Inhibition of Transglutaminase 2, a Novel Target for Pulmonary Fibrosis, by Two Small Electrophilic Molecules

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Abstract

Idiopathic pulmonary fibrosis (IPF) is characterized by progressive fibrotic destruction of normal lung architecture. Due to a lack of effective treatment options, new treatment approaches are needed. We previously identified transglutaminase (TG)2, a multifunctional protein expressed by human lung fibroblasts (HLFs), as a positive driver of fibrosis. TG2 catalyzes crosslinking of extracellular matrix proteins, enhances cell binding to fibronectin and integrin, and promotes fibronectin expression. We investigated whether the small electrophilic molecules 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and 15-deoxy-delta-12,14-prostaglandin J₂ (15d-PGJ₂) inhibit the expression and profibrotic functions of TG2. CDDO and 15d-PGJ₂ reduced expression of TG2 mRNA and protein in primary HLFs from control donors and donors with IPF. CDDO and 15d-PGJ₂ also decreased the *in vitro* profibrotic effector functions of HLFs including collagen gel contraction and cell migration. The decrease in TG2 expression did not occur through activation of the peroxisome proliferator activated receptor γ or generation of reactive oxidative species. CDDO and 15d-PGJ₂ inhibited the extracellular signal-regulated kinase pathway, resulting in the suppression of TG2

expression. This is the first study to show that small electrophilic compounds inhibit the expression and profibrotic effector functions of TG2, a key promoter of fibrosis. These studies identify new and important antifibrotic activities of these two small molecules, which could lead to new treatments for fibrotic lung disease.

Keywords: pulmonary fibrosis; fibroblast; transglutaminase 2; 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; 15-deoxy-delta-12,14-prostaglandin J₂

Clinical Relevance

Using normal and fibrotic primary human lung fibroblasts, we demonstrate that two different small electrophilic compounds, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid and 15-deoxy-delta-12,14-prostaglandin J₂, reduce TG2 expression and fibrogenic activities. These compounds are potent antifibrotic drugs, and we have identified TG2 as a novel target for the therapy of pulmonary fibrosis.

Idiopathic pulmonary fibrosis (IPF) is a deadly disease with a median survival of 2 to 3 years after diagnosis (1). IPF is characterized pathologically by increased numbers of lung fibroblasts and

accumulation of excess extracellular matrix (ECM) components, such as collagen and fibronectin. These changes destroy the normal lung architecture and result in impaired lung function (2). Fibroblasts and

myofibroblasts are major sources of these excess ECM components and are key effector cells in lung fibrosis (3). The overall prognosis after diagnosis with IPF is generally grim because the disease often

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progresses steadily, ultimately resulting in death. There are no therapies that have been proven to reverse or stop the progression of IPF (4). A better understanding of the pathogenesis of IPF will aid the discovery of new therapeutic targets for drug development.

Transglutaminase (TG)2 is a widely expressed, multifunctional, profibrotic protein. TGs catalyze the formation of posttranslational bonds between glutamine and lysine residues. This activity requires high calcium concentrations, which can be found in the extracellular space, and renders target proteins more resistant to degradation (5). TG2 plays a key role in lung fibrosis and other fibrotic diseases by crosslinking and stabilizing ECM proteins including

collagen, fibronectin, and elastin (6, 7). When present on the cell surface, TG2 binds to integrins, enhancing cellular binding to fibronectin and promoting fibronectin production (6–9). Prior studies, including ours, have shown the importance of TG2 in the development of hepatic (10, 11), renal (12, 13), cardiac (14), and pulmonary fibrosis (15, 16). We recently demonstrated that TG2 expression was increased in histologic lung sections from patients with IPF and that TG2 knockout mice developed reduced lung scarring in the bleomycin fibrosis model (15). Several inhibitors of TG2 have been developed, but systemic administration of nonselective inhibitors can result in bleeding complications due to homology

between TG2 and clotting factor XIII (17). Therefore, we are interested in targeting TG2 expression to reduce fibrosis.

