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Electrophilic PPAR γ ligands inhibit corneal fibroblast to myofibroblast differentiation *in vitro*: A potentially novel therapy for corneal scarring

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Abstract

A critical component of corneal scarring is the TGF β -induced differentiation of corneal keratocytes into myofibroblasts. Inhibitors of this differentiation are potentially therapeutic for corneal scarring. In this study, we tested the relative effectiveness and mechanisms of action of two electrophilic peroxisome proliferator-activated receptor gamma (PPAR γ) ligands: cyano-3,12-dioxolean-1,9-dien-28-oic acid-methyl ester (CDDO-Me) and 15-deoxy- Δ -12,14-prostaglandin J₂ (15d-PGJ₂) for inhibiting TGF β -induced myofibroblast differentiation *in vitro*. TGF β was used to induce myofibroblast differentiation in cultured, primary human corneal fibroblasts. CDDO-Me and 15d-PGJ₂ were added to cultures to test their ability to inhibit this process. Myofibroblast differentiation was assessed by measuring the expression of myofibroblast-specific proteins (α SMA, collagen I, and fibronectin) and mRNA (α SMA and collagen III). The role of PPAR γ in the inhibition of myofibroblast differentiation by these agents was tested in genetically and pharmacologically manipulated cells. Finally, we assayed the importance of electrophilicity in the actions of these agents on TGF β -induced α SMA expression via Western blotting and immunofluorescence. Both electrophilic PPAR γ ligands (CDDO-Me and 15d-PGJ₂) potently inhibited TGF β -induced myofibroblast differentiation, but PPAR γ was only partially required for inhibition of myofibroblast differentiation by either agent. Electrophilic PPAR γ ligands were able to inhibit myofibroblast differentiation more potently than non-electrophilic PPAR γ ligands, suggesting an important role of electrophilicity in this process. CDDO-Me and 15d-PGJ₂ are strong inhibitors of TGF β -induced corneal fibroblast to myofibroblast differentiation *in vitro*, suggesting this class of agents as potential novel therapies for corneal scarring warranting further study in pre-clinical animal models.

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1. Introduction

Corneal scarring, the second most common cause of blindness in the world, can be due to ocular infections (Whitcher, et al., 2001), trauma (Farjo, et al., 2008), and surgeries (Marchini, et al., 2006; Saini, et al., 2003; Wilson, et al., 2001). Safe and efficacious medical therapies for the treatment of corneal scarring are limited.

Keratocytes are transparent cells of the corneal stroma that are involved in corneal wound healing and scarring (Fini and Stramer, 2005; Fini, 1999; Jalbert, et al., 2003; Muller, et al., 1995). Corneal wounds result in the release of several cytokines and chemokines that drive keratocyte activation, migration, and differentiation into fibroblasts and myofibroblasts (West-Mays and Dwivedi, 2006). Corneal myofibroblasts provide wound contraction and produce extracellular matrix molecules (ECMs), such as Type I and III collagen (Funderburgh, et al., 2001) and fibronectin (FN) (Welch, et al., 1990), which are important in regenerative wound repair (Jester, et al., 1995; West-Mays and Dwivedi, 2006). However, myofibroblasts also contribute to corneal haze and corneal shape change through decreased crystallin production (Jester, et al., 1999; 2008) and excessive and disordered production of collagen and other extracellular matrix molecules (Marchini, et al., 2006; Netto, et al., 2006; Pal-Ghosh, et al., 2004; Saini, et al., 2003; Sosne, et al., 2002; Wilson, et al., 2001). Both haze and changes in corneal shape decrease visual acuity (Marchini, et al., 2006; Netto, et al., 2006; Pal-Ghosh, et al., 2004; Saini, et al., 2003; Sosne, et al., 2002; West-Mays and Dwivedi, 2006; Wilson, et al., 2001).

Differentiation of keratocytes to myofibroblasts *in vitro* and *in vivo* is driven primarily by transforming growth factor beta (TGF β) (Jester, et al., 1996), a cytokine released by corneal epithelial cells, corneal fibroblasts, the lacrimal gland, and conjunctival cells under inflammatory conditions (Buehren, et al., 2008; Wilson, et al., 1992). Netto and colleagues (2006) demonstrated that by damaging the corneal epithelial basement membrane, penetrating keratectomy in mice causes the release of TGF β into the corneal stroma, stimulating keratocyte to myofibroblast differentiation. While factors other than TGF β are involved in the corneal scarring phenomena, including platelet-derived growth factor (PDGF) (Kaur, et al., 2009a; 2009b) and integrin signaling (Jester, et al., 2002), pharmaceutical inhibitors of TGF β have been shown to decrease myofibroblast differentiation and corneal opacification in several animal models of corneal scarring (Buehren, et al., 2008; Moller-Pedersen, et al., 1998; Jester, et al., 1997).

