Electrophilic Peroxisome Proliferator-Activated Receptor- γ Ligands Have Potent Antifibrotic Effects in Human Lung Fibroblasts

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Pulmonary fibrosis is a progressive scarring disease with no effective treatment. Transforming growth factor (TGF)- β is up-regulated in fibrotic diseases, where it stimulates differentiation of fibroblasts to myofibroblasts and production of excess extracellular matrix. Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor that regulates adipogenesis, insulin sensitization, and inflammation. We report here that a novel PPAR γ ligand, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), is a potent inhibitor of TGF- β -stimulated differentiation of human lung fibroblasts to myofibroblasts, and suppresses up-regulation of α -smooth muscle actin, fibronectin, collagen, and the novel myofibroblast marker, calponin. The inhibitory concentration causing a 50% decrease in α SMA for CDDO was 20-fold lower than the endogenous PPAR γ ligand, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15 d-PGJ₂), and 400-fold lower than the synthetic ligand, rosiglitazone. Pharmacologic and genetic approaches were used to demonstrate that CDDO mediates its activity via a PPAR γ -independent pathway. CDDO and 15 d-PGJ₂ contain an α/β unsaturated ketone, which acts as an electrophilic center that can form covalent bonds with cellular proteins. Prostaglandin A₁ and diphenyl diselenide, both strong electrophiles, also inhibit myofibroblast differentiation, but a structural analog of 15 d-PGJ₂ lacking the electrophilic center is much less potent. CDDO does not alter TGF- β -induced Smad or AP-1 signaling, but does inhibit acetylation of CREB binding protein/p300, a critical coactivator in the transcriptional regulation of TGF- β -responsive genes. Overall, these data indicate that certain PPAR γ ligands, and other small molecules with electrophilic centers, are potent inhibitors of critical TGF- β -mediated profibrogenic activities through pathways independent of PPAR γ . As the inhibitory concentration causing a 50% decrease in α SMA for CDDO is 400-fold lower than that in rosiglitazone, the translational potential of CDDO for treatment of fibrotic diseases is high.

Keywords: myofibroblast; peroxisome proliferator-activated receptor γ ; 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; α -smooth muscle actin

Idiopathic pulmonary fibrosis affects approximately 5 million people worldwide. The mechanism of disease initiation and progression is poorly understood, and the disease is refractory to current therapies (1). Fibrotic remodeling of lung tissue is

CLINICAL RELEVANCE

This article describes a novel mechanism by which a peroxisome proliferator-activated receptor γ agonist blocks myofibroblast differentiation in lung fibroblasts. This has applications for the design of novel treatments for lung fibrosis.

also an important facet to the pathology of other lung diseases, including sarcoidosis and asthma (2). Fibrosis is hypothesized to be a defect in regulation of wound healing, resulting in the production of excess extracellular matrix components (3, 4). Fibroblasts are major effector cells in the development of pulmonary fibrosis; they differentiate to myofibroblasts and produce excess extracellular matrix components, including collagen and fibronectin (4–6). The increased extracellular matrix leads to alteration of normal lung architecture and impaired gas exchange.

The cytokine, transforming growth factor (TGF)- β 1, induces the differentiation of fibroblasts to myofibroblasts *in vitro* (1, 6), and is implicated as a key cellular inducer of fibrotic diseases (reviewed in References 2, 7), including idiopathic pulmonary fibrosis (8–10). Overexpression of TGF- β in rodent lung leads to severe, irreversible pulmonary fibrosis (11, 12), whereas inhibiting TGF- β *in vivo* decreases the development and progression of pulmonary fibrosis (13–15). TGF- β gene transcription is initiated by binding of TGF- β to its receptor, which leads to phosphorylation of the signaling proteins, Smad2/3, recruitment of Smad4, and translocation of the Smad2/3/4 complex to the nucleus, where it interacts with coactivator proteins, such as CREB (cAMP response element binding protein) binding protein (CBP)/p300. CBP/p300 is a histone acetyl transferase (HAT) implicated in transcriptional regulatory pathways, including TGF- β (16).

Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor. After ligand binding, PPAR γ heterodimerizes with the retinoid X receptor (RXR), and is translocated to the nucleus, where it binds to PPAR γ response elements (PPREs) (14, 17). PPAR γ agonists regulate fat metabolism and adipogenesis (18). Endogenous PPAR γ ligands include prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15 d-PGJ₂), as well as lysophosphatidic acid and 15S-hydroxyeicosatetraenoic acid. The most well studied synthetic agonists are the thiazolidinedione class of drugs, such as rosiglitazone and pioglitazone, which are currently prescribed as insulin sensitizers in type II diabetes (19). PPAR γ agonists have potentially important roles as anti-inflammatory and antifibrotic agents (20, 21), and are also involved in the regulation of cellular differentiation (22, 23). We and others have reported that PPAR γ agonists, including thiazolidinediones, have antifibrotic effects on human lung fibroblasts *in vitro* (24, 25). Interestingly, rosiglitazone is

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reported to be 100-fold more effective at promoting fibroblast-to-adipocyte differentiation than 15 d-PGJ₂ (26), but is much less effective than 15 d-PGJ₂ at inhibiting differentiation of lung fibroblasts to myofibroblasts (24). This suggests that the proadipogenesis and antifibrotic properties of PPAR γ ligands may act via different mechanisms.