We and others have recently identified antifibrotic activities of the small electrophilic compounds 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) and 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) (18–21). 15d-PGJ₂ is an endogenously produced member of the prostaglandin (PG) family, whereas CDDO is a synthetic derivative of the naturally occurring triterpenoid oleanolic acid (22, 23). These compounds are potent agonists of peroxisome proliferator activated receptor (PPAR) γ (24, 25) but also have biological effects that are independent of

Figure 1. Small electrophilic compounds decrease transglutaminase (TG)2 protein expression. Primary human lung fibroblasts (HLFs) were treated with the indicated concentrations of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) (A–C) or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) (D, E) for 48 hours (A, B, D, E) or 72 hours (C) and analyzed by Western blot. Blots are representative of at least three independent experiments. (B) Densitometry of three technical replicates for one cell strain treated for 48 hours normalized to the untreated control. (C) Densitometry of three technical replicates for one cell strain treated for 72 hours normalized to the untreated control. (E) Densitometry of four biological replicates per group. Error bars represent mean $\pm$ SD. *** P < 0.001; ** P < 0.01; * P < 0.05 by ANOVA. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

PPAR γ (19, 20, 26). CDDO and 15d-PGJ₂ inhibit fibroblast to myofibroblast differentiation and profibrotic effector functions *in vitro* independently of PPAR γ (19, 20) and reduce fibrosis in the

bleomycin mouse fibrosis model (21 and unpublished observations).

We hypothesized that one mechanism by which CDDO and 15d-PGJ₂ inhibit fibrogenesis is by inhibiting the expression

of TG2. Here, we report that CDDO and 15d-PGJ₂ inhibit TG2 expression in a dose-dependent manner that is independent of PPAR γ or antioxidant pathway activation but is dependent on inhibition of

Figure 2. CDDO and 15d-PGJ₂ reduce TG2 expression in multiple normal and fibrotic HLF strains. (A) Three control (N) and three IPF fibroblast strains (F) were treated with 1 μ M CDDO for 24 hours, and RNA was collected. TG2 mRNA was determined by RT-qPCR and normalized to 18 s mRNA ($n = 3$ replicates per strain). Error bars represent mean $\pm$ SD. (B–E) Fibroblast strains as above were treated with 1 μ M CDDO (B, C) or 5 μ M 15d-PGJ₂ (D, E) for 48 hours, and TG2 protein was analyzed by Western blot. (B, D) Representative blots are shown. (C, E) Densitometry of TG2 expression from the cells treated in B and D. Three replicates per cell strain were analyzed. Error bars represent mean $\pm$ SD. (F) Average baseline TG2 expression from the three normal lines and the three fibrotic lines taken from C. (G, H) Percent inhibition from the baseline was calculated from A for mRNA (G) and for protein (H). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by Student's t test. For the Western blot shown in D, the cell strains were analyzed on a single blot but in a different loading order than in A and B. The lanes have been reordered for clarity.

extracellular signal-regulated kinase (ERK) signaling. These new data identify a novel mechanism by which CDDO and 15d-PGJ₂ blunt fibrogenesis and bolster their potential as a new class of antifibrotic therapeutic agents.

Materials and Methods

Primary Human Lung Fibroblasts

Primary human lung fibroblasts (HLFs) from normal and fibrotic lungs were isolated from patients and cultured as described (18–20). Written informed consent was obtained from all patients. All studies were approved by the University of Rochester Institutional Review Board.

Reagents

CDDO was from the NIH-RAID Program. 15d-PGJ₂, CAY10410, PGA₁, and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂-biotin (biotinylated 15d-PGJ₂) were from Cayman Chemical (Ann Arbor, MI). GW9662 and n-acetyl cysteine (NAC) were from Sigma (St. Louis, MO). U0126 was from Cell Signaling (Danvers, MA). FR148083 [(3S,5Z,8S,9S,11E)-3,4,9,10-tetrahydro-8,9,16-trihydroxy-14-methoxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione, (5Z)-7-Oxozaenol] was from Tocris (Minneapolis, MN). The compounds were dissolved in DMSO at a concentration of 10 mM and diluted in culture medium immediately before use. Control cell cultures were treated with the same final concentration of DMSO (0.1% or less).