PPAR γ ligands have anti-fibrotic properties and have been studied as agents capable of inhibiting TGF β -induced myofibroblast differentiation in different tissues (Ferguson, et al., 2009; Galli, et al., 2002; Kawai, et al., 2009), including the cornea (Pan, et al., 2009; Pan, et al., 2010). PPAR γ gene transfer decreased corneal opacification in an alkali burn mouse model of corneal scarring (Saika, et al., 2007). A non-electrophilic PPAR γ ligand, pioglitazone, was found to inhibit TGF β -induced keratocyte to myofibroblast differentiation *in vitro* (Pan, et al., 2010). However, a recent *in vitro* study found two electrophilic peroxisome proliferator-activated receptor gamma (PPAR γ) ligands, cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO) and 15-deoxy- Δ -12,14-prostaglandin J₂ (15d-PGJ₂), to inhibit TGF β -induced lung myofibroblast differentiation more potently than non-electrophilic PPAR γ ligands (Ferguson, et al., 2009). 15d-PGJ₂ (Kliwer, et al., 1995), CDDO, and CDDO derivatives bind PPAR γ with high affinity (Wang, et al., 2000). Once a PPAR γ ligand binds to PPAR γ , the latter forms a heterodimer with the retinoid X receptor (RXR) and its ligand (Ferguson, et al., 2009; Liby, et al., 2007; Rizzo and Fiorucci, 2006). The heterodimer then translocates to the nucleus and interacts with PPAR response elements (PPRE), leading to PPAR γ -induced gene transcription (Ferguson, et al., 2009; Liby, et al., 2007; Rizzo and Fiorucci, 2006). Electrophilic PPAR γ ligands have α/β -unsaturated ketone

rings with electrophilic carbons (Brookes, et al., 2007; Ferguson, et al., 2009; Shi and Greaney, 2005). These electrophilic carbons are highly susceptible to Michael addition reactions (Shi and Greaney, 2005), which enables them to form covalent bonds with intracellular nucleophiles, such as thiol groups or cysteine residues (Brookes, et al., 2007 and Chintharlapalli, et al., 2005; Ferguson, et al., 2009; Ray, et al., 2006; Straus, et al., 2000; Suh, et al., 1999). For this reason, electrophilic PPAR γ ligands, such as CDDO (Brookes, et al., 2007; Chintharlapalli, et al., 2005; Ferguson, et al., 2009; Suh, et al., 1999) and 15d-PGJ₂ (Ray, et al., 2006; Straus, et al., 2000), can also exert PPAR γ -independent effects.

The present experiments examine the ability of two electrophilic PPAR γ ligands, a CDDO derivative, CDDO-methyl ester (–Me), and 15d-PGJ₂ to inhibit differentiation of corneal fibroblasts to myofibroblasts *in vitro*. The anti-scarring properties of these agents have been studied in fibroblasts in the lung (Ferguson et al. 2009). However, due to the heterogeneity of fibroblasts between tissues (Smith, et al., 1995), this study is an essential first step towards assessing the potential effectiveness of these molecules for treating corneal scarring. CDDO in particular has already been used systemically in Phase I and II clinical trials as a chemotherapeutic agent (Dezube, et al., 2007). Since it was shown to have a favorable safety profile when administered systemically in humans (Dezube, et al., 2007), this significantly increases its translational potential for a topical application such as eye drops in the treatment of corneal scarring.

2. Methods

2.1 Cells and reagents

Human corneal fibroblast cell strains were derived from anatomically normal donor rim corneal tissue donated to the Rochester/Finger Lakes Eye and Tissue Bank. Human tissue was obtained and handled in accordance with the tenets of the Declaration of Helsinki. The cells derived from these explants were isolated and then cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO), gentamicin (0.05 mg/ml; Invitrogen, Carlsbad, CA), amphotericin (.25 mg/ml; Invitrogen), and 25 mM HEPES at pH 7.4 at 37°C in 7% CO₂. The cells were morphologically consistent with fibroblasts, expressing vimentin, but not CD45, factor VIII, or cytokeratin. Cells from three different patient strains were used at passages 4–10. The PPAR γ ligands, 15d-PGJ₂ (Biomol, Plymouth Meeting, PA), 9,10-dihydro-15-deoxy-D^{12,14}-PGJ₂ (CAY10410; Cayman Pharmaceuticals, Ann Arbor, MI), rosiglitazone (Cayman Pharmaceuticals), CDDO-Me (a kind gift from Dr. Michael Sporn, Dartmouth University), were prepared as 10 mM stocks in dimethyl sulfoxide (DMSO) and added to cell cultures to the final concentration indicated. GW-9662 (Cayman Pharmaceuticals), an irreversible, small-molecule PPAR γ antagonist, was prepared in the same manner. This molecule has been used by us (Burgess, et al., 2005; Feldon, et al., 2006; Ferguson, et al., 2009) and others (Fu, et al., 2001; Mix, et al., 2009) and been shown to block PPAR γ activity. Recombinant human TGF β 1 (#240B) was purchased from R&D systems (Minneapolis, MN).