2-Cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO) is a novel PPAR γ ligand. Although both 15 d-PGJ₂ and CDDO bind PPAR γ with high affinities (K_i [inhibition constant] 1.2×10^{-9} and 10^{-8} , respectively) (26, 27), and regulate adipogenesis in a PPAR γ -dependent manner, there are emerging data that they mediate some of their biological activities via a PPAR γ -independent pathway (22, 24, 28, 29). 15 d-PGJ₂ is the final cyclopentenone metabolite of PGD₂ in the cyclooxygenase pathway. The cyclopentenone prostaglandins have an α/β -unsaturated ketone ring with electrophilic carbons that are highly susceptible to Michael addition reactions, forming covalent bonds with free reduced thiols, such as glutathione and accessible cysteine residues of intracellular proteins (*see* Figure E3 in the online supplement) (30). For example, 15 d-PGJ₂ mediates its anti-inflammatory effects in part via covalent modification of cysteine residues in the NF- κ B components I κ B and p65 (31). CDDO also has an α/β -unsaturated ketone, and is potentially reactive in modifying cellular proteins (22, 29, 32). Recent data from our laboratory and others suggest that the electrophilic carbon on 15 d-PGJ₂ is important for mediating its PPAR γ -independent effects (22, 29, 32).

Here, we report that CDDO has profound antifibrotic effects on human lung fibroblasts *in vitro* at 20-fold lower concentrations than 15 d-PGJ₂ and 400-fold lower concentration than rosiglitazone. Moreover, the effects are independent of PPAR γ -mediated transcribed and dependent on the presence of a strong electrophilic center. This independent mechanism does not involve the classic TGF- β /Smad signaling pathway or the alternate activator protein (AP)-1 and mitogen-activated protein kinase pathways, but does involve alterations of TGF- β -mediated acetylation of CBP/p300.

MATERIALS AND METHODS

Cells and Reagents

Normal human lung fibroblast cell strains were derived from tissue explants and maintained in Eagle's minimum essential medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Sigma, St. Louis, MO), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin (0.25 μ g/ml; Invitrogen, Carlsbad, CA) at 37°C in 7% CO₂. These explants were derived from anatomically normal lung tissue from patients undergoing surgical resection for benign hamartoma or for small peripheral nodules. Patient samples were obtained with informed consent under the approval of the Institutional Review Board of the University of Rochester. These cells are morphologically consistent with fibroblasts, they express collagen and vimentin, and they do not express CD45, factor VIII, or cytokeratin (33). Cells were used at low passages, typically 4–8. The PPAR γ agonists, 15 d-PGJ₂ (Biomol, Plymouth Meeting, PA), 9,10-dihydro-15-deoxy- $\Delta^{12,14}$ -PGJ₂ (CAY10410; Cayman Chemical Co., Ann Arbor, MI), and CDDO (NIH-RAID Program and Reata Pharmaceuticals, Dallas, TX), were prepared as 10-mM stocks in DMSO and added to cell cultures to the final concentrations indicated. GW9662 and prostaglandin A₁ (PGA₁; Cayman Chemical), and diphenyl diselenide (DSPS; Sigma) were prepared in the same manner. Recombinant human TGF- β 1 was purchased from R&D Systems (Minneapolis, MN).

Western Blots for α -Smooth Muscle Actin, Calponin, Fibronectin, Smad 2/3, and Acetylated CBP/p300

Primary human lung fibroblasts (100,000 cells/well) were plated in triplicate in six-well plates (Falcon/Becton Dickinson, Franklin Lakes,

NJ) and treated with TGF- β and/or PPAR γ agonists, as described subsequently here. In some experiments, the fibroblasts were infected with a lentiviral vector expressing a dominant negative PPAR γ or the empty vector (*see below*) for 24 hours before treatment with TGF- β and PPAR γ agonists. Cell lysates were prepared using either an NP-40-based lysis buffer or the commercial ActiveMotif Nuclear Extract kit (Active Motif, Carlsbad, CA). Lysates containing 2 or 5 μ g of protein were separated by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and proteins of interest were detected by Western blotting and chemiluminescence (Western Lighting, Perkin-Elmer, Wellesley, MA). To analyze collagen and fibronectin levels, precast 4–15% SDS-PAGE gradient gels (Bio-Rad, Hercules, CA) were used. Lysates were examined for expression of α -smooth muscle actin (SMA; Sigma), calponin (DAKO, Carpinteria, CA), fibronectin (Sigma), Smad2/3, pSmad2/3, Smad4, or acetylated CBP/p300 (Cell Signaling, Beverly, MA) or collagen I (Santa Cruz, Carlsbad, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, MA), β -actin (Abcam), and/or vinculin (Sigma) were used as loading controls. Densitometry of the resulting bands was performed using Kodak Molecular Imaging Software (Kodak, Rochester, NY), and normalized to the loading control.

RT-PCR

RNA was isolated from homogenized primary human lung fibroblast cultures using RNeasy, according to the manufacturer's protocol (Qiagen, Valencia, CA). RNA (1.0 μ g) was incubated with PCR buffer, 0.5 μ g of oligo (dT)_{12–18} primer (Invitrogen), 10 mM deoxynucleotide-triphosphate (dNTP) for 10 minutes at 70°C and 5 minutes in ice water, followed by addition of 40 U of recombinant RNasin RNase inhibitor (Promega, Madison, WI), 0.1 mM DTT, and 200 U of Superscript III reverse transcriptase (RT; Invitrogen). The mixture was further incubated for 5 minutes at room temperature, 60 minutes at 50°C, and 15 minutes at 70°C. The reaction contents were diluted to 80- μ l volume and stored at –20°C. Negative controls contained no RT enzyme.

