PPAR-γ Ligands Repress TGFβ-Induced Myofibroblast Differentiation by Targeting the PI3K/Akt Pathway: Implications for Therapy of Fibrosis

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Abstract

Transforming growth factor beta (TGFβ) induced differentiation of human lung fibroblasts to myofibroblasts is a key event in the pathogenesis of pulmonary fibrosis. Although the typical TGFβ signaling pathway involves the Smad family of transcription factors, we have previously reported that peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands inhibit TGFβ-mediated differentiation of human lung fibroblasts to myofibroblasts via a Smad-independent pathway. TGFβ also activates the phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) pathway leading to phosphorylation of AktS473. Here, we report that PPAR-γ ligands, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and 15-deoxy-12,14-15d-prostaglandin J2 (15d-PGJ2), inhibit human myofibroblast differentiation of normal and idiopathic pulmonary fibrotic (IPF) fibroblasts, by blocking Akt phosphorylation at Ser473 by a PPAR-γ-independent mechanism. The PI3K inhibitor LY294002 and a dominant-negative inactive kinase-domain mutant of Akt both inhibited TGFβ-stimulated myofibroblast differentiation, as determined by Western blotting for α-smooth muscle actin and calponin. Prostaglandin A1 (PGA1), a structural analogue of 15d-PGJ2 with an electrophilic center, also reduced TGFβ-driven phosphorylation of Akt, while CAY10410, another analogue that lacks an electrophilic center, did not; implying that the activity of 15d-PGJ2 and CDDO is dependent on their electrophilic properties. PPAR-γ ligands inhibited TGFβ-activated fibrillogenesis via both post-translational and post-transcriptional mechanisms. This inhibition is independent of MAPK-p38 and PTEN but is dependent on TGFβ-induced phosphorylation of FAK, a kinase that acts upstream of Akt. Thus, PPAR-γ ligands inhibit TGFβ signaling by affecting two pro-survival pathways that culminate in myofibroblast differentiation. Further studies of PPAR-γ ligands and small electrophilic molecules may lead to a new generation of anti-fibrotic therapeutics.

Citation: Kulkarni AA, Thatcher TH, Olsen KC, Maggirwar SB, Phipps RP, et al. (2011) PPAR-γ Ligands Repress TGFβ-Induced Myofibroblast Differentiation by Targeting the PI3K/Akt Pathway: Implications for Therapy of Fibrosis. PLoS ONE 6(1): e15909. doi:10.1371/journal.pone.0015909

Editor: Ramy K. Aitiz, Cairo University, Egypt

Received October 12, 2010; Accepted November 26, 2010; Published January 6, 2011

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Funding: This research was supported by National Institutes of Health Grants: HL075432, HL075432-04S1, HL075432-04S2, HL095402-02, T32 HL66988, F30 HL097596, T32 ES07026 and P30 ES01247. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a progressive disease of unknown etiology that can result in respiratory failure [1,2]. IPF is anatomically characterized by scarring of lung tissues owing to excessive deposition of extracellular matrix proteins (ECM). This excessive and uncontrolled deposition of ECM compromises normal lung function and structure [1,3]. Fibroblasts are structural cells that show plasticity and ability to differentiate into myofibroblasts upon tissue injury or inflammation [4,5]. Myofibroblasts are characterized by expression of alpha smooth muscle actin (αSMA), calponin and extracellular matrix (ECM) proteins including Type 1 and III collagen (Col1A1 and Col3A1), fibronectin and proteoglycan [4]. Deposition of ECM and other proteins produced by myofibroblasts plays an important role in normal physiologic processes such as wound healing. However, in pathologic conditions such as IPF, myofibroblasts accumulation and matrix deposition is excessive leading to scarring [1,2].

Transforming Growth Factor β (TGFβ) is a pleiotropic cytokine that promotes myofibroblast differentiation and plays a major role in both wound healing and fibrosis [4,6,7]. Binding of active TGFβ to its receptor triggers several signaling pathways [8,9], including the well-characterized Smad pathway and the PI3K/Akt (phosphotidylinositol-3-kinase/Protein Kinase B/PKB) pathway [7,10,11]. PI3K activates Akt by phosphorylation at two sites, Thr308 and Ser473 [10]. Once active, Akt functions as a serine/threonine kinase, which is involved in multiple cellular processes including cell proliferation, inflammation, survival and glucose metabolism. Akt is inactivated by a specific phosphatase enzyme, PTEN (the phosphatase and tensin homologue deleted on chromosome 10) [12]. Multiple upstream signaling events can activate Akt via PI3K. In fetal lung fibroblasts [13], and other organs [14,15], TGFβ activates Akt signaling via p38 Mitogen
Prostaglandins (e.g. 15d-PGJ2) and Triterpenoids (e.g. CDDO: 2-cyano-3,12-dioxoolean-1,9-diene-23-oic-acid) are ligands of peroxisome proliferator-activated receptor-γ (PPAR-γ) [29]. Three of the main classes of PPAR-γ ligands include; Thiazolidinediones or TZDs (e.g. Rosiglitazone), transcriptional response [29]. Three of the main classes of PPAR-γ ligands include; Thiazolidinediones or TZDs (e.g. Rosiglitazone), Prostaglandins (e.g. 15d-PGJ2: 15-deoxy-Smooth muscle actin (α-SMA) and calponin. Pretreatment of cells with LY294002 markedly reduced TGFβ-induced phosphorylation of Akt (Fig 1A) and almost completely inhibited myofibroblast differentiation (Fig 1A and 1B). LY294002 treatment alone significantly reduced basal level of Akt phosphorylation and expression of calponin (Fig 1A). These results indicate that TGFβ induces phosphorylation of Akt and myofibroblast differentiation via the P38 pathway. Primary HLF cells were transfected with an empty vector or a dominant negative Akt construct, treated with TGFβ (50ng/ml) followed by TGFβ (5ng/ml) for 48 hours and A, immunoblots were performed to detect expression of the indicated proteins, and B, immunofluorescence for α-SMA (green) was performed to assess the effects of P38 inhibition on TGFβ-induced myofibroblast differentiation. DAPI (blue) was used to visualize nuclei. C, HLF cells were transfected with an empty vector or a dominant negative kinase-dead (KD) Akt construct, treated with TGFβ, and assayed for myofibroblast differentiation by Western blot. Protein lysates from all the indicated samples were electrophoretically separated on the same gel, and representative lanes from a single experiment are shown here. These data indicate that a functional P38-Akt pathway is essential for the TGFβ-induced myofibroblast differentiation in primary human lung fibroblast.