2.2 Cell viability assay

The calcein AM/EthD-I Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) was used to assess cell viability. Calcein AM specifically stains live cells via their intracellular esterase activity, and EthD-I specifically stains dead cells that have lost plasma membrane integrity. Corneal fibroblasts were plated in 25 ml flasks (Greiner Bio-One) in 10% FBS media and were either untreated, treated with TGF β alone, or with PPAR γ ligands with or without TGF β for 72 hours. PPAR γ ligands were added 30 minutes prior to TGF β . Prior to harvesting the cells, one of corneal fibroblast samples were killed using 0.1%

saponin treatment for 15 minutes. At harvest, cells were trypsinized and washed with PBS twice and then stained with 1 μ M calcein AM and 8 μ M EthD-1 for 10 minutes. Samples were analyzed on a FACSCanto II (BD Biosciences). The live cell gate was set based on cells staining with calcein AM in the untreated corneal fibroblast culture. The dead cell gate was set based on cells staining with EthD-1 in the 0.1% saponin treated culture. Treated samples were analyzed using these gates to assess percent of live and dead cells.

2.3 Western blots for α SMA, fibronectin, collagen I

Primary human corneal fibroblasts were plated in 60 or 100 mm dishes (Falcon/Becton Dickinson, Franklin Lakes, NJ) or 6 well plates (Falcon/Becton) in 10% FBS media and were either untreated, treated with TGF β alone, or treated with PPAR γ ligands with or without TGF β for 72 hours. PPAR γ ligands were added 30 minutes prior to TGF β . For certain experiments, the fibroblasts were infected with an adenovirus vector expressing a dominant negative PPAR γ or an empty vector for 24 hours before treatment with TGF β and PPAR γ ligands. In other experiments, the fibroblasts were treated with GW9662 for 4 hours before treatment with TGF β and PPAR γ ligands. Cell lysates were prepared using either a 1 $\times$ SDS lysis buffer or with the commercial ActiveMotif Nuclear Extract kit (Carlsbad, CA). Proteins were separated by 10% SDS-PAGE under reducing conditions, transferred to a polyvinylidene fluoride (PVDF) membrane and examined for expression of α SMA (1:4000 dilution, Sigma). Precast 4–15% SDS-PAGE gradient gels (Bio-Rad, Hercules, CA) were used to examine expression of panfibronectin (1:1000 dilution, Sigma), and collagen I (1:1000 dilution, Santa Cruz, Carlsbad, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000 dilution, Abcam, Cambridge, MA) was used as a loading control. Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Wellesley, MA) was used to visualize protein bands. Densitometry of the resulting bands was performed using QuantityOne Software (Biorad) and normalized to GAPDH loading control.

2.4 Immunofluorescence microscopy for α SMA

Primary corneal fibroblasts were cultured at a density of 1,500 cells per well on eight-well chamber slides (Nalge Nunc International, Naperville, IL) in 10% FBS media and either untreated, treated with TGF β alone, or treated with PPAR γ ligands with or without TGF β for 72 hours. Slides were fixed with 2% paraformaldehyde for 15 minutes and sequentially treated with mouse monoclonal α SMA antibody (1:800 dilution, Sigma) and anti-mouse-Alexafluor 555 (Sigma). Mounting media containing 4,6-diamidino-2-phenylindole (DAPI; Sigma) was used to mount the slides and stain nuclei. Slides were viewed using a Zeiss Axioplan microscope (Zeiss, Thornwood, NY).

2.5 Imaging Flow Cytometry

Primary human corneal fibroblasts were plated in 25ml flasks (Greiner Bio-One, Frickenhausen, Germany) in 10% FBS media and treated as described above. The cells were harvested, stained for α SMA (Sigma) and Draq5 (Axxora, San Diego, CA), and analyzed using Imagestream System 100 (Amnis Corporation, Seattle, WA) as described in Ferguson et al. (2009). Briefly, both the intensity and aspect ratio of the nuclear stain Draq5 were used in a gating strategy to isolate true single cells from clumps and debris. Subsequently, objects identified as single cells were analyzed for α SMA expression, which was defined by both the intensity of the α SMA signal and the fractional area of the cell covered by α SMA signal. A gate was created encompassing 1% of cells in the untreated sample with high α SMA signal intensity and area, and this was identified as the myofibroblast region in order to compare relative myofibroblast differentiation across samples.