Quantitative real-time RT-PCR reactions were performed using a Bio-Rad iCycler with SYBR Green Supermix (Bio-Rad) according to the supplier's recommended protocol, with the following modifications. For amplification of the collagen I (COL1A1) message, the reaction contained 4 mM MgCl₂ and 0.1 μ M of each primer, whereas, for collagen III (COL3A1), α -SMA (ACTA2), and GAPDH, the reactions contained 3 mM MgCl₂ and 0.2 μ M of each primer. Oligomer primers were ordered from Real Time Primers (Elkins Park, PA) (COL1A1) and Integrated DNA Technologies (Coralville, IA) (COL3A1, ACTA2, and GAPDH). The primer sequences were as follows: COL1A1 sense, 5'-GGA CAC AGA GGT TTC AGT GG-3'; COL1A1 anti-sense, 5'-CCA GTA GCA CCA TCA TTT CC-3'; GAPDH sense, 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3'; GAPDH anti-sense, 5'-AGC CTT GAC GGT GCC ATG GAA TTT-3'; ACTA2 sense, 5'-TCT GGA GAT GGT GTC ACC CAC AAT-3'; ACTA2 anti-sense, 5'-AAT AGC CAG GCT CAG TCA GG-3'; COL3A1 sense, 5'-AGC AA AGT TTC CTC CGA GGC CAG-3'; COL3A1 antisense, 5'-GGA GAA TGT TGT GCA GTT TGCCCA-3'.

Imaging Flow Cytometry

Primary human lung fibroblast strains were cultured and treated as described above. At harvest, cells were trypsinized, washed twice with PBS, and fixed with 100% methanol for 30 minutes at 4°C. The cells were then washed twice with PBS/0.1% Tween-20 (PBS-T) and blocked with 5% normal mouse serum for 20 minutes at room temperature. Blocked cells were washed once with PBS-T and incubated with fluorescently labeled (Cy3) mouse anti- α -SMA antibody (Sigma) at a 1:400 dilution in PBS-T plus 1% BSA, overnight at 4°C. The next day, cells were washed twice with PBS-T, once with PBS, and stained with Draq5 (Exira, San Diego, CA) at a 1:200 dilution in PBS. Samples were analyzed directly on an ImageStream System 100 (Amnis Corporation, Seattle, WA). A gating strategy was employed to discriminate live single cells from debris and clumps of cells based on the intensity and aspect ratio of nuclear staining (Draq5). Single cells were identified as fibroblasts or myofibroblasts based on the intensity and fractional area of α -SMA staining. *See the online supplemental MATERIALS AND METHODS for more details.*

Construction of Lentiviral Vectors

Plasmids encoding a Flag-tagged wild-type PPAR γ (pcDNA-Flag-PPAR γ 1) and a Flag-tagged dominant-negative PPAR γ (pcDNA-Flag-PPAR γ 1-L466A/E469A) were a kind gift from V.K.K. Chatterjee (University of Cambridge, Cambridge, UK). The cDNA sequences were amplified by PCR using primers that added a restriction site (*NheI* and *NotI*) at each end. The PCR product was then digested with *NheI* and *NotI* and subcloned into a *NheI*-*NotI*-digested pCDH1-MCS1-EF1-copGFP vector (System Bioscience, Mountain View, CA). These vectors were named LV-PPAR γ and LV-PPAR γ -DN, and the vector without PPAR γ cDNA was termed LV-empty.

Lentiviral Vector Production

Human embryonic kidney-293FT cells (Invitrogen) were grown to 50–70% confluency in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA) supplemented with 10% FBS in T-175 flasks. Subsequently, the vesicular stomatitis virus G (VSV-G)-pseudotyped HIV vector was generated by cotransfecting with 5 μ g of envelope vector (pomp-VSV-G), 14 μ g of transfer vector, and 14 μ g of packaging vector (pomp- Δ 89.2), using Lipofectamine LTX (Invitrogen) following the manufacturer's recommendations. Cells were split into two new T-175 flasks 6 hours after transfection. Supernatants were collected 48 and 72 hours after transfection. Virus was harvested by ultracentrifugation at 50,000 $\times$ g for 2 hours at 4°C using a Beckman SW28 rotor (Beckman Coulter, Inc., Fullerton, CA). The concentrated virus stocks were titrated on human embryonic kidney-293FT cells based on green fluorescent protein (GFP) expression.

AP-1 Luciferase Reporter Assay

Primary lung fibroblasts cultured in six-well plates were cotransfected using Fugene6 (Roche Applied Science, Indianapolis, IN) with an AP-1 luciferase reporter (a gift from Dr. Sanjay Maggirwar, University of Rochester) (34) and a CMV- β -galactosidase control construct (a gift from Dr. T. Gasiewicz, University of Rochester) (35). After 24 hours, the cells were washed and then treated with TGF- β (5 ng/ml) with or without 15 d-PGJ $_2$ (5 μ M), or CDDO (1 μ M) in medium (10% FBS), and harvested after a further 24-hour incubation. Luciferase activity was measured using a luciferase assay system (Promega) in a luminometer (Packard Instruments, Meriden, CT) and normalized to β -galactosidase activity, determined by a colorimetric assay (Promega). The experiments were performed in triplicate wells.