Western Blot

Whole cell lysates were prepared in NP-40 lysis buffer, and protein concentration was determined by BCA (Thermo, Fremont, CA). Proteins were detected with antibodies to TG2 (Thermo), β -tubulin, GAPDH (Abcam, Cambridge, MA), heme oxygenase (HO)-1 (Stressgen, Ann Arbor, MI), PPAR γ (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ERK, total ERK, and MEK (Cell Signaling). Blots probed for phospho-ERK were stripped and probed again for total ERK. Densitometry was performed as described (20) and normalized to loading controls.

Real-Time qPCR

RNA was collected, and real-time PCR was performed on cDNA as described (27) using primers to 18S rRNA (27) and TG2 (28).

Collagen Gel Contraction and Migration Assay

The collagen gel contraction assay was performed as described (15). For the Transwell migration assay, cells were placed in the top of an insert (Transwell Permeable Support; Corning Life Sciences, Corning, NY) in serum-free media with indicated treatments. The lower well contained culture media with serum and indicated treatments. Cells were allowed to migrate for 24 hours, after which the membranes were fixed in methanol and stained with crystal violet, and cells were counted in 10 high-power fields per well.

PPAR γ Lentivirus

Cells were incubated with the dominant-negative (DN) PPAR γ lentiviral vector (29) and polybrene in serum-free media for 2 hours. Growth media was then added,

and transfection was assessed by green fluorescence protein (GFP) fluorescence 48 hours later. Cells were used for experiments as indicated.

Transfection

Cells were transfected with the constitutively active mitogen-activated protein kinase kinase 1-Glu 217/Glu 221 (MEK-EE) plasmid (30) or a control plasmid expressing GFP using Fugene (Roche, Indianapolis, IN) transfection reagent as directed by the manufacturer. Three days after transfection, protein was collected for Western blot analysis.

Biotin-15d-PGJ₂ Precipitation

Primary HLFs were serum starved for 24 hours and pretreated with FR148083 for 1 hour. Fresh media containing biotinylated 15d-PGJ₂ was added for 5 hours. The cells

Figure 3. CDDO and 15d-PGJ₂ reduce collagen gel contraction and cell migration. (A) Primary HLFs were seeded into collagen gels and floated in media containing the indicated concentrations of small electrophilic compound. Percent contraction was calculated by gel weight after 24 hours ($n = 4$ per group). (B) HLFs were plated in the top well of a Transwell insert in serum-free media. Cells were allowed to migrate for 24 hours toward 10% serum and treated with 1 μ M CDDO or 5 μ M 15d-PGJ₂ ($n = 3$ per group). Error bars represent mean $\pm$ SD. *** $P < 0.001$ by ANOVA.

were lysed with radio immunoprecipitation assay buffer and precipitated with streptavidin coated Dynabeads (Invitrogen, Carlsbad, CA) at 4°C overnight. After magnetic separation and washing in radio immunoprecipitation assay buffer, the beads were boiled in 2× Laemmli buffer (Bio-Rad, Hercules, CA), and the precipitated proteins were subjected to Western blot analysis.

Statistics

Differences between data sets were determined using ANOVA analysis with Tukey's post test or Student's *t* test using Prism Software (Graphpad, La Jolla, CA). *P* values are as listed in the legends.

Results

CDDO and 15d-PGJ₂ Decrease TG2 Expression

We previously demonstrated that CDDO and 15d-PGJ₂ inhibit myofibroblast differentiation and production of ECM proteins (19, 20). We were thus interested in determining the effects of these compounds on the expression of TG2 (15). CDDO and 15d-PGJ₂ decreased TG2 protein levels in normal primary HLFs. Western blot analysis of TG2 protein expression showed a dose-dependent decrease in TG2 expression in HLFs treated with CDDO (Figures 1A–1C) or 15d-PGJ₂ (Figures 1D and 1E). These PPAR γ ligands do not exhibit cytotoxic effects in primary HLFs at the doses used for these experiments (20).