doi:10.1371/journal.pone.0015909.g001

**Results**

**TGFβ-Stimulated Myofibroblast Differentiation of Primary Human Lung Fibroblasts Requires the Phosphatidylinositol 3-Kinase Pathway**

TGFβ is known to induce cell-type specific actions that are context-specific and microenvironment-dependent [9]. We previously reported that inhibition of TGFβ-stimulated myofibroblast differentiation of primary human lung fibroblast (HLF) by PPAR-γ ligands was largely Smad-independent [33]. To investigate potential non-Smad signaling pathways, we examined whether TGFβ drives myofibroblast differentiation via the P38/Akt pathway. Primary HLF cells were treated with TGFβ in presence or absence of LY294002, a highly selective and potent inhibitor of P38. Following treatment, whole-cell lysates were subjected to specific Western blot analyses. As evident in Fig 1A, TGFβ potently induced phosphorylation of Akt. TGFβ was also able to induce myofibroblast differentiation as determined either by Western blot analysis (Fig 1A) or indirect immunofluorescence (Fig 1B) for α-SMA and calponin. Pretreatment of cells with LY294002 markedly reduced TGFβ-induced phosphorylation of Akt (Fig 1A) and almost completely inhibited myofibroblast differentiation (Fig 1A and 1B). LY294002 treatment alone significantly reduced basal level of Akt phosphorylation and expression of calponin (Fig 1A). These results indicate that TGFβ induces phosphorylation of Akt and myofibroblast differentiation via the P38 pathway in primary HLF.

To investigate if functional Akt kinase is required for TGFβ-induced myofibroblast differentiation, primary HLF cells transfected with either an empty vector or a dominant negative kinase-dead Akt (KD-AktK179A) plasmid that encodes a mutant form of Akt [34] and lacks kinase activity [35]. Overexpression of the KD-AktK179A markedly inhibited TGFβ-induced expression of α-SMA and calponin (Fig 1C) indicating that the intact kinase domain of

**Figure 1. Inhibition of PI3K-Akt pathway by LY294002 inhibits myofibroblast differentiation.** Primary HLFs were treated with the P38 inhibitor LY294002 (50μM) followed by TGFβ (5ng/ml) for 48 hours.
Akt is essential for TGFβ-induced myofibroblast differentiation. These results firmly establish that TGFβ induces myofibroblast differentiation of primary human lung fibroblasts via a PI3K-Akt-dependent mechanism.

PPAR-γ Ligands Block TGFβ-induced Phosphorylation of Akt in a Dose-Dependent Manner

After establishing that Akt activity is essential for TGFβ-induced myofibroblast differentiation, we examined whether PPAR-γ ligands CDDO and 15d-PGJ2 inhibit Akt phosphorylation. Primary HLF cells were treated with TGFβ alone or in combination with varying pharmacological concentrations of PPAR-γ ligands. The efficacy of PPAR-γ ligands to repress TGFβ-induced Akt phosphorylation at various concentrations was assessed by Western blot analysis.

Both CDDO and 15d-PGJ2 inhibited Akt phosphorylation as well as myofibroblast differentiation (Fig 2), but CDDO was about five times more potent than 15d-PGJ2 (IC50 of 0.5 μM and 2.5 μM, respectively). The potency of PPAR-γ ligands to inhibit TGFβ-induced myofibroblast differentiation correlates extremely well with their relative efficacy in inhibiting Akt phosphorylation under the same physiological conditions (Fig 2 and [33]). Taken together, our results obtained thus far demonstrate that a functional PI3K-Akt pathway is essential for TGFβ-driven myofibroblast differentiation, and PPAR-γ ligands target this pathway through inhibition of AktSer473 phosphorylation.

Since Akt is involved in the cell survival pathway next, we examined if 15d-PGJ2 and CDDO are cytotoxic at the concentrations used in these experiments. Neither 15d-PGJ2 ([27] and Fig 2B) nor CDDO (Fig 2B) were found to be cytotoxic as measured by LDH release assay. Additionally, we confirmed that 15d-PGJ2 and CDDO both were able to induce PPAR-γ-dependent transcription as measured by their ability to induce PPRE-luciferase (PPAR-γ Response Elements-luciferase) (Fig 2C and [27]).