Cell Viability Assay

Cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (36). Fibroblasts were plated in triplicate at a density of 5,000 cells per well in 96-well plates, and treated with TGF- β and PPAR γ agonists for 72 hours at the same concentrations previously used here. Production of the colored reaction product was measured at 560 nm, and the results were normalized to the negative control wells.

Statistical Analysis

All data are expressed as means ($\pm$ SD). A Student's unpaired *t*-test and ANOVA were used to establish statistical significance. Results were considered significant if *P* was less than 0.05.

RESULTS

15 d-PGJ $_2$, CDDO, and Rosiglitazone Inhibit TGF- β -Stimulated Differentiation of Fibroblasts to Myofibroblasts

To determine the efficacy of CDDO, 15 d-PGJ $_2$, and rosiglitazone at inhibiting TGF- β -driven differentiation of fibroblasts to myofibroblasts, human lung fibroblasts were treated with 5 ng/ml of TGF- β and cotreated with either CDDO, 15 d-PGJ $_2$, or rosiglitazone. The maximum concentrations used (5 μ M 15 d-PGJ $_2$, 1 μ M CDDO, and 20 μ M rosiglitazone) were chosen for having the strongest inhibitory effect on TGF- β -stimulated α -SMA up-regulation without affecting cell viability (3-[4,5-

dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; data not shown). Myofibroblast differentiation was assessed using the common myofibroblast marker, α -SMA, by immunocytochemistry, Western blot, Imagestream flow cytometry, and RT-PCR. In addition, the expression of a novel myofibroblast

Figure 1. Both 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$ (15 d-PGJ $_2$) potentially suppress transforming growth factor (TGF)- β -induced α -smooth muscle actin (SMA) and calponin expression at very low concentrations, whereas rosiglitazone requires higher concentrations. (A) Primary lung fibroblasts were treated with 5 ng/ml TGF- β for 72 hours with the indicated concentrations of 15 d-PGJ $_2$, CDDO, and rosiglitazone. CDDO potentially inhibited α -SMA expression at 10- to 40-fold lower concentrations than 15 d-PGJ $_2$; however, rosiglitazone was only moderately effective at high concentrations. (B) 15 d-PGJ $_2$ (5 μ M) and CDDO (1 μ M) also inhibit TGF- β -induced expression of the smooth muscle marker, calponin. The experiment was performed three times, each with triplicate samples. The triplicate samples were loaded in adjacent lanes, and representative lanes from a single experiment are shown here. RE, relative expression as determined by densitometry and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (untreated cells = 1). (C) CDDO and 15 d-PGJ $_2$ also inhibit TGF- β -induced accumulation of α -SMA mRNA. Primary human lung fibroblasts were treated with TGF- β alone (5 ng/ml) or with TGF- β and CDDO (1 μ M), 15 d-PGJ $_2$ (5 μ M), or rosiglitazone (20 μ M), and RNA was harvested after 24 hours. CDDO and 15 d-PGJ $_2$, but not rosiglitazone, significantly inhibited TGF- β -stimulated α -SMA message. The results shown are from a single experiment performed in triplicate, and are representative of three independent experiments. *Significant increase compared with untreated; †significant decrease compared with TGF- β alone (*P* < 0.05, ANOVA).

marker, calponin, was examined. Calponin is a calmodulin-, F-actin-, and tropomyosin-binding protein that is involved in smooth muscle contraction (37). CDDO, 15 d-PGJ₂, and rosiglitazone all inhibited myofibroblast differentiation, as determined by Western blot for α -SMA (Figure 1A) and by immunocytochemistry (Figure E1). CDDO and 15 d-PGJ₂ also efficiently inhibited the novel pulmonary myofibroblast marker, calponin (Figure 1B). The inhibitory concentration causing a 50% decrease in α -SMA CDDO was 20-fold lower than that for 15 d-PGJ₂ with respect to α -SMA expression (0.04 and 0.7 μ M, respectively) (Figure 1A), and sixfold lower with respect to calponin (0.2 and 1.3 μ M, respectively) (Figure 1B and data not shown). We also measured α -SMA mRNA levels. Similar to the protein data, α -SMA mRNA was induced by TGF- β , and was significantly decreased by CDDO and 15 d-PGJ₂ (Figure 1C). By all three measures of myofibroblast differentiation, rosiglitazone was much less effective at inhibiting TGF- β -driven myofibroblast differentiation than either CDDO or 15 d-PGJ₂.

We also examined lung fibroblasts treated with TGF- β and CDDO or 15 d-PGJ₂ by Imagestream analysis, a new flow cytometry technique that images cells in addition to determining their fluorescence emissions. We have refined this technique and used intracellular staining to identify single-cell expression and changes in α -SMA staining with TGF- β treatment. This technique is valuable, because it is an objective measure of α -SMA on an individual cell basis. Myofibroblasts were distinguished from fibroblasts based on the area and intensity of α -SMA staining, as described in MATERIALS AND METHODS. TGF- β induced myofibroblast differentiation; after 72 hours, 35% of lung fibroblasts were defined as myofibroblasts by our gating

