- β 1-induced myofibroblast differentiation, Tro, but not Cig, resulted in significantly decreased activation of the α -SMA promoter in rat fibroblasts. The modest inhibition of myofibroblast differentiation by Cig may have occurred through induction of apoptosis, inasmuch as this agonist induces apoptosis in a variety of cell types (52, 60) through mechanisms that may be independent of PPAR- γ (60).

Thus, although PPAR- γ ligands are potent inhibitors of fibroblast activation, there appear to be differences in effectiveness between the different ligands. Previous results have demonstrated that Tro is a more potent activator of PPAR- γ than Cig (7, 11), as we also found. In our study, Tro appears to be the most potent ligand in inhibiting fibroblast/myofibroblast activation, an important issue to consider in designing human clinical trials. Although cases of idiosyncratic hepatic dysfunction have occurred with the use of Tro in the treatment of diabetes mellitus (12, 68), the dismal short-term prognosis of diseases such as

IPF outweighs the potential risks posed by this drug. Additionally, for lung-specific diseases, it may be possible to bypass systemic administration and, thus, lower the potential for toxicity. For example, in a murine model of asthma, TZD drug administration via inhalation exhibited potent effectiveness (4, 24).

In conclusion, our results demonstrate that PPAR- γ agonists significantly reduce the response of human lung fibroblasts to mitogenic and differentiation signals. We also show that PPAR- γ agonists inhibit fibrosis in the bleomycin-induced murine fibrosis model. Significantly, they do so even when administered solely during the postinflammatory phase, which corresponds to the period during which IPF patients are likely to present clinically. Our findings thus suggest that PPAR- γ agonists result in inhibition of fibroblast/myofibroblast activation and collagen production or accumulation in vitro and in vivo and, therefore, may be useful in the treatment of human fibrotic lung diseases.

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Fig. 1.

Human lung fibroblasts express peroxisome proliferator-activated receptor- γ (PPAR- γ). *A*: Western immunoblot for PPAR- γ on cells from patients with idiopathic interstitial pneumonia [usual interstitial pneumonia (UIP), respiratory bronchiolitis-associated interstitial lung disease (RBILD), and nonspecific interstitial pneumonia (NSIP)] and normal human fibroblasts. Each lane represents a different patient sample. Equal loading was confirmed by stripping and probing with antibody against β -actin. Blot is representative of a larger group of patient samples ($n = 16$). *B*: densitometric analysis of Western immunoblots in *A* ($n = 4$ /group). *C*: immunofluorescence staining for PPAR- γ in IMR-90 fibroblasts.

Fig. 2.

Activation of PPAR- γ in human lung fibroblasts. IMR-90 cells were simultaneously transfected with a PPAR-dependent luciferase reporter (pFATP-luc) and pRL-SV40 and then stimulated with vehicle (DMSO) or 1–20 μ M troglitazone (Tro) or ciglitazone (Cig) for 24 h. Relative luciferase activity was calculated by normalization of firefly luciferase activity to *Renilla* luciferase activity. Data are averages from 3 independent experiments. * $P < 0.05$ vs. DMSO.

Fig. 3.

PPAR- γ ligands inhibit proliferative responses of human lung fibroblasts. *A*: IMR-90 cells were plated in 5% FBS and then treated with 0 μ M (■), 1 μ M (●), 5 μ M (▲), 10 μ M (▼), or 20 μ M (◆) Tro or Cig in DMSO. At 24-h intervals, cells were trypsinized and counted using a Coulter counter. Data are averages from 3 independent experiments. *B*: cultured fibroblasts from normal lungs or patients with different forms of idiopathic interstitial pneumonia (NSIP, RBILD, and UIP) were plated in 5% FBS. At 24-h intervals, cells were trypsinized and counted using a Coulter counter. Data from normal and IIP fibroblasts are

from 4 individual patients in 2 separate experiments with Tro treatment. $*P < 0.05$; $**P < 0.01$ vs. 0 μM at 72 h.

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Fig. 4.

PPAR- γ ligands inhibit proliferative responses of human lung fibroblasts to mitogenic growth factors. **A:** IMR-90 cells were treated with Tro or Cig in the presence of 5% FBS followed by stimulation with the mitogen PDGF (5 ng/ml) for 24, 48, and 72 h. In both panels, concentration of drugs in DMSO increases from top line to bottom line: 0 μ M (—■—), 1 μ M (—▲—), 5 μ M (—▼—), 10 μ M (—◆—), and 20 μ M (—●—). At 24-h intervals, cells were trypsinized and counted using a Coulter counter. Data are averages from 3 independent experiments. * P < 0.05; ** P < 0.01 vs. 0 μ M at 72 h. **B:** IMR-90 cells were seeded on dishes and allowed to grow to 80% confluence in medium containing 10% FBS

(Ctrl); then their growth was arrested by change to serum-free medium for 48 h. Cells were treated with transforming growth factor (TGF)- β 1 (2 ng/ml) for 24 h (SS) before treatment with 1–20 μ M Tro or vehicle (DMSO) and then stimulated with fibroblast growth factor (FGF, 10 ng/ml) for 48 h. Cells were trypsinized and cell counts were assessed as previously described. Data are averages from 3 independent experiments. ** $P < 0.01$ vs. 0 μ M + TGF- β 1 + FGF.

Fig. 5.