Inhibition of TGFβ-Stimulated Activation of Akt by PPAR-γ Ligands is Independent of PPAR-γ Activity

Typically, PPAR-γ agonists bind to the ligand binding site of PPAR-γ, causing its nuclear translocation and resulting in a

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**Figure 2. PPAR-γ ligands inhibit TGFβ-induced phosphorylation of Akt and myofibroblast differentiation in a dose-dependent manner.** A, Primary HLFs were grown until 70–80% confluent, serum starved for 24 hours and treated with the indicated concentrations of PPAR-γ ligands for 48 hours. Total cell lysates were prepared, and subjected to SDS-PAGE followed by immunoblotting. The blot was probed with antibodies against phospho-AktSer473, stripped and probed to detect total Akt, αSMA and loading control GAPDH. The relative changes in the ratio of phospho-AktSer473/total Akt (R.P.) and relative changes in the expression of αSMA/GAPDH (R.E.) are as indicated in the figure. The experiment was performed in triplicate and a representative blot is shown here. B, LDH release does not increase in response to 15d-PGJ2 or CDDO. Primary human lung fibroblasts were treated with either 5 μM 15d-PGJ2 or 1 μM CDDO for 72 hours and LDH release was measured (nmol/min/mL). C, Primary human lung fibroblasts were transfected with a PPRE luciferase reporter and a CMV β-galactosidase construct. Cells were treated with either 5 μM 15d-PGJ2 or 1 μM CDDO for 48 hrs and luciferase activity was measured. Background was subtracted and data normalized to β-galactosidase transfection efficiency and reported as fold induction of luciferase units over the untreated samples. These data represent three independent experiments (mean ± S.E. shown, **p≤0.01, *** p≤0.001, compared to untreated).

doi:10.1371/journal.pone.0015909.g002
transcriptional response at target genes [10]. However, we [27,20,33] and others [32], have shown that PPAR-γ ligands also have effects that are independent of PPAR-γ activity. To examine if inhibition of Akt phosphorylation by PPAR-γ ligands was also independent of the functional activity of PPAR-γ, we used a pharmacological approach involving a PPAR-γ antagonist GW9662, an irreversible PPAR-γ antagonist that covalently binds to the active site of PPAR-γ [36].

Primary HLF were pretreated with GW9662 followed by treatment with PPAR-γ ligands and TGFβ. After 48 hours, total protein lysate was subjected to Western blot analysis to assess the level of changes in Akt phosphorylation. TGFβ alone increased phosphorylation of Akt while addition of CDDO or 15d-PGJ2 blocked TGFβ-induced Akt phosphorylation (Fig 3A). However, treatment with GW9662 did not restore either TGFβ-induced phospho-Akt levels in PPAR-γ-ligand-treated cultures or myofibroblast differentiation (Fig 3A) suggesting that CDDO and 15d-PGJ2 block TGFβ-induced Akt phosphorylation in a PPAR-γ-independent mechanism.

The Electrophilic Center Present in PPAR-γ Ligands is Critical to Their Ability to Block TGFβ-induced Phosphorylation of Akt

CDDO and 15d-PGJ2 contain electrophilic carbons (Fig 3B) that can modify sulfhydryl groups in proteins via the ‘Michael addition reaction’ [37,38]. To determine whether the ability of PPAR-γ ligands to inhibit TGFβ-induced Akt phosphorylation is dependent on the presence of an electrophilic carbon, we used two different structural analogues of 15d-PGJ2, prostaglandin A1 (PGA1), which has an electrophilic center, and CAY10410, which does not have an electrophilic carbon but binds to PPAR-γ [39]. Because the effects of CDDO and 15d-PGJ2 are PPAR-γ-dependent on the functional activity of PPAR-γ, we hypothesized that their effect would be independent of PPAR-γ. Additionally, we used another potent electrophilic compound, diphenyl disulfide (DSPS), which has an electrophilic center. CDDO, CAY10410, and DSPS contain electrophilic carbons (Fig 3B). Interestingly, only the compounds with electrophilic centers, PGA1 and DSPS, reduced TGFβ-driven phosphorylation of Akt, while CAY10410, did not (Fig 3C). Based on these results, we concluded that the electrophilic carbons present in the structures of CDDO and 15d-PGJ2 have an essential role in blocking TGFβ-mediated activation of Akt.

PPAR-γ Ligands Inhibit TGFβ-induced Phosphorylation of Akt in a Transcription-Independent Manner

Because the effects of CDDO and 15d-PGJ2 are PPAR-γ independent, we hypothesized that their effect would be independent of transcription as well. To investigate if PPAR-γ ligands require de novo transcription for their inhibition of Akt phosphorylation we treated primary HLF cells with a transcription inhibitor Actinomycin D (ActD), followed by the PPAR-γ ligands and TGFβ. Western blot analysis was performed to detect phospho-Akt in total Akt and the ratio of phospho-Akt/Akt was calculated. Inhibition of Akt by PPAR-γ ligands was independent of transcription (Fig 4A and 4B, open bars) suggesting that these compounds directly inhibit Akt kinase activity and upregulate a phosphatase in a post-translational mechanism that is independent of de novo transcription.

PPAR-γ Ligands Block TGFβ-Stimulated Phosphorylation of Akt by Inhibiting FAK but not PTEN and MAPK-p38 Phosphorylation

To investigate proteins that could potentially phosphorylate or dephosphorylate Akt, we performed a time-course of action of PPAR-γ ligands and TGFβ on the phosphorylation of PTEN, p38-MAPK and FAK.