Figure 2. Imaging flow cytometry demonstrates that PPAR γ ligands attenuate TGF- β -induced myofibroblast differentiation. Human lung fibroblasts were treated with TGF- β in combination with the indicated PPAR γ ligands (1 μ M CDDO, 5 μ M 15 d-PGJ₂) for 72 hours, and were then fixed with methanol and stained for α -SMA using a Cy3-conjugated anti- α -SMA antibody, and analyzed on an Amnis Imagestream imaging flow cytometer. Live single cells were identified as described in the online supplemental MATERIALS AND METHODS. Myofibroblasts were distinguished from fibroblasts based on the intensity (*y* axis) and fractional area (*x* axis) of α -SMA staining. The numerical percentage value on each dot-plot refers to the percent of cells contained within the myofibroblast region. Note that TGF- β significantly increases the percentage of myofibroblasts, and this effect is inhibited by both 15 d-PGJ₂ and CDDO. See Figure E2 in the online supplement for representative images of cells from both outside and inside the myofibroblast region.

strategy. Both 15 d-PGJ₂ and CDDO inhibited this differentiation by approximately 50%. (Figure 2 and Figure E2).

We examined the effects of 15 d-PGJ₂ and CDDO on extracellular matrix production, an important profibrotic effector function of lung fibroblasts and myofibroblasts, by determining the expression of fibronectin and collagen. Both CDDO and 15 d-PGJ₂ suppressed TGF- β -induced fibronectin expression, as determined by Western blotting (Figure 3A). In addition, TGF- β stimulated up-regulation of collagen I and III protein (data not shown), and mRNA (Figures 3B and 3C) was

Figure 3. CDDO and 15 d-PGJ₂ potently inhibit the expression of fibronectin and collagen. (A) Primary lung fibroblasts were treated with 5 ng/ml TGF- β and either 5 μ M 15 d-PGJ₂ or 1 μ M CDDO for 72 hours, and expression of fibronectin was determined by Western blotting. Vinculin, constitutively expressed in fibroblasts, was used as a loading control. RE, relative expression of fibronectin as determined by densitometry and normalized to vinculin (untreated cells = 1). The results shown are representative of three independent experiments, each performed on triplicate wells. (B and C) Primary human lung fibroblasts were treated for 24 hours with TGF- β alone or TGF- β plus CDDO (1 μ M) or 15 d-PGJ₂ (5 μ M) or rosiglitazone (20 μ M). RNA was harvested and analyzed by RT-PCR using collagen I (COL1A1)- and collagen III (COL3A1)-specific primers, and normalized to GAPDH mRNA. CDDO, 15 d-PGJ₂, and rosiglitazone all inhibited TGF- β -induced up-regulation of collagen I and III. Results shown are the mean ($\pm$ SD) for triplicate wells from a single experiment and are representative of three independent experiments (each performed in triplicate). *Significant reduction compared with TGF- β alone ($P < 0.05$, ANOVA).

significantly decreased by CDDO as well as 15 d-PGJ₂ and rosiglitazone.

Inhibition of Myofibroblast Differentiation Is Largely PPAR γ Independent

We used complimentary pharmacological and genetic approaches to analyze the mechanism of inhibition of TGF- β -stimulated α -SMA expression by CDDO and 15 d-PGJ₂. GW9662 is a highly specific PPAR γ antagonist that covalently binds to a cysteine residue within the binding pocket of PPAR γ , permanently altering its ability to bind PPAR γ ligands. PPAR γ agonist-driven adipogenesis is PPAR γ dependent, and is completely inhibited by GW9662 (18). Primary lung fibroblasts were pretreated with GW9662, followed by the addition of TGF- β and

either CDDO or 15 d-PGJ₂. After 72 hours, myofibroblast differentiation was determined by Western blot for α -SMA. GW9662 did not prevent the inhibition of TGF- β -stimulated myofibroblast differentiation by CDDO or 15 d-PGJ₂ (Figure 4A).

Using a genetic approach, we overexpressed a dominant-negative PPAR γ (LV-PPAR γ -DN) in primary human lung fibroblasts using a lentiviral vector that results in stable coexpression of the DN PPAR γ and GFP. Greater than 90% of cells infected with the construct expressed GFP and the Flag-tagged DN PPAR γ (data not shown). Overexpression of LV-PPAR γ -DN did not significantly reduce the ability of either CDDO or 15 d-PGJ₂ to inhibit TGF- β -induced myofibroblast differentiation. α -SMA expression was 45% inhibited by 15 d-PGJ₂ in fibroblasts infected with LV-PPAR γ -DN compared with 39% inhibition in fibroblasts infected with the control vector (LV-empty). Similarly, CDDO reduced α -SMA expression by 75% in LV-PPAR γ -DN-infected fibroblasts compared with 77% in the control cells (Figure 4B). Interestingly, LV-PPAR γ -DN plus TGF- β resulted in a strong up-regulation of α -SMA compared with fibroblasts treated with LV-empty and TGF- β . This suggests that, in the absence of exogenous PPAR γ ligands, α -SMA expression and myofibroblast differentiation are regulated by the PPAR γ transcription factor, perhaps involving endogenous or media-derived PPAR γ ligands. However, as the LV-PPAR γ -DN did not alter the ability of 15 d-PGJ₂ and CDDO to inhibit α -SMA expression, their effects must be due largely to PPAR γ -independent effects.