2.6 RT-PCR

Primary human corneal fibroblasts were plated in 100 ml dishes (Falcon/Becton Dickinson, Franklin Lakes, NJ) in 10% FBS media and either untreated, treated with TGF β alone, or treated with PPAR γ ligands with or without TGF β for 24 hours. RNA was isolated using RNeasy (Qiagen, Valencia, CA), following the manufacturer's protocol. RNA (1.0 μ g) was incubated with PCR buffer, 0.5 μ g of oligo (dT)_{12–18} primer (Invitrogen), and 10 mM deoxy-nucleotide-triphosphate (dNTP) for 10 minutes at 70°C and 5 minutes in ice water. Subsequently, 40 U of recombinant RNasin RNase inhibitor (Promega, Madison, WI), 0.1 mM DTT, and 200 U of Superscript III reverse transcriptase (RT; Invitrogen) were added to the mixture and 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 did not contain RT enzyme.

Quantitative real-time RT-PCR reactions were performed using a Bio-Rad iCycler with SYBR Green Supermix (Bio-Rad) according to the manufacturer's recommended protocol, which was modified such that the reactions contained 3 mM MgCl₂ and 0.2 μ M of each primer. Oligomer primers were ordered from Integrated DNA Technologies (Coralville, IA) (COL3A1, ACTA2, and GAPDH). The primer sequences were as follows: 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 CAC 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 TGC CCA-3'.

2.7 Dominant Negative (DN) PPAR γ Adenovirus Infection

Primary human corneal fibroblasts were plated in 6 well plates (Falcon/Becton) in 10% FBS media and infected with DN PPAR γ (a kind gift from Drs. E. J. Lee and J. L. Jameson (Northwestern University)) or control adenovirus, AdDL70-3, at a multiplicity of infection (MOI) of 30 plaque forming units (pfu)/cell for 24 hours. The infected cells were then either untreated, treated with TGF β alone, or treated with CDDO-Me with or without TGF β . DN PPAR γ adenovirus vector construction and production has been previously described by us and others (Burgess, et al., 2005; Park, et al., 2003). The DN PPAR γ is able to bind the ligand; however, it does not activate transcription at the PPRE (Burgess, et al., 2005; Park, et al., 2003). Control adenovirus construction and production has been previously described (Bett, et al., 1994; Sime, et al., 1997). The control adenovirus has no inserted transgene.

2.8 Luciferase reporter assays to monitor PPAR γ activity

Primary human corneal fibroblasts were plated in 75 cm² flasks (Falcon/Becton) in 10% FBS media and infected with DN PPAR γ or control adenovirus as described above. After a 24 hour infection, cells were harvested and PPRE $\times$ 3-firefly luciferase (Ray, et al., 2006) and SV40-renilla luciferase (Promega) plasmid DNAs were introduced using an Amaxa nucleofector device (Lonza AG) following the manufacturer's protocol. Nucleofected cells were plated into 6 well plates and allowed to grow for 6–8 hrs. Either DMSO (vehicle) or 5 β -M 15d-PGJ₂ was then added to the cultures for an additional 14–18 hrs. Following incubation, cells were washed two times in 1XPBS and lysed directly in plates using the Dual-Glo luciferase assay buffer (Promega). Firefly and renilla luciferase readings were read on a Varioskan Flash luminometer (Thermo Fisher) following manufacturer's instructions. Luciferase readings were normalized to the control adenovirus, vehicle treated samples for statistical analysis.

2.9 Collagen Contraction Assay

Collagen gels were prepared from lyophilized rat tail collagen (Roche Diagnostics GmbH, Mannheim, Germany) as previously described (Mio, et al., 1996; Skold et al., 1999). Collagen was dissolved in 0.2% acetic acid (v/v) and mixed with sterile 2× MEM and 0.1M NaOH. 1× DMEM media with or without cells was added to this mixture, and 667 µl of the collagen mixture with or without 15,000 corneal fibroblasts per well was added to BSA coated 24 well plates (Falcon). The plates were incubated at 37°C for 2 hours to polymerize, forming a 3-dimensional collagen gel. The gels were separated from the wells using a 200 µl pipette tip. The gels were then either untreated, treated with TGFβ alone, or treated with PPARγ ligands with or without TGFβ in 10% FBS media. The gels were imaged and weighed after 48 hours of treatment. Since measuring the two-dimensional surface area of the gels fails to encompass the three-dimensional changes occurring in each gel, quantitative analysis of this assay was accomplished using gel weight. Based on the weight of the gels, the percent contraction of the gels with cells, compared to the gel without cells, was calculated as previously described by Lehmann and colleagues (2011).

2.10 Statistical analysis

One-way ANOVA with post-hoc Tukey HSD test was used for statistical analysis. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, LaJolla, CA) software. A p value less than 0.05 was considered statistically significant.