First, we investigated the time-course of TGFβ-mediated phosphorylation of Akt and its repression by CDDO and 15d-PGJ2 (Fig 5A). Next, we examined changes in phosphorylation of PTEN, which is a negative regulator of Akt phosphorylation. PTEN is more stable but less active when it is phosphorylated at T308, and less stable but more active when it is dephosphorylated at the same site [39]. If PPAR-γ ligands inhibit Akt phosphorylation via upregulation of phosphatase activity of PTEN, we would expect to see a decrease in the ratio of phospho-PTEN/T308 to total PTEN. We found that although TGFβ slightly increased levels of phospho-PTEN after two hours (Fig 5B, filled squares), treatment with PPAR-γ ligands was unable to cause any significant deviation in the ratio of phospho-PTEN to total PTEN as compared to the TGFβ-alone-treated samples (Fig 5B, open squares and circles).

It has been reported that TGFβ induces p38-MAPK in some cell types [17]. We also observed an increase in the phospho-p38Ser180/Thr182 to total p38 ratio in response to TGFβ, but, phosphorylation of TGFβ-induced p38 was not reduced upon treatment with either CDDO or 15d-PGJ2 (Fig 5C), indicating that the mode of action of PPAR-γ ligands is likely independent of p38-MAPK activity. Finally, we examined the effect of PPAR-γ ligands on FAK [37,38] phosphorylation, which is required for FAK kinase activity. We observed TGFβ-induced phosphorylation of FAK repressed by both the PPAR-γ ligands 24 hours after the treatment (Fig 5D). CDDO inhibited FAK [37,38] phosphorylation more potently than 15d-PGJ2 (Fig 5D). Since PPAR-γ ligands inhibit FAK phosphorylation but do not change either p38-MAPK or PTEN phosphorylation, we suggest that PPAR-γ ligands inhibit Akt pathway by inhibiting the upstream FAK kinase.

Pharmacological Inhibition of FAK Activity Inhibits the PI3K-Akt Pathway and Myofibroblast Differentiation

To determine whether TGFβ-mediated myofibroblast differentiation mediated through PI3K-Akt signaling also requires FAK activity in our cell strain, we treated primary HLF cells with a FAK-kinase inhibitor (AG1296) and its inactive analogue, PP3. Western blot analysis was used to measure phospho-FAK, total FAK, phospho-Akt, total Akt, αSMA, calponin and GAPDH. We observed that inhibition of FAK inhibited not only Akt phosphorylation but also myofibroblast differentiation (Fig 6). Although, inhibition of FAK inhibited αSMA expression, we did not observe complete inhibition of expression of αSMA under the conditions tested. These results indicate that FAK activity is important for TGFβ-mediated Akt activation and myofibroblast differentiation of primary HLF.

Next, we used a complimentary genetic approach to ascertain involvement of FAK in myofibroblast differentiation of primary HLF. We found that over-expression of FAK resulted in marked up-regulation of Akt phosphorylation and myofibroblast differentiation (Fig 6B).

PPAR-γ Ligands Inhibit Myofibroblast Differentiation of Primary IPF Fibroblasts by Inhibiting FAK and PI3K-Akt Pathways

To ascertain the therapeutic potential of PPAR-γ ligands we examined their ability to suppress Akt and FAK in *bona fide* diseased primary lung fibroblasts obtained from patients with IPF. First, we investigated whether TGFβ-induced myofibroblast differentiation of IPF fibroblasts via activation of Akt and FAK pathways. IPF fibroblasts were treated with two highly potent and
Figure 3. PPAR-γ ligands inhibit TGFβ-induced Akt phosphorylation and myofibroblast differentiation in a PPAR-γ-independent but electrophilic carbon-dependent manner. HLF cells were treated with indicated compounds and/or TGFβ (5ng/ml) for 48 hours. Immunoblots were performed to assess the expression of indicated proteins. Protein lysates from all the indicated samples were electrophoretically separated on the same gel, and representative lanes from a single experiment are shown here.

A, the ability of PPAR-γ ligands (CDDO (1μM) and 15d-PGJ2 (5μM)) to reduce p-Akt was not altered upon GW9662-mediated inhibition of PPAR-γ. GW9662 (5μM) inhibits PPAR-γ activity by a covalent bond formation with PPAR-γ protein [36]. R.P. indicates relative changes in Akt phosphorylation compared to control sample, and R.E., relative changes in expression compared to control sample. B, PPAR-γ ligands contain electrophilic carbons. Here, positions of the electrophilic carbons in the structures of the compounds are marked. CAY10410 and PGA1 are structural analogues of 15-d-PGJ2. PGA1 has an electrophilic center but CAY10410 does not. DSPS, like CDDO, has two electrophilic centers. Cells were pre-treated with CAY10410 (5μM), PGA1 (10μM) and DSPS (10μM) for 30 minutes C, only compounds with an electrophilic carbon are able to reduce Akt phosphorylation, indicating that presence of an electrophilic carbon is essential for the observed reduction in the phosphorylation of Akt. All the experiments were performed in triplicate and representative images are shown here.

doi:10.1371/journal.pone.0015909.g003
selective inhibitors of PI3K pathway, LY294002 and wortmannin (Fig 7A) or increasing concentrations of AG1879 (PP2), a FAK inhibitor (Fig 7B) followed by TGFβ. Inhibition of both the pathways potently blocked phosphorylation of Akt and myofibroblast differentiation (Fig 7A and B). Finally, we treated IPF fibroblasts with CDDO and 15d-PGJ2 followed by TGFβ. These data firmly establish that CDDO and 15d-PGJ2 are both capable of blocking myofibroblast differentiation via Akt and FAK pathways (Fig 7C) as measured by the expression of αSMA and calponin.