Inhibition of Myofibroblast Differentiation by 15 d-PGJ₂ Is Dependent on Its Electrophilic Centers

CDDO and 15 d-PGJ₂ contain an α/β -unsaturated ketone that can form covalent bonds with free sulfhydryls, such as occur in glutathione and accessible cysteine residues in cellular proteins

Figure 4. Neither the irreversible PPAR γ antagonist, GW9662, nor overexpression of a dominant-negative PPAR γ , inhibits 15 d-PGJ₂ and CDDO suppression of α -SMA expression. (A) Primary lung fibroblasts were pretreated with 5 μ M GW9662 for 4 hours or left untreated, and were then treated with 5 ng/ml TGF- β and either 5 μ M 15 d-PGJ₂ or 1 μ M CDDO for 72 hours. α -SMA expression was analyzed by Western blot. GW9662 did not restore TGF- β -stimulated expression of α -SMA in cells treated with 15 d-PGJ₂ or CDDO. RE, relative expression of α -SMA normalized to GAPDH (untreated cells = 1). Results shown are representative of three independent experiments. (B) Lung fibroblast cultures were infected with a lentiviral vector that coexpresses GFP and a dominant-negative PPAR γ (LV-PPAR γ -DN, shaded bars), or the GFP-expressing vector only (LV-Empty, solid bars). At 24 hours after infection, the media were changed and TGF- β with or without 5 μ M 15 d-PGJ₂ or 1 μ M CDDO was added to the wells. At 72 hours after treatment, cells were lysed and α -SMA protein levels were analyzed by Western blotting and densitometry. Results shown are the mean ($\pm$ SE) for two independent experiments with triplicate cultures in each experiment. CDDO and 15 d-PGJ₂ significantly reduced TGF- β -driven expression of α -SMA in the presence of both wild-type and DN PPAR γ . *Significant reduction compared with TGF- β alone ($P < 0.05$, ANOVA).

Figure 5. Inhibition of TGF- β -stimulated α -SMA expression requires an electrophile. (A) Primary lung fibroblasts were treated with TGF- β and 5 μ M 15 d-PGJ₂, 5 μ M CAY10410, or 1 μ M CDDO for 72 hours. Cells were lysed, harvested, and analyzed for α -SMA expression by Western blot. CAY10410 did not inhibit α -SMA expression at the same concentration as 15 d-PGJ₂. (B) Primary lung fibroblasts were treated with TGF- β as described in MATERIALS AND METHODS. With the addition of either 10 μ M diphenyl diselenide (DSPS) or 15 μ M prostaglandin A₁ (PGA₁), both potent electrophiles that do not activate PPAR γ -dependent transcription. DSPS and PGA₁ both inhibited TGF- β -induced α -SMA expression. RE, relative expression of α -SMA normalized to GAPDH (untreated cells = 1). The experiment was performed in triplicate, with the triplicate samples loaded in adjacent lanes of the Western blot. Representative lanes from each condition are shown here.

(Figure E3) (38, 39). There are reports that the PPAR γ -independent effects of 15 d-PGJ₂ may be mediated via this active site (31). CAY10410 (9,10-dihydro-15-deoxy- $\delta^{12,14}$ -PGJ₂) is an analog of 15 d-PGJ₂ that lacks the unsaturated ketone, and is resistant to metabolism by glutathione addition (39). CAY10410 can activate PPRE-dependent transcription (40), but has significantly diminished PPAR γ -independent activity compared with 15 d-PGJ₂ (22). CAY10410 is a much less effective inhibitor of TGF- β -stimulated myofibroblast differentiation than 15 d-PGJ₂ at the same concentration (Figure 5A), demonstrating that the electrophilic center of 15 d-PGJ₂ is important in mediating this bioactivity. To further investigate the importance of the electrophilic center, we tested two potent electrophiles, DSPS and PGA₁. Both of these compounds significantly reduced TGF- β -induced α -SMA expression (Figure 5B).

CDDO and 15 d-PGJ₂ Do Not Inhibit TGF- β -Induced Signaling along the Smad or AP-1 Pathways

Our data suggest that CDDO and 15 d-PGJ₂ mediate their antifibrotic effect by a PPAR γ -independent pathway. One mechanism by which these ligands may mediate their activity is by interference with TGF- β -mediated Smad signaling. Primary human lung fibroblasts were pretreated with CDDO and 15 d-PGJ₂ for 1 hour, then treated with TGF- β and harvested after 15 or 30 minutes. Total and phospho-Smad2/3 were determined in whole-cell lysates and nuclear extracts, and Smad4 was determined in nuclear extracts by Western blot. TGF- β treatment of human primary lung fibroblasts resulted in rapid phosphorylation of Smad2/3 within 15 minutes, with a slight further increase by 30 minutes. There was no significant difference in Smad2/3 phosphorylation between TGF- β alone and TGF- β plus 15 d-PGJ₂ or CDDO (Figure 6A). TGF- β treatment also induced rapid nuclear translocation of Smad4 that was unaffected by cotreatment with CDDO or 15 d-PGJ₂ (Figure 6B). Another important TGF- β -mediated signaling pathway involves the transcription factor, AP-1 (41). Primary lung fibroblasts were transfected with an AP-1 reporter construct and treated with TGF- β and 15 d-PGJ₂ or CDDO. TGF- β alone induced an eightfold increase in AP-1 activity. Interestingly, both 15 d-PGJ₂ and CDDO alone also significantly induced AP-1 activity, and the combination of TGF- β and CDDO was twice as potent as TGF- β alone (Figure 6C), demonstrating that 15 d-PGJ₂ and CDDO do not inhibit myofibroblast differentiation by inhibiting AP-1 signaling.