CDDO and 15d-PGJ₂ Decrease Expression of TG2 in Normal and Fibrotic Fibroblasts

To determine whether the decrease in TG2 caused by CDDO and 15d-PGJ₂ occurs in fibroblasts from normal subjects and subjects with IPF, primary HLFs derived from three control subjects and three patients with IPF were examined. CDDO decreased TG2 mRNA levels (Figure 2A) and protein expression (Figures 2B and 2C) in all six fibroblast strains tested. 15d-PGJ₂ also decreased TG2 protein expression in most of the fibroblast strains, although it was less effective than CDDO (Figures 2D and 2E). Baseline TG2 expression was significantly higher in the fibroblasts grown from fibrotic patients compared with control strains

(Figure 2F). CDDO was equally effective at inhibiting TG2 mRNA but was significantly less effective at inhibiting TG2 protein from IPF fibroblast strains compared with control strains (Figures 2G and 2H), even accounting for the higher baseline expression.

Small Electrophilic Compounds Decrease Fibroblast Contraction and Migration

Because CDDO and 15d-PGJ₂ decreased TG2 levels, we hypothesized that they would decrease profibrotic functions of these fibroblasts. We previously demonstrated that TG2 protein was necessary for *in vitro* collagen gel contraction and fibroblast migration (15). The collagen gel contraction assay measures the ability of fibroblasts seeded into collagen gels to organize the collagen matrix, resulting in contraction over time (31). Primary HLFs seeded in collagen gels and treated with CDDO or 15d-PGJ₂ showed dose-dependent inhibition of gel contraction (Figure 3A). Second, we used a Transwell migration assay to assess the

ability of these compounds to reduce serum-induced fibroblast migration. Fibroblasts treated with CDDO or 15d-PGJ₂ exhibited 45 and 30% decreased migration, respectively (Figure 3B). In both of these assays, CDDO was more effective than 15d-PGJ₂ at decreasing fibroblast contraction and migration. These results show that treatment with CDDO or 15d-PGJ₂ decreased properties of HLFs that are associated with a fibrotic phenotype.

An Electrophilic Carbon Is Necessary to Decrease TG2 Expression

Although CDDO and 15d-PGJ₂ differ structurally, they share the common feature of an electrophilic carbon (Figure 4A). This electrophilic carbon can form a covalent bond with cellular proteins through the thio-Michael addition reaction involving a cysteine residue in the target protein (32). We previously showed that these electrophilic carbons are involved in the inhibition of myofibroblast differentiation by CDDO and 15d-PGJ₂ (19, 20). To determine if this electrophilic carbon was

Figure 4. An electrophilic center is necessary to reduce TG2 expression. (A) Chemical structures of compounds used. Circles indicate electrophilic centers. (B) HLFs were treated with the indicated compounds for 48 hours and analyzed by Western blot. (C) HLFs were treated with the indicated compounds for 24 hours and analyzed by RT-qPCR (*n* = 3 per group). Error bars represent mean $\pm$ SD. **P* < 0.05; ****P* < 0.001 by ANOVA. PGA₁, prostaglandin A₁.

important in decreasing TG2 expression, a structural analog of 15d-PGJ₂ that lacks an electrophilic carbon CAY10410 was used. Unlike 15d-PGJ₂, CAY10410 did not reduce TG2 mRNA or protein expression (Figures 4B and 4C). This indicates that the electrophilic carbon is necessary for the ability of 15d-PGJ₂ to decrease TG2 expression. We also tested another PG, PGA₁, which contains an electrophilic carbon. Similar to 15d-PGJ₂, PGA₁ treatment decreased TG2 expression (Figures 4B and 4C).

CDDO and 15d-PGJ₂ Do Not Decrease TG2 through PPAR γ or Activation of the Glutathione Pathway

To examine if CDDO and 15d-PGJ₂ decrease TG2 expression through PPAR γ -dependent or PPAR γ -independent pathways, we used complimentary genetic and pharmacological methods. GW9662 is an irreversible inhibitor of PPAR γ that potently blocks PPAR γ activity (19). Cells were pretreated for 2 hours with GW9662 before addition of CDDO or 15d-PGJ₂. When PPAR γ was blocked by GW9662, CDDO and 15d-PGJ₂ still inhibited TG2 expression (Figure 5A). Next we used a genetic approach to reduce the activity of PPAR γ with transfection of a DN receptor (19). We previously reported the use of this DN-PPAR γ lentiviral vector construct to block PPAR γ (19). Cells were transduced with the DN-PPAR γ lentiviral vector or a control lentiviral vector. After confirmation of transduction by GFP fluorescence (data not shown), fibroblasts were treated with CDDO or 15d-PGJ₂. Even when the PPAR γ pathway was blocked by the DN-PPAR γ lentiviral vector, CDDO and 15d-PGJ₂ decreased expression of TG2 (Figure 5B). This further confirms that PPAR γ is not necessary for the action of CDDO and 15d-PGJ₂ in reducing TG2 expression.