Discussion

Idiopathic pulmonary fibrosis (IPF) can lead to respiratory failure and death due to deteriorating respiratory function [1,4]. There are currently few if any effective therapies. Therefore, novel antifibrotic drugs are urgently needed for the treatment of IPF and other scarring diseases.

PPAR-γ ligands are emerging as exciting potential therapeutics for inflammatory and fibrotic and other diseases [29,40,41]. PPAR-γ activation induces adipogenesis and differentiation, and represses inflammation [27,29,33]. PPAR-γ ligands including rosiglitazone and other members of TZD family are used for the treatment of type II diabetes [39]. Clinical trials for CDDO are in progress, and it has been found to be orally active for the treatment of solid tumors and lymphoma [42]. However, the mechanisms of anti-fibrotic actions of PPAR-γ ligands remain poorly understood. Therefore, we investigated the underlying molecular pathways targeted by distinct PPAR-γ ligands to explore the potential of PPAR-γ agonists as anti-fibrotic therapies.

Our previous work identified CDDO, a novel PPAR-γ ligand, as a potent anti-fibrotic agent of TGFβ-driven pro-fibrotic activity in vivo [27,33]. TGFβ induces lung fibrosis in vivo [43] and also stimulates phosphorylation of Akt in animal models [44] and other human organs [10,45]. Studies in fetal lung fibroblasts demonstrate the role of TGFβ-induced Akt pathway in myofibroblast differentiation [13]. Here, we report that in both normal and IPF primary human lung fibroblasts, PPAR-γ ligands potently block myofibroblast differentiation via a PPAR-γ-independent mechanism by targeting the TGFβ-induced PI3K-Akt pathway involving FAK.

We investigated the role of PI3K-Akt pathway in TGFβ-stimulated myofibroblast differentiation using LY294004, a specific PI3K activity inhibitor, and by using a kinase-dead (KD-Akt) construct of Akt. Both LY294002 and the Akt mutant strongly blocked TGFβ-stimulated myofibroblast differentiation, confirming the central role of PI3K-Akt pathway in TGFβ-mediated myofibroblast differentiation in adult human normal and “diseased” IFP lung fibroblasts (Fig 1 and 7). Although, CDDO [46,47] and 15d-PGJ2 [48] have been reported to reduce Akt phosphorylation in some studies, their mechanism of reduction of TGFβ-induced myofibroblast differentiation through Akt pathway is not yet reported. Here, we show that the suppression of TGFβ-induced phosphorylation of Akt by PPAR-γ ligands is the central mechanism of action of CDDO and 15d-PGJ2 that leads to their anti-fibrotic activity. CDDO suppresses TGFβ-induced phospho-Akt more potently than 15d-PGJ2, which correlates very well with the abilities of CDDO and 15d-PGJ2 to reduce TGFβ-induced myofibroblast differentiation (Fig 2). Compared to CDDO and 15d-PGJ2, rosiglitazone was relatively poorly effective at inhibiting TGFβ-induced Akt phosphorylation (data not shown). Interestingly, Kilter et al. reported that rosiglitazone facilitates rephosphorylation of Akt in rat cardiomyocytes [49]. We and others have reported that rosiglitazone has some anti-fibrotic effects in vitro [27,32,33,50], but is much less potent than either CDDO or 15d-PGJ2. If rosiglitazone indeed facilitates re-phosphorylation of Akt, then it would result in a pro-fibrotic response that undercuts its anti-fibrotic effects, suggesting that rosiglitazone is not an optimal choice for treating fibrotic lung diseases. We did not investigate rosiglitazone further in this study.

CDDO and 15d-PGJ2 have electrophilic properties that rosiglitazone does not, and we have previously identified the electrophilic centers as important in the anti-fibrotic activity of these compounds. Building on these observations, here we demonstrate that the PPAR-γ-independent effects of CDDO and 15d-PGJ2 on Akt phosphorylation are dependent on the electrophilic properties (Fig 3). These observations offer an additional possibility that CDDO and 15d-PGJ2 could directly bind to the active site of a signaling molecule involved in the Akt pathway.
One study has shown that biotinylated CDDO is capable of binding and thus inactivating the active site of PTEN [37], and 15d-PGJ2 is capable of inhibiting the activities of proteins through direct covalent modification [51,52]. Further studies involving biochemical approaches should help us understand the exact nature of action of compounds involving electrophilic carbon. To generate newer anti-fibrotic therapeutics, further investigation of the electrophilic properties of PPAR-\(\gamma\) ligands and similar compounds is necessary.