CDDO Decreases Acetylation of CBP/p300

Recruitment of CBP/p300 to the Smad2/3/4 complex is required for maximal TGF- β -driven up-regulation of α -SMA (42). Primary human lung fibroblasts were treated with TGF- β alone or with TGF- β plus 1 μ M CDDO for 72 hours, then harvested for Western blotting. TGF- β treatment resulted in significant acetylation of CBP/p300 that was completely inhibited by CDDO (Figure 7).

DISCUSSION

PPAR γ ligands have both anti-inflammatory and antiscarring effects. In primary human lung fibroblasts, rosiglitazone and 15 d-PGJ₂ inhibit expression of α -SMA and collagen (24, 25). Others have demonstrated in animal models of lung fibrosis, as well as in other organs, that PPAR γ ligands have antifibrotic effects (20, 25, 43). Here, we report that CDDO is a novel antifibrotic agent that potently inhibits TGF- β -stimulated differentiation of human lung fibroblasts to myofibroblasts via a largely PPAR γ -independent pathway, with an inhibitory

Figure 6. CDDO and 15 d-PGJ₂ did not inhibit Smad2/3 phosphorylation, Smad nuclear translocation, or AP-1 signaling. Primary lung fibroblasts were pretreated with 5 μ M 15 d-PGJ₂ or 1 μ M CDDO for 1 hour, followed by treatment with 5 ng/ml TGF- β . Cells were harvested after 15 and 30 minutes and analyzed by Western blot. (A) TGF- β stimulated rapid phosphorylation of Smad 2/3, which was not inhibited by pretreatment with CDDO or 15 d-PGJ₂. (B) TGF- β also stimulated translocation of Smad 4 to the nucleus, which was not inhibited by 15 d-PGJ₂ or CDDO. Data shown are representative of two independent experiments. PGJ₂ indicates 15 d-PGJ₂. (C) Primary lung fibroblasts were transfected with an AP-1 luciferase reporter construct and treated with 5 ng/ml TGF- β plus or minus 5 μ M 15 d-PGJ₂ or 1 μ M CDDO for 24 hours, at which time the cells were harvested and luciferase activity was determined. Not only did 15 d-PGJ₂ and CDDO not inhibit AP-1 transcriptional activity, they stimulated it. Two independent experiments were performed, each in triplicate. The data shown are means ($\pm$ SD) for a single representative experiment. *Significant increase over untreated cells; †significant increase over TGF- β alone ($P < 0.05$, ANOVA).

concentration causing a 50% drop (40 nM) that is 20-fold lower than 15 d-PGJ₂ and 400-fold lower than rosiglitazone.

Because of the exciting potential of PPAR γ ligands as antifibrotic agents, it is important to understand their mechanism of action. The anti-inflammatory and antifibrotic effects of the both synthetic and natural PPAR γ ligands have been reported to involve both PPAR γ -dependent and -independent mechanisms (22, 28, 44). The PPAR γ -dependent mechanism requires ligand binding to PPAR γ , heterodimerization with RXR and its ligand, nuclear translocation, binding to the PPRE, and induction of gene transcription, whereas the PPAR γ -independent mechanisms have not yet been fully determined.

To investigate whether the antifibrotic effects of CDDO and 15 d-PGJ₂ are PPAR γ dependent or independent, we used

Figure 7. CDDO suppresses TGF- β -induced acetylation of CBP/p300. Primary lung fibroblasts were treated with or without TGF- β (5 ng/ml) and 1 μ M CDDO. After 72 hours, the cells were harvested, and expression of acetylated CBP/p300 (aCBP/p300) was determined by Western blotting. Levels of aCBP/p300 were determined by densitometry normalized to GAPDH (untreated cells = 1). The data shown are the mean ($\pm$ SE) for three independent experiments, each performed in triplicate. CDDO significantly inhibited TGF- β -induced acetylation of CBP/p300 (* P < 0.05, ANOVA). Unt, untreated.

a small molecule inhibitor of PPAR γ and overexpression of a dominant-negative PPAR γ construct. GW9662, an irreversible PPAR γ antagonist, did not restore myofibroblast differentiation in fibroblasts treated with TGF- β and CDDO or 15 d-PGJ₂ (Figure 4A). Similarly, when lung fibroblasts were transfected with LV-PPAR γ -DN, CDDO and 15 d-PGJ₂ were still able to significantly inhibit TGF- β -driven myofibroblast differentiation (Figure 4B). Interestingly, when PPAR γ activity was blocked with either GW9662 or the DN construct, treatment with TGF- β resulted in significantly greater induction of α -SMA than in cells with intact PPAR γ signaling cascades treated with TGF- β . This suggests that, in the absence of potent exogenous agonists, PPAR γ opposes TGF- β -stimulated myofibroblast differentiation via a PPAR γ -dependent mechanism involving endogenous or media-derived PPAR γ ligands, such as eicosanoids and other fatty acids (21, 45). Blocking PPAR γ function removes this opposing regulatory pathway, increasing the profibrotic effects of TGF- β . Note that, although rosiglitazone is a strong PPAR γ -dependent promoter of adipogenesis, it exhibits little PPAR γ -independent activity, and is a relatively poor inhibitor of myofibroblast differentiation compared with other agents (24, 26, 46, 47). We hypothesize that myofibroblast differentiation is regulated by both PPAR γ -dependent and PPAR γ -independent pathways, but that the PPAR γ -independent mechanisms are much stronger, and compounds that access the independent pathways are more effective at inhibiting TGF- β -stimulated myofibroblast differentiation at lower concentrations. Therefore, compounds that access PPAR γ -independent pathways are likely to have greater clinical potential as antifibrotic agents.