3.0 Results

3.1 Pre-treatment with electrophilic PPARγ ligands inhibits TGFβ-induced differentiation of corneal fibroblasts to myofibroblasts

To study the ability of different electrophilic PPARγ ligands to inhibit TGFβ-induced corneal myofibroblast differentiation, human corneal fibroblasts were treated with 5 ng/ml TGFβ after 30 mins pretreatment with varying doses of the electrophilic PPARγ ligands, CDDO-Me and 15d-PGJ₂. The maximum concentrations used (0.350 µM CDDO-Me and 10 µM 15d-PGJ₂) were chosen for having the strongest inhibitory effect on TGFβ-stimulated αSMA up-regulation without effect on cell viability (data not shown). Although there were slight differences in sensitivity to TGFβ and effectiveness of the PPARγ ligands at reducing TGFβ-induced αSMA expression between the three human corneal fibroblast strains, pre-treatment with CDDO-Me and 15d-PGJ₂ inhibited TGFβ-induced αSMA expression in all three strains (Figure 1A and B). Subsequent experiments were completed in the strain of corneal fibroblasts with the greatest sensitivity to TGFβ (strain 2). CDDO-Me and 15d-PGJ₂ were also able to significantly inhibit TGFβ-induced αSMA mRNA expression (Figure 1C). CDDO-Me and 15d-PGJ₂ treatment alone led to a non-significant decrease of αSMA mRNA compared to untreated cells (Figure 1C).

Further evidence about the ability of electrophilic PPARγ ligands to inhibit TGFβ-induced myofibroblast differentiation came from imaging flow cytometry, a technique that allows thousands of labeled cells to be imaged while measuring their fluorescent emissions (Figure 2). TGFβ alone increased the percentage of cells within the myofibroblast region to 29% compared to 1% in the untreated sample (Figure 2B). Pretreatment with CDDO-Me and 15d-PGJ₂ reduced the percent of cells within the myofibroblast region from 29% to 9% and 19%, respectively.

In addition to reducing TGFβ-induced αSMA expression, CDDO-Me and 15d-PGJ₂ also inhibited TGFβ-induced collagen I and fibronectin expression in cultured human corneal fibroblasts (Figure 3A). Furthermore, TGFβ-induced collagen III mRNA expression was inhibited by these electrophilic PPARγ ligands (Figure 3B). CDDO-Me and 15d-PGJ₂

treatment alone led to a non-significant decrease of collagen III mRNA compared to untreated cells (Figure 3B).

A collagen gel contraction assay was used to assess whether electrophilic PPAR γ ligands were effective at not only decreasing TGF β -induced production of extracellular matrix molecules, but also at decreasing TGF β -induced contraction of collagen gels by corneal myofibroblasts. Due to the increased contractile properties of myofibroblasts relative to fibroblasts, collagen gels with myofibroblasts are expected to be smaller in size and weigh less (Kurosaka, et al., 1998; Lehmann, et al., 2011; Skold, et al., 1999; Tingstrom, et al., 1992; Xi-Wen, et al., 2007). The percent contraction of the gels with cells was calculated based on the weight of the gels with cells compared to the gel without cells. As predicted, collagen gels containing corneal fibroblasts exhibited greatly reduced surface area (Figure 4A) and weight (Figure 4B) following treatment with TGF β . CDDO-Me ($p<0.001$) and 15d-PGJ $_2$ ($p<0.05$) treatment attenuated the collagen gel surface area contraction and weight reduction induced by TGF β . As expected, collagen gels without cells did not change in size over time (Figure 4A).

3.2 Inhibition of myofibroblast differentiation by electrophilic PPAR γ ligands is largely PPAR γ independent

We used both a pharmacological and a genetic approach to study the role of PPAR γ in inhibition of TGF β -induced myofibroblast differentiation by electrophilic PPAR γ ligands. Pharmacologic inhibition of PPAR γ was accomplished by using GW9662, a highly specific, irreversible PPAR γ antagonist that renders PPAR γ unable to bind with other ligands by forming a covalent bond with a cysteine residue within the PPAR γ binding pocket (Feldon, et al., 2006; Guo, et al., 2011). Corneal fibroblasts were pre-treated with GW9662 prior to treatment with TGF β and/or CDDO-Me or 15d-PGJ $_2$. Western blotting was used to measure levels of α SMA after 72 hours. GW9662 treatment did not prevent CDDO-Me or 15d-PGJ $_2$ from inhibiting TGF β -induced α SMA expression (Figure 5A).