We previously demonstrated that CDDO and 15d-PGJ₂ up-regulate expression of antioxidant proteins in a glutathione-dependent manner (29). Therefore, to determine if the decrease in TG2 caused by CDDO treatment was due to activation of this pathway, HLFs were pretreated with NAC. NAC is an antioxidant and a precursor for the intracellular antioxidant glutathione (29). The effectiveness of NAC in reducing antioxidant pathway activation is demonstrated by examination of the level

of expression of HO-1, a protein induced by oxidative stress. NAC strongly reduced the up-regulation of HO-1 by CDDO but did not alter the ability of CDDO to inhibit TG2 expression (Figure 5C). This shows that CDDO does not likely reduce TG2 expression through the previously identified glutathione-dependent pathway.

The ERK Pathway Regulates TG2 Expression

In a transformed mouse fibroblast strain, TG2 expression has been shown to be positively and negatively regulated by

ERK activation (33, 34). We therefore examined the ability of the ERK pathway to control TG2 expression in primary HLFs. To examine if targeting the ERK pathway changes TG2 expression, we used pharmacologic and genetic approaches. First we used U0126, an inhibitor of the upstream ERK kinase MEK1, which results in potent suppression of ERK phosphorylation (Figure 6A). Decreased ERK phosphorylation was directly associated with a 50% decrease in TG2 protein expression (Figure 6A). Next, we wanted to test if activation of the ERK pathway

Figure 5. CDDO does not reduce TG2 expression through peroxisome proliferator activated receptor (PPAR γ) or activation of antioxidant pathways. (A) HLFs were pretreated for 2 hours with the PPAR γ antagonist GW9662. Cells were then treated with 1 μ M CDDO or 5 μ M 15d-PGJ₂ for 48 hours. Protein was analyzed by Western blot. (B) Cells were transduced with a lentiviral vector containing GFP or GFP and a dominant negative PPAR γ . After 48 hours, cells were treated with 1 μ M CDDO or 5 μ M 15d-PGJ₂ for 48 hours and analyzed by Western blot. (C) HLFs were pretreated with 5 mM NAC for 2 hours. Cells were then treated with 1 μ M CDDO for 48 hours and analyzed by Western blot. Blots are representative samples from three separate experiments.

could lead to an increase in TG2 expression. We used a plasmid to express a constitutively active mutant of MEK1, MEK-EE, and transfected it into HLFs (30). When the MEK-EE was expressed in HLFs, there was an increase in ERK

phosphorylation and TG2 expression (Figure 6B). CDDO is still capable of suppressing TG2 expression in cells transfected with MEK-EE (Figure 6C), confirming that CDDO acts in the ERK pathway to inhibit TG2.

Figure 6. The extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway controls TG2 expression levels. (A) HLFs were cultured in modified Eagle's medium (MEM) with 10% FBS for 24 hours and treated with the indicated doses of the MEK1/2 inhibitor U0126 in serum-free MEM for 48 hours. TG2 expression was analyzed by Western blot ($n = 3$ per group). Error bars represent mean $\pm$ SD. * $P < 0.05$ by ANOVA compared with untreated. (B) HLFs were transfected with a constitutively active MEK1 plasmid. TG2 expression was analyzed by Western blot. (C) HLFs were transfected with a constitutively active MEK1 plasmid. After 24 hours, cells were treated with 1 μ M CDDO for 48 hours, and TG2 expression was analyzed by Western blot. Representative examples of three separate experiments are shown. pERK, phospho-ERK.