Because CDDO and 15d-PGJ2 block Akt phosphorylation in the absence of new transcription (Fig 4), this suggests that CDDO and 15d-PGJ2 act to directly inhibit a kinase or activate a phosphatase that acts on Akt. We examined the role of three important upstream regulators of Akt phosphorylation: p38-MAPK, PTEN and FAK (Fig 8). We chose to examine p38-MAPK because it is previously known to be a part of the PI3K/Akt pathway [13] while other MAP kinases are involved in separate [53] or even antagonistic pathways [54]. TGF\(\beta\) activates a number of signaling pathways including Smads, Akt and the MAPK-ERK, and while our data does not completely rule out that PPAR-\(\gamma\) ligands may act via other pathways, the ability of LY294002 and KD-Akt to almost completely block myofibroblast differentiation shows that the PI3K/Akt pathway is the most important pathway. Since TGF\(\beta\) stimulates phosphorylation of p38-MAPK to activate myofibroblast differentiation by up-regulating Akt phosphorylation we examined involvement of p38-MAPK in primary HLF.

In agreement with a previous report [13], we determined that p38-MAPK is phosphorylated following TGF\(\beta\) treatment of HLF (Fig 5C), and MAPK inhibitor SB203580 reduced phosphorylation of Akt (data not shown). However, neither CDDO nor 15d-PGJ2 altered TGF\(\beta\)-induced p38-MAPK phosphorylation, indicating that MAPK is likely not involved in inhibition of Akt by CDDO and 15d-PGJ2. We were also able to exclude PTEN as a major mediator of the effects of PPAR-\(\gamma\) ligands in HLFs. PTEN can inhibit the Akt pathway by dephosphorylating Akt, and PTEN phosphatase is itself activated by dephosphorylation at Thr308. Thus, if CDDO or 15d-PGJ2 blocked Akt phosphorylation via PTEN, we would expect levels of phospho-Akt to increase in presence of CDDO; instead we observed the opposite.

Multiple reports show that TGF\(\beta\) stimulates autophosphorylation-dependent activation of focal adhesion kinase (FAK) [23,55]. For example in CCL20 lung fibroblasts, Xia et al demonstrated that \(\beta\)1-integrin signaling upregulates FAK phosphorylation and its physical interaction with PI3K-p85 resulting in phosphorylation of Akt [24]. Although FAK is a widely accepted upstream regulator of Akt phosphorylation, it has been reported that FAK

Figure 5. PPAR-\(\gamma\) ligands inhibit TGF\(\beta\)-induced phosphorylation of Akt and FAK but not MAPK-p38 and PTEN. Primary HLFs were pretreated with PPAR-\(\gamma\) ligands; CDDO (1\(\mu\)M) and 15d-PGJ2 (5\(\mu\)M) followed by TGF\(\beta\) (5ng/ml). Cells were harvested and lysates analyzed by immunoblots at the indicated time. The ratio of phospho-protein to total protein was measured by densitometric analysis and normalized to untreated cells (untreated = 1.0). TGF\(\beta\)-induced phosphorylation of A, AktS473 and D, FAKY397 was inhibited significantly by the PPAR-\(\gamma\) ligands but the phosphorylation of B, PTENT308 and C, p38-MAPK T180/Y182 was not affected. The statistical significance over TGF\(\beta\)-treatment alone was calculated either by one way ANOVA on triplicate samples (A, B and C) or using unpaired t-test on duplicate samples (D) and is indicated as * where P<0.05. doi:10.1371/journal.pone.0015909.g005
FAK overexpression induces myofibroblast differentiation of primary human lung fibroblasts. doi:10.1371/journal.pone.0015909.g006

Figure 6. Pharmacological inhibition of FAK activity inhibits the PI3K-Akt pathway and myofibroblast differentiation. A, Primary HLFs were treated in presence or absence of TGFβ and 10μM specific Src-FAK kinase inhibitor AG1879 (PP2) or its analogue, PP3, that does not inhibit FAK activity and immunoblots were performed to analyze expression of the indicated proteins. The FAK inhibitor AG1879, but not its analogue PP3, inhibited TGFβ-induced phosphorylation of FAK and Akt and reduced myofibroblast differentiation as determined by expression of αSMA and calponin. B, HLF cells were transfected with the empty vector (V) or FAK overexpressing construct (HA-FAK) and assayed for myofibroblast differentiation by Western blot. Protein lysates from all the indicated samples were electrophoretically separated on the same gel, irrelevant lanes excluded and representative lanes from a single experiment are shown here. These data indicate that FAK overexpression induces myofibroblast differentiation of primary human lung fibroblasts. does not act upstream of Akt during TGFβ signaling in IMR90 human fetal lung fibroblasts [17]. However, it is widely accepted that integrins are able to activate TGFβ [56] and FAK [57,58], both of which are involved in myofibroblast differentiation. Our results demonstrate that PPAR-γ ligands are able to inhibit phosphorylation of FAK and limit the TGFβ-mediated fibrotic response in adult primary HLFs (Fig 5D). We confirmed both in our normal (Fig 6) and IPF (Fig 7) primary human cell strains that blocking FAK activity inhibited not only phosphorylation of Akt, but also expression of αSMA, confirming that FAK indeed regulates myofibroblast differentiation under normal and diseased conditions through activation of Akt pathway. To establish the therapeutic potential of PPAR-γ ligands we treated fibroblasts obtained from IPF patients, in parallel, with PPAR-γ ligands, two PI3K inhibitors and a FAK inhibitor and confirmed that CDDO and 15d-PGJ2 potently block myofibroblast differentiation of not only normal HLF but also diseased IPF fibroblasts (Fig 7) via PI3K-Akt and FAK pathways. Although by using a complimentary genetic approach we confirmed that overexpression of FAK is capable of upregulating Akt phosphorylation and myofibroblast differentiation (Fig 6B), at this point, we cannot rule out an additional mechanism of action of PPAR-γ ligands that would result in reduction of Akt phosphorylation (Fig 8).