Genetic inhibition of PPAR γ was accomplished by infection of corneal fibroblasts with replication-deficient adenovirus expressing a mutated dominant negative (DN) PPAR γ gene. Corneal fibroblasts were treated with the DN PPAR γ or empty adenovirus (AdDL70-3) for 24 hours and then treated with TGF β or TGF β and CDDO-Me. 72 hours after treatment, Western blotting for α SMA demonstrated that CDDO-Me inhibited TGF β -induced α SMA expression in both the control virus infected fibroblasts and in the DN PPAR γ infected fibroblasts (Figure 5B and C). The ability of the DN PPAR γ adenovirus to block PPAR γ -dependent transcriptional activity in corneal fibroblasts was demonstrated using a PPAR γ reporter luciferase assay (Supplementary Figure 1). Corneal fibroblasts infected with AdDL70-3 and a PPAR γ ligand, displayed a two-fold increase in PPAR γ -dependent transcriptional activity as demonstrated by luciferase reporter assays. Corneal fibroblasts infected with the DN PPAR γ adenovirus displayed almost no PPAR γ dependent transcriptional activity in the presence or absence of a PPAR γ ligand.

The ability of CDDO-Me to inhibit TGF β -induced α SMA expression in the absence of PPAR γ activity suggests that the inhibitory effects of this molecule on myofibroblast differentiation are largely PPAR γ independent. However, an interesting observation was that corneal fibroblasts treated with GW9662 had a 1.43-fold increased TGF β -induced α SMA compared to non-GW9662 treated cells. Similarly, corneal fibroblasts infected with the DN-PPAR γ virus had a 1.36-fold increased TGF β -induced α SMA compared to control virus infected cells. However, these differences were not statistically significant.

3.3 The ability of PPAR γ ligands to inhibit TGF β -induced myofibroblast differentiation of corneal fibroblasts depends on their electrophilicity

An important question in the context of the present studies is whether electrophilicity of CDDO-Me and 15d-PGJ₂ was a critical factor in their ability to inhibit the differentiation of corneal fibroblasts into myofibroblasts. To answer this question, we contrasted the effects of these two electrophilic PPAR γ ligands with two non-electrophilic PPAR γ ligands (rosiglitazone and CAY10410), on TGF β -induced α SMA expression. CAY10410 is a non-electrophilic structural analog of 15d-PGJ₂. Western blotting and immunohistochemistry showed that all of the PPAR γ ligands were capable of inhibiting TGF β -induced α SMA expression (Figure 6A and B). However, the natural electrophilic PPAR γ ligand 15d-PGJ₂ inhibited α SMA expression much more potently than both non-electrophilic PPAR γ ligands (Figure 6A). The synthetic electrophilic PPAR γ ligand CDDO-Me inhibited α SMA expression even more potently than 15d-PGJ₂, with 0.175 μ M CDDO-Me and 2.5 μ M 15d-PGJ₂ reducing α SMA expression to about the same extent (Figure 6A). Thus, the electrophilic property of PPAR γ ligands appears to be important for their inhibition of TGF β -induced α SMA expression in human corneal fibroblasts.

4. Discussion

PPAR γ and its ligands have been previously studied in the context of corneal wound healing and have been shown to have anti-inflammatory and anti-scarring properties (Pan, et al., 2009; 2010; Saika, et al., 2007). Electrophilic PPAR γ ligands, such as CDDO and 15d-PGJ₂ have been shown to inhibit TGF β -induced myofibroblast differentiation in lung fibroblasts more potently than non-electrophilic PPAR γ ligands (Ferguson, et al., 2009). Here, we report for the first time, that the CDDO derivative, CDDO-Me, and 15d-PGJ₂ can also function as potent anti-fibrotic agents for cultured human corneal fibroblasts stimulated to differentiate into myofibroblasts by TGF β . In fact, CDDO-Me was able to inhibit TGF β -induced myofibroblast differentiation to the same extent as 15d-PGJ₂, but at a 14-fold lower concentration.

In order to elucidate the mechanism through which CDDO-Me and 15d-PGJ₂ inhibit TGF β -induced myofibroblast differentiation, we first investigated the PPAR γ -dependence of their anti-fibrotic properties. Using pharmacologic and genetic methods to block PPAR γ , we demonstrated that the anti-fibrotic properties of CDDO-Me and 15d-PGJ₂ are largely PPAR γ -independent. Ferguson and colleagues (2009) demonstrated similar findings with CDDO (rather than CDDO-Me) and 15d-PGJ₂ in human lung fibroblasts and hypothesized that both PPAR γ -dependent and PPAR γ -independent pathways (Bishop-Bailey and Wray, 2003; Rizzo and Fiorucci, 2006) may generally modulate myofibroblast differentiation in that tissue. Both CDDO-Me and CDDO bind to PPAR γ with high affinity and activate PPARE (Chintharlapalli, et al., 2005).