CDDO and 15d-PGJ₂ Reduce ERK Phosphorylation

To determine how CDDO interacts with the ERK pathway, we analyzed ERK phosphorylation by Western blot. CDDO inhibited ERK phosphorylation in normal human fibroblasts after 30 minutes of treatment (Figure 7A). We next determined whether CDDO could inhibit ERK phosphorylation at longer time points of treatment and in cells grown from patients with fibrosis. CDDO decreased ERK phosphorylation at 48 hours in fibroblasts grown from three normal patients and three patients with IPF (Figure 7B). To determine if an electrophilic center was necessary to decrease ERK phosphorylation, cells were treated with the 15d-PGJ₂ analog CAY10410. 15d-PGJ₂, but not CAY10410, decreased ERK phosphorylation suggesting that the electrophilic carbon is necessary for these compounds to inhibit ERK phosphorylation (Figure 7C).

15d-PGJ₂ Directly Interacts with MEK and ERK

Because CDDO and 15d-PGJ₂ block ERK phosphorylation, they might interact with MEK, ERK, or both. To test for interactions with MEK and ERK, HLFs were incubated with biotinylated 15d-PGJ₂, and streptavidin-coated magnetic beads were used to pull down interacting proteins for Western blot analysis. We found that biotin-15d-PGJ₂ bound ERK and MEK (Figure 7D). As a control, we probed for p38 mitogen-activated protein kinase (MAPK), which is not inhibited by 15d-PGJ₂ (20). As expected, biotin 15d-PGJ₂ did not interact with p38 (data not shown). A review of the literature identified a putative binding site for small electrophilic compounds in the ATP binding domain of MEK and ERK and a commercially available small molecule inhibitor, FR148083, known to target this binding site (35). We therefore pretreated HLFs with FR148083 before adding biotin-15d-PGJ₂, and associated proteins were precipitated as described above. FR148083 inhibited biotin 15d-PGJ₂ interaction with MEK but not with ERK (Figure 7D). These data reveal that 15d-PGJ₂ specifically interacts with the ERK pathway and suggest that 15d-PGJ₂ modulates ERK signaling by its direct interaction with MEK and ERK proteins.

Figure 7. CDDO and 15d-PGJ₂ inhibit ERK phosphorylation. (A) HLFs were cultured in MEM with 10% FBS for 24 hours and then treated with 1 μ M CDDO in serum-free MEM for the indicated time periods and analyzed by Western blot for ERK phosphorylation ($n = 3$ per group). Error bars represent mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by ANOVA. (B) Three normal and three fibrotic primary HLF strains were treated with 1 μ M CDDO for 48 hours and analyzed for ERK phosphorylation by Western blot. (C) HLFs were treated as indicated with CDDO, 15d-PGJ₂, and CAY10410 for 48 hours and analyzed by Western blot. (D) HLFs were pretreated with FR148083 for 1 hour, followed by biotinylated 15d-PGJ₂ for 5 hours. Biotin 15d-PGJ₂ was precipitated using streptavidin-coupled beads, and the bound protein was analyzed by Western blot for MEK and ERK expression. Representative examples of three separate experiments are shown.

Discussion

TG2 is a multifunctional protein that plays a key role in the pathogenesis of fibrosis in the lung and is a newly recognized target for therapy of fibrotic diseases (36). Here, we

report that CDDO and 15d-PGJ₂ decreased expression and inhibited profibrotic effector functions of TG2 in primary HLFs. TG2 baseline activity in IPF fibroblasts was significantly higher compared with control fibroblasts, consistent with our previous

observation that TG2 expression is elevated in samples from patients with IPF (15). However, CDDO and 15d-PGJ₂ were less efficient at inhibiting TG2 expression in IPF compared with control fibroblasts. We hypothesize that longer treatments might be needed to fully reverse the IPF phenotype or that TG2 inhibitors would need to be used in combination with other treatments for maximum effectiveness.