PPAR- γ ligands result in cell cycle arrest and inhibition of cyclin D expression in human lung fibroblasts. **A:** number of propidium iodide-stained IMR-90 cells in G₀/G₁, S, and G₂/M phases at 72 h determined by flow cytometry. Proportion of cells in G₀/G₁, S, and G₂/M phases was determined relative to cells in the absence of Tro. Actual values used to calculate relative percentage were obtained from mean of 3 independent experiments. * $P < 0.05$ vs. 0 μ M. **B:** Western blot analysis of cyclin D expression in IMR-90 cells after exposure to 20 μ M Tro. Equal loading was confirmed by stripping and probing with antibody against β -actin. Blot is representative of 2 separate experiments.

Fig. 6.

PPAR- γ activation inhibits fibroblast-to-myofibroblast differentiation. **A:** cells from IPF patients or normal human controls were grown to 80% confluence, and growth was arrested for 48 h. Cells were treated for 1–2 h with 1 or 5 μ M Tro or Cig and then for 24 h with TGF- β 1 (TGF; 2 ng/ml), and α -smooth muscle actin (α -SMA) was assessed by Western immunoblotting. Equal loading was confirmed by stripping and probing with antibody against β -actin. **B:** densitometric analysis of Western immunoblots in **A** ($n = 4$ /group). * $P < 0.05$ vs. TGF. **C:** IMR-90 cells were treated as described in **A** or, before TGF- γ 1 stimulation, transiently transfected with pcDNA3-VP16-PPAR- γ construct or empty vector

(Ctrl). Cells were then assessed for α -SMA levels as described in *A*. *D*: Tro, but not Cig, decreases α -SMA transcriptional activity in rat fibroblasts. Rat fibroblasts were simultaneously transfected with an α -SMA reporter plasmid (pGAL3- α -SMAp-luc) and pRL-SV40 and then treated with 10 or 20 μ M Tro or Cig and TGF- β 1 (2 ng/ml) for 24 h. Relative luciferase activity was calculated by normalization of firefly luciferase activity to *Renilla* luciferase activity. Data are averages from 3 independent experiments. * P < 0.05; ** P < 0.01 vs. DMSO.

Fig. 7.

PPAR- γ activation inhibits TGF- β 1-induced collagen secretion. *A*: Fibroblasts constitutively produce collagen, so baseline collagen secretion was set to be 100%. Cells from normal human controls (shaded bars) or patients with idiopathic pulmonary fibrosis (IPF, solid bars) were grown to 80% confluence in standard medium and then placed in serum-free medium for 48 h. Fresh serum-free medium was added, and cells were pretreated for 1–2 h in triplicate with 10 or 20 μ M Tro or vehicle and then with TGF- β 1 (2 ng/ml). After 24 h, cell culture supernatants were assayed for total soluble collagen on the basis of specific binding of Sirius red dye with the [Gly-X-Y] $_n$ helical structure of collagen. * P < 0.05; ** P < 0.01

vs. 0 μ M. *B*: IMR-90 cells were grown and treated as described in *A*. Other IMR-90 cells (solid bars) were transiently transfected with the pcDNA3-VP16-PPAR- γ (VP16-PPAR- γ) construct or empty vector (E. Vector). After 24 h, cell culture supernatants were assayed for total soluble collagen as described in *A*. * P < 0.05; ** P < 0.01 vs. 0 μ M. # P < 0.01 vs. E. Vector.

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Fig. 8.

Tro inhibits lung hydroxyproline and collagen accumulation in response to bleomycin (Bleo) treatment in vivo. All mice ($n = 10/\text{group}$) received 0.025 U of bleomycin intratracheally as a single dose. *A* and *B*: mice received Tro by oral gavage at 0, 200, or 400 mg·kg body wt⁻¹·day⁻¹ beginning 3 days before bleomycin instillation. *C* and *D*: mice received Tro at 400 mg·kg body wt⁻¹·day⁻¹ beginning on *day 11* following bleomycin instillation. On *day 21* following bleomycin instillation, mice were killed and amounts of hydroxyproline (*A* and *C*) and collagen (*B* and *D*) in their lungs were measured. * $P < 0.05$; ** $P < 0.01$ vs. vehicle + bleomycin-treated mice.

Fig. 9.

Tro inhibits collagen deposition in lungs of bleomycin-treated mice. Saline (*A*) or bleomycin (0.025 U, *B–D*) was administered intratracheally as a single dose. Bleomycin-treated mice were also treated with vehicle (*B*), Tro by oral gavage at $400 \text{ mg} \cdot \text{kg body wt}^{-1} \cdot \text{day}^{-1}$ beginning 3 days before bleomycin instillation (*C*), or Tro at $400 \text{ mg} \cdot \text{kg body wt}^{-1} \cdot \text{day}^{-1}$ beginning on *day 11* following bleomycin instillation (*D*). On *day 21* following bleomycin or saline instillation, mice were killed and their lungs were fixed, sectioned, and stained for collagen with Masson's trichrome. Original magnification $\times 20$.

Fig. 10.

Tro decreases TGF-β1 levels in lungs of bleomycin-treated mice. Mice ($n = 4/\text{group}$) were treated with 0.025 U of bleomycin intratracheally as a single dose. Tro was administered by oral gavage at 0 or 400 mg·kg body wt⁻¹·day⁻¹ beginning 3 days before bleomycin instillation. On *day 14* following bleomycin instillation, mice were killed and TGF-β1 in lung homogenates was measured by ELISA.