Current models of pulmonary fibrosis suggest that TGFβ, mechanical stress, or adhesion and integrin mediated activation of myofibroblast differentiation all contribute to upregulation of a fibrotic response. One very critical, central and shared event in all of these pathways involves activation of Akt, defined by its phosphorylation at Tyr397. It is conceivable that once TGFβ activates myofibroblast differentiation, the increased deposition of extracellular matrix proteins would cause additional mechanical stress on the cell surface leading to sustained and continual activation of FAK. Since FAK itself upregulates myofibroblast differentiation, once TGFβ initiates this process, sustained activation of Akt would be able to perpetuate the fibrotic response even in the absence of active TGFβ. Our work is the first report in any biological system demonstrating that PPAR-γ ligands reduce FAK activity by reducing FAK phosphorylation at Tyr397. Since FAK plays a cardinal role in myofibroblast differentiation, drugs that target the catalytic activity of FAK could be very valuable in the treatment of pulmonary fibrosis.

This study highlights a very important mechanism of action of CDDO and 15d-PGJ2 that involves down-regulation of PI3K-Akt pathway in both normal and IPF fibroblasts. Knowing that Akt is a central regulator of multiple cellular pathways including cell proliferation, cell cycle progression, inflammation and apoptosis [7,10], interfering with the Akt pathway can have multiple cellular and organ-wide effects. Although, we have noted sustained basal activity of Akt in untreated cells, the nature of Akt activation is largely inducible and dependent on upstream signaling molecules. Therefore, the use of Akt-inhibition as a potential therapy for pulmonary fibrosis is a very novel and exciting concept.

Overall, we propose that certain PPAR-γ ligands have tremendous translational potential as therapeutics for pulmonary fibrosis by not only inhibiting Akt but also FAK activation. Future in vivo studies involving PPAR-γ ligands will be pivotal in exploring the promising potential of PPAR-γ ligands as therapeutics for pulmonary fibrosis as well as other scarring diseases.

Materials and Methods

Cells and reagents

Normal primary human lung fibroblasts (HLFs) were derived as previously described [33], grown in MEM supplemented with 10% FBS, L-Glutamine, antibiotic and antimycotic (Gibco, Carlsbad, CA) and used between passages 7–10. They were grown until 70–80% confluent and serum-starved for 24 hrs before treatment, unless otherwise mentioned. Primary human idiopathic pulmonary fibrotic (IPF) fibroblasts were derived from lung tissues obtained from patients with IPF undergoing wedge biopsy. A written informed consent was obtained from all the subjects in accordance with the University of Rochester Medical Center Institutional Review Board. Explant technique was used to isolate primary fibroblasts as described previously [33] and fibroblasts were used between passages 5–9 and maintained as described above.

Treatments

PPAR-γ agonists were used at the following concentrations; 1μM of CDDO (NIH-RAID Program and Reata Pharmaceuticals, Dallas, TX), 5μM 15d-PGJ2 and 9,10-dihydro-13-deoxy-A12,14-PGJ2 (Cayman Pharmaceuticals, Ann Arbor, MI). Ligands were dissolved in DMSO to make 10mM stock solution and diluted in serum-free media before treatment.
GW9662 (Sigma, St. Louis, MO), 15-deoxy-D12,14-Prostaglandin A1 (Cayman Pharmaceuticals, Ann Arbor, MI) and diphenyl diselenide (DSPS) (Sigma, St. Louis, MO) were prepared in the same manner as described above. GW9662 (5 μM) was added two hours prior to any other treatment. Human recombinant TGFβ1 (R&D Systems, Minneapolis, MN) was used at a final concentration of 5 ng/ml. 50 μM LY294002 (Cell Signaling Technology, Danvers, MA), 10 or 20 μM AG1879 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, (PP2)) and its analogue, 4-Amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3) (EMD Chemicals Inc. Gibbstown, NJ), 100 nM wortmannin and, 1 μg/ml Actinomycin D (Sigma, St. Louis, MO) were used to pretreat cells 30 min prior to TGFβ treatment.

Western blots
Primary human lung fibroblasts were plated (1×10^5 cells/well) in six-well plates (Falcon/Becton Dickson, Franklin Lakes, NJ) for all experiments and allowed to grow for 48 hours prior to any other treatment. Crude cellular protein lysates were prepared using NP-40 lysis buffer supplemented with protease inhibitor, phosphatase inhibitor and 1mM PMSF (Sigma, St. Louis, MO). Total proteins (5 to 10 μg) were resolved by 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and specific proteins were detected by standard Western blotting and chemiluminescence (Western Lightning, Perkin-Elmer, Wellesley, MA). Kodak Molecular Imaging Software (Rochester, NY) was used to perform densitometry on Western blot films and the band intensities were normalized to the loading control. The following primary antibodies were used: phospho-Akt S473, total Akt, phospho-PTEN S380, total PTEN, phospho-p38 MAPK T180/Y182, p38 MAPK, total FAK (Cell Signaling Technologies, Danvers, MA), αSMA (Sigma, St. Louis MO), calponin (DAKO, Carpinteria, CA), GAPDH (Abcam, Cambridge, MA) and phospho-FAK Y397 (Invitrogen Corporation Camarillo, CA). The secondary antibodies used were; goat anti-rabbit (sc-2004), goat anti-mouse (sc-2031, Santa Cruz Biotechnology, Inc. Santa Cruz, CA. 95060 U.S.A) and donkey anti-rabbit (NA 934, Amersham/GE Health Care Life Sciences Piscataway, NJ 08855-1327).