CDDO-Me has two electrophilic α/β -unsaturated ketones, while 15d-PGJ₂ has one. The electrophilic α/β -unsaturated ketone is susceptible to Michael addition reactions (Shi and Greaney, 2005) with intracellular nucleophiles, such as glutathione and cysteine residues (Brookes, et al., 2007). Non-electrophilic PPAR γ ligands such as rosiglitazone and CAY10410 (the non-electrophilic structural analog of 15d-PGJ₂), were much less potent inhibitors of TGF β -induced myofibroblast differentiation than 15d-PGJ₂ and CDDO-Me. This suggests that the ability of CDDO-Me and 15d-PGJ₂ to inhibit TGF β -induced myofibroblast differentiation is related to the electrophilic nature of these two compounds. Furthermore, CDDO-Me was a more potent inhibitor of TGF β -induced proteins at a much lower concentration than 15d-PGJ₂, which may be attributable to the presence of an additional α/β -unsaturated ketone. Other studies have also demonstrated that CDDO and its derivatives are effective inhibitors of TGF β -induced proteins at much lower concentrations

than 15d-PGJ₂ (Ferguson, et al., 2009; Kulkarni, et al., 2011). Reactivity with intracellular nucleophiles may be a potential mechanism through which these compounds are able to inhibit myofibroblast differentiation. Indeed, data show other electrophilic agents, such as PGA₂ and DSPS to also inhibit TGFβ-induced lung myofibroblast differentiation (Ferguson, et al., 2009).

It should be noted here that we did not examine the ability of 15d-PGJ₂ and CDDO-Me to inhibit TGFβ-induced myofibroblast differentiation using keratocytes grown in serum-free media. Serum free conditions enable maintenance of the keratocyte phenotype *in vitro*, while culturing keratocytes in serum results in their activation, entry into the cell cycle, and phenotypic changes (spindle shape, actin stress fiber assembly, etc.) that are consistent with those seen in activated, repair fibroblasts in a wounded cornea. (Fini and Stramer, 2005; Jester, et al., 1996) Our decision to culture cells in serum was based partly on the fact that CDDO-Me requires a significant amount of protein in the media in order to remain soluble and partly because we wanted to test the agents of interest in a setting that mimicked the wounded cornea *in vitro*, rather than a non-inflammatory setting, generated by using serum free media.

Finally, a limitation of our study is related to the capability of bone-marrow derived cells to differentiate into αSMA expressing cells (Barbosa, et al., 2010). Since we used corneal fibroblast cultures, we cannot draw conclusions on the capability of CDDO-Me or 15d-PGJ₂ to inhibit TGFβ-induced differentiation of bone-marrow derived cells to myofibroblasts. Our ongoing experiments examining the ability of electrophilic PPARγ ligands to inhibit corneal scarring *in vivo* are designed to directly answer this question.

In summary, we have shown that CDDO-Me and 15d-PGJ₂ are potent inhibitors of TGFβ-induced corneal myofibroblast differentiation by examining their ability to decrease levels of multiple myofibroblast proteins and mRNAs. We show that the ability of these PPARγ ligands to inhibit myofibroblast differentiation is largely independent of PPARγ, but dependent on electrophilicity. Further studies are needed to determine the exact mechanisms of action of CDDO-Me and 15d-PGJ₂ in corneal fibroblasts. For example, these electrophilic PPARγ ligands may alter the function of intracellular mediators of TGFβ signaling. Due to its established favorable safety profile in human trials, CDDO-Me appears promising as a proximate therapeutic agent for corneal scarring. However, preclinical animal models of corneal scarring should be utilized to assess its efficacy, as well as that of 15d-PGJ₂ and other electrophilic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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6.0 References

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Highlights

- CDDO-Me and 15d-PGJ₂ are electrophilic PPAR γ ligands.
- CDDO-Me and 15d-PGJ₂ inhibit TGF β -induced corneal myofibroblast differentiation.
- Inhibition of myofibroblast differentiation is independent of PPAR γ ligand function.
- Electrophilic PPAR γ ligands are more potent inhibitors than non-electrophilic ones.
- Further study of these agents in animal models of corneal scarring is warranted.

Figure 1.

CDDO-Me and 15d-PGJ₂ inhibited TGFβ-induced αSMA in corneal fibroblasts from 3 separate individuals. (A) Corneal fibroblasts from 3 individuals (i.e., strain 1, strain 2, and strain 3) were treated with TGFβ alone or in combination with 350 nM CDDO-Me or 10 μM 15d-PGJ₂. (B) Results shown are the mean (±SD) relative expression (RE) of αSMA in corneal fibroblasts from three different individuals. RE was determined by densitometry and normalized to GAPDH. (C) Primary human corneal fibroblasts were treated with 5 ng/ml TGFβ alone or with 350 nM CDDO-Me or 10 μM 15d-PGJ₂ for 24 hours. RNA was harvested and analyzed by quantitative RT-PCR using αSMA (ACTA)-specific primers and normalized to GAPDH mRNA. The results shown are the mean (±SD) for a single experiment performed in triplicate.