Inspection of the structures of the PPAR γ ligands suggests that the strong PPAR γ -independent effects of 15 d-PGJ₂ and CDDO might be mediated by their electrophilic α/β -unsaturated ketone. These compounds are susceptible to Michael addition reactions by nucleophiles of free sulfhydryls, such as occur in glutathione and accessible cysteine residues in cellular proteins (Figure E3B) (30, 31), and it has been reported that 15 d-PGJ₂ modifies the activity of the estrogen receptor by covalently binding to its DNA binding domain (48). We tested this hypothesis using CAY10410, an analog of 15 d-PGJ₂ that lacks the α/β -unsaturated ketone and is unable to undergo this reaction (Figure E3A). CAY10410 was a much less effective inhibitor of

α -SMA expression than 15 d-PGJ₂ at the same concentration (Figure 5A). We further demonstrated the importance of the electrophilic carbon by using two other strong electrophilic compounds (DSPS and PGA₁), which potently decreased TGF- β -induced expression of α -SMA (Figure 5B). These new findings support the concept that the electrophilic carbon has a central role in mediating inhibition of TGF- β -stimulated myofibroblast differentiation by a PPAR γ -independent pathway.

PPAR γ -independent effects of PPAR γ ligands have been reported in several papers; however, no common pathway applicable to all cell types has been identified. For example, 15 d-PGJ₂ decreases Smad2/3 phosphorylation in human hepatic stellate cells (49), whereas it inhibits nuclear localization without affecting phosphorylation in human glomerular mesangial cells (50). Our data demonstrate that, in primary human lung fibroblasts, neither 15 d-PGJ₂ nor CDDO decrease TGF- β -induced phosphorylation of Smad2/3, or alter Smad localization. It has been reported that Smad3, but not Smad2, is required for the fibrotic response (51, 52); however, the antibodies used in this study detect both Smad2 and Smad3. Therefore, a role for Smad3 alone is not completely ruled out.

Alternately, pioglitazone inhibits profibrotic changes in mouse glomerular mesangial cells by suppressing AP-1 activity (41). However, neither 15 d-PGJ₂ nor CDDO inhibits AP-1 activation in human lung fibroblasts (Figure 6C). In fact, both ligands alone activated an AP-1 reporter, and cotreatment with TGF- β and either ligand resulted in more AP-1 activity than TGF- β alone (Figure 6C). We have found that 15 d-PGJ₂ and CDDO induce heme oxygenase-1 (HO-1), an enzyme involved in protecting cells from oxidative stress (H. E. Ferguson and colleagues, unpublished data), and it has been reported that HO-1 induces AP-1 activity (53). We hypothesize that, because 15 d-PGJ₂ and CDDO are strong electrophiles and can react with and consume glutathione (Figure E3), they induce oxidative stress and HO-1 in lung fibroblasts, leading to enhanced AP-1 activity.

We report here the novel finding that CDDO inhibits acetylation of CBP/p300. CBP/p300 is a critical transcriptional cofactor, the activity of which is regulated in part by acetylation (54). CBP/p300 contains a HAT domain that can acetylate the core histones, H3 and H4, which relaxes the chromatin, making it accessible to other transcription factors. The HAT domain of CBP/p300 can acetylate other transcription factors as well, and it has been shown that acetylation of Smad3 is required for the expression of TGF- β -responsive genes, including α -SMA (16, 42, 55). Upon binding of TGF- β to its receptor, Smad3 is phosphorylated, recruits Smad4, and the Smad3/4 complex is translocated to the nucleus, where it binds to the promoters of TGF- β -responsive genes (Figure E4). Smad3 recruits binding of the CBP/p300 cofactor, which becomes acetylated itself by an unknown mechanism. The HAT domain of CBP/p300 acetylates Smad3, which is required for transcriptional activation, and the core histones, H3 and H4, which converts the chromatin from a closed, transcriptionally inactive conformation to an open, transcriptionally active conformation. Acetylation of CBP/p300 results in enhanced HAT activity (54). We have shown that CDDO dramatically inhibits acetylation of CBP/p300 (Figure 7). Therefore, we hypothesize that CDDO inhibits myofibroblast differentiation by inhibiting acetylation of CBP/p300, which, in turn, inhibits acetylation of Smad3 and histones, thereby blocking transcription from TGF- β -responsive genes.

Overall, we have demonstrated for the first time that PPAR γ ligands, such as CDDO and other small molecules with electrophilic carbons, are potent inhibitors of TGF- β -induced myofibroblast differentiation and extracellular matrix production in human lung fibroblasts that act via a PPAR γ -independent

dent pathway. This pathway may involve a novel mechanism of action for CDDO, inhibiting α -SMA expression by dysregulating acetylation of CBP/p300. CDDO is an orally active agent, with a long half-life, and is in clinical trials for the treatment of several forms of cancer, including leukemia, solid tumors, and myelomas. The translational potential of this compound is thus high. Our results also suggest that developing other electrophilic compounds that target PPAR γ -independent pathways may be of great clinical utility for scarring diseases of the lungs and other tissues.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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