Cell cytotoxicity assay
Cell cytotoxicity was measured by lactate dehydrogenase release assay (LDH5 assay) using an optimized LDH assay kit (Sigma, Cat # DG1340-K). Briefly, fibroblasts were plated in triplicate at a density of 1×10^5 cells per well in 6 well plates and treated with either CDDO (1 μM) or 15d-PGJ2 (5 μM) followed by TGFβ (5 ng/ml) for 48 hours. Protein lysates were prepared and immunoblots were performed to detect expression levels of the indicated proteins. The experiment was performed in triplicate on three independent IPF fibroblast strains. Protein lysates from all the indicated samples were electrophoretically separated on the same gel, and representative lanes from one representative set of data are shown here. These data confirm that CDDO and 15d-PGJ2 inhibit both, FAK and PI3K-Akt pathways to inhibit TGFβ-induced myofibroblast differentiation of primary IPF fibroblasts.

doi:10.1371/journal.pone.0015909.g007

Figure 7. PPARγ ligands block myofibroblast differentiation of primary human IPF fibroblasts. Primary IPF fibroblasts were treated with either A, two PI3K inhibitors LY294002 (50 μM) or wortmannin (100 nM) or B, a Src-FAK inhibitor AG1879 (10 and 20 μM) or C, CDDO (1 μM) or 15d-PGJ2 (5 μM) followed by TGFβ (5 ng/ml) for 48 hours. Protein lysates were prepared and immunoblots were performed to detect expression levels of the indicated proteins. The experiment was performed in triplicate on three independent IPF fibroblast strains. Protein lysates from all the indicated samples were electrophoretically separated on the same gel, and representative lanes from one representative set of data are shown here. These data confirm that CDDO and 15d-PGJ2 inhibit both, FAK and PI3K-Akt pathways to inhibit TGFβ-induced myofibroblast differentiation of primary IPF fibroblasts.

doi:10.1371/journal.pone.0015909.g007
PPARγ Ligands Target TGFβ-Induced PI3K-Akt Pathway

Figure 8. A proposed model showing the mechanism of action of electrophilic PPAR-γ ligands on TGFβ-induced myofibroblast differentiation. TGFβ induces myofibroblast differentiation by activating SMAD, FAK and PI3K-Akt pathways. However, PPAR-γ ligands inhibit the TGFβ-induced PI3K-Akt pathway, partly by targeting FAK induced activation of Akt.

doi:10.1371/journal.pone.0015909.g008

Using a spectrophotometer as per the manufacturer’s protocol. The results were normalized to untreated control samples and plotted as fold change over untreated samples.

PPRE luciferase reporter assay

Primary lung fibroblasts cultured in 6-well plates were co-transfected using FuGene6 (Roche Applied Science, Indianapolis, IN) with a PPAR-γ-luciferase reporter construct containing three PPREs (a gift from Dr. Brian Seed, Harvard University) [59]. A CMV-β-galactosidase control construct was included as control. After 24 h, the cells were washed and then treated with 15l-PGJ2 (5μM) or CDDO (1μM) in medium and harvested after a further 48-h incubation. Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) in a luminometer (Packard Instruments, Meriden, CT) and normalized to β-galactosidase activity, determined by a colorimetric assay (Pro-mega). The experiments were carried out in triplicate wells.

Transfections

Primary HLF cells were plated (5x10⁴ cells/well) in 12 well plates (Falcon/Becton Dickson, Franklin Lakes, NJ) and Eugeen 6 transfection kit was used as per the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN) for transfection. Transfection reactions were carried out using either empty vector pcDNA3.1 or a dominant negative kinase dead Akt (KD-Akt) (a kind gift from Dr. Robert Freeman, University of Rochester, NY USA [34]). Upon transfection, cells were allowed to grow for 16–24 hours, and were then supplemented with 10% FBS for 24 hours followed by treatment. Cells were lysed using NP-40 lysis buffer and subjected to further analysis as described above. Transfections with HA-FAK (a kind gift from Dr. William Cance, Roswell Park Cancer Institute, Buffalo, NY USA) were performed in a similar manner.

Indirect immunofluorescence assay

Cells were grown in four well chamber slides and treated as outlined above. Cells were fixed in methanol and stained with an antibody to α-SMA (St. Louis, MO, USA) and with anti-mouse AlexaFluor 488 (Invitrogen Corporation Carlsbad, CA, USA). Slides were mounted with Prolong Gold supplemented with DAPI (Invitrogen Corporation Carlsbad, CA, USA) to visualize the nuclei and analyzed by fluorescence-microscopy using a Zeiss Axio Imager Z.1 Microscope.

Acknowledgments

We would like to thank Dr. Robert Freeman (University of Rochester, Rochester NY) for providing a KD-Akt construct and Dr. William Cance (Roswell Park Cancer Institute, Buffalo, NY) for providing a HA-FAK construct, Dr. Heather Ferguson, Katherine Smolnycki and Marta Ekstrom for their technical help, and Dr. R.M. Kottmann for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: AAK THT RPP PJS. Performed the experiments: AAK KLO. Analyzed the data: AAK THT KLO SBM PJS. Wrote the paper: AAK.

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