CDDO and 15d-PGJ₂ have potent antifibrotic activity *in vitro* and *in vivo* (19, 20, 26, 37), although the mechanisms remain under active investigation. CDDO and 15d-PGJ₂ are ligands for PPAR γ , and it is known that PPAR γ is involved in regulating inflammation and cell differentiation (22, 26, 38). However, CDDO and 15d-PGJ₂ inhibit TGF- β -stimulated differentiation of primary HLFs to myofibroblasts by a predominantly PPAR γ -independent mechanism (19, 20). Here, we used pharmacologic and genetic approaches to demonstrate that CDDO and 15d-PGJ₂ also reduce TG2 expression primarily through a PPAR γ -independent mechanism.

CDDO is a known activator of oxidative stress responses (39, 40). We have previously shown that CDDO potently up-regulates expression of oxidative stress-response proteins in a glutathione-dependent manner and that NAC inhibits this up-regulation (29, 41). Here, we show that NAC blocks up-regulation of HO-1 but does not inhibit the ability of CDDO to reduce TG2 expression (Figure 5C). Therefore, although we cannot rule out a role for oxidative stress in the effect of CDDO on TG2, we believe we can exclude the previously reported glutathione-mediated oxidative stress response as being the mechanism for inhibition of TG2. Similarly, we and others have reported that NAC does not inhibit the ability of CDDO to block TGF- β -stimulated differentiation of primary HLFs to myofibroblasts (29, 42). This is of particular interest because antioxidants in general, and NAC in particular, have been investigated as antifibrotic therapies, and NAC is currently in clinical trials for IPF (42). Taken together, these results suggest that novel therapies based on small electrophilic molecules are not likely to compromise the potential therapeutic use of NAC.

TG2 is a multifunctional protein with several potential roles in lung fibrosis. As a cell adhesion molecule, TG2 is important

for proper cell migration and can enhance cell migration on fibronectin *in vitro* in fibroblasts and monocytic cells (7, 43). TG2 also promotes organization of the matrix and collagen cross-linking. We previously reported that TG2 is up-regulated in patients with pulmonary fibrosis and that TG2-deficient mice are protected from lung fibrosis in the bleomycin model (15). We also showed that inhibition of TG2 expression with a shRNA reduced cell migration and collagen gel contraction in primary HLFs (15). Here, we show that CDDO and 15d-PGJ₂ not only reduce TG2 expression but also inhibit migration and collagen gel contraction. It is possible that the reductions in migration and gel contraction are due in part to effects of CDDO and 15d-PGJ₂ on other cellular targets, and this will be the subject of future studies. However, CDDO and 15d-PGJ₂ inhibit multiple profibrotic fibroblast phenotypes, including expression of TG2.

Expression of TG2 can be regulated by the ERK pathway (33, 34), and ERK activity is up-regulated in patients with pulmonary fibrosis and in mouse models of fibrosis (44–46). Blockade of the ERK pathway by a small molecule inhibitor reduced inflammation and fibrosis in the mouse bleomycin model (47), and Yoshida and colleagues reported that, compared with control subjects, activated ERK signaling is significantly increased in lung homogenates from patients with IPF (44). It has also been reported that the methyl ester derivative of CDDO can inhibit the ERK pathway in leukemia cells (48) and that 15d-PGJ₂ can inhibit the ERK pathway in human $\gamma\delta$ T cells (49).

Here, we report that ERK activity regulates TG2 expression in primary HLFs. Inhibition of MEK with U0126 decreases TG2 expression, whereas constitutive activation of ERK via overexpression of MEK-EE increases TG2 expression. Furthermore, CDDO and 15d-PGJ₂ inhibit ERK phosphorylation, providing a mechanism by which these compounds can down-regulate TG2 through the ERK pathway. Our results suggest that CDDO and 15d-PGJ₂ inhibit ERK activity by

directly interacting with MEK and ERK proteins. CDDO and 15d-PGJ₂ are small molecule electrophilic compounds that can covalently bind to the thiol groups of free (reduced) cysteine residues in intracellular proteins via the Michael addition reaction (32, 50). Such binding has the potential to alter or inhibit protein structure and function. A review of the literature identified a reactive cysteine residue within the ATP binding domain of MEK, C166, that is a target for Michael addition by other small electrophilic compounds, including FR148083 (35). The ATP binding domain of ERK is highly conserved with that of MEK and contains a cysteine residue in the same position (35). Here, we demonstrate that biotinylated 