Figure 2.

The electrophilic PPAR γ ligands CDDO-Me and 15d-PGJ $_2$ inhibited TGF β -induced myofibroblast differentiation. Human corneal fibroblasts were treated with 5 ng/ml TGF β alone or in combination with 350 nM CDDO-Me or 10 μ M 15d-PGJ $_2$ for 72 hours. Fibroblasts were fixed with methanol and stained for α SMA using a Cy3-conjugated anti- α SMA antibody and for nuclear material using Draq5. Samples were analyzed on an Amnis Imagestream imaging flow cytometer, which enabled imaging of each cell as its fluorescence was analyzed. (A) The left panel shows corneal fibroblasts; these did not stain for α SMA. The right panel shows α SMA-positive myofibroblasts. (B) A gate was created encompassing 1% of cells in the untreated sample with high α SMA signal intensity and area, and this was identified as the myofibroblast region in order to compare relative myofibroblast differentiation across samples. The percentage of cells within the myofibroblast region in each sample is shown.

Figure 3.

CDDO-Me and 15d-PGJ₂ inhibited the expression of TGF β -induced extracellular matrix proteins. (A) Primary human corneal fibroblasts were treated with 5 ng/ml TGF β alone or in combination with 350 nM CDDO-Me or 10 μ M 15d-PGJ₂ for 72 hours. Expression of collagen I and fibronectin (FN) was determined by Western blotting. Relative expression (RE) of fibronectin and collagen I was determined by densitometry and normalized to GAPDH (untreated cells = 1). (B) Primary human corneal fibroblasts were treated with TGF β alone or in combination with 350 nM CDDO-Me or 10 μ M 15d-PGJ₂ for 24 hours. RNA was harvested and analyzed by quantitative RT-PCR using collagen III (COL3A1)-specific primers and normalized to GAPDH mRNA. The results shown are the mean ($\pm$ SD) for triplicate wells in a single experiment.

Figure 4. CDDO-Me inhibited both TGFβ-induced collagen gel contraction by corneal fibroblasts. (A) Collagen gels were prepared with primary human corneal fibroblasts and treated with 5 ng/ml TGFβ alone or in combination with 350 nM CDDO-Me or 10 μM 15d-PGJ2. Images of the collagen gels were taken at baseline and 48 hours after treatment. (B) Collagen gels were weighed after 48 hours. The percent contraction was calculated based on the weight of the collagen gels with cells compared to the collagen gel without cells. The results shown are the mean (±SD) for triplicate wells of a single experiment.

Figure 5.

Electrophilic PPAR γ ligands inhibited TGF β -induced α SMA expression in corneal fibroblasts in which PPAR γ activity was inhibited by either an irreversible PPAR γ antagonist or expression of a dominant-negative (DN) PPAR γ . (A) Primary human corneal fibroblasts were pretreated with 5 μ M GW9662 for 4 hours or left untreated. The fibroblasts were then treated with 5 ng/ml TGF β alone or in combination with 350 nM CDDO-Me or 10 μ M 15d-PGJ₂ for 72 hours. Western blotting was used to assess α SMA expression. The blot shown is representative of a single experiment performed in triplicate. Relative expression (RE) was determined by densitometry and normalized to GAPDH (untreated cells = 1). (B) Primary human corneal fibroblasts were infected with either an adenovirus vector that expressed a DN PPAR γ or a control adenovirus with no inserted transgene. At 24 hours after infection, corneal fibroblasts were treated with TGF β alone or in combination with 350 nM CDDO-Me. α SMA expression was assessed using Western blotting 72 hours after treatment. (C) Results shown are the mean ($\pm$ SD) RE of α SMA levels for one experiment in triplicate. RE was determined by densitometry and normalized to GAPDH (untreated cells = 1).

Figure 6. The electrophilic PPAR γ ligands, CDDO-Me and 15d-PGJ₂, are much more potent inhibitors of TGF- β -induced α SMA expression than the non-electrophilic ligands, rosiglitazone and CAY10410. (A) Primary human corneal fibroblasts were treated with 5 ng/ml TGF- β alone or with the indicated concentrations of CDDO-Me, 15d-PGJ₂, rosiglitazone, or CAY10410 (a non-electrophilic structural analog of 15d-PGJ₂) for 72 hours. Relative expression (RE) was determined by densitometry and normalized to GAPDH (untreated cells = 1). (B) Primary human corneal fibroblasts were treated with 5 ng/ml TGF- β alone or with 350 nM CDDO-Me, 10 μ M 15d-PGJ₂, 10 μ M rosiglitazone, or 10 μ M CAY10410 for 72 hours. α SMA expression (red staining) was detected by immunofluorescence microscopy. DAPI stain was used to stain nuclei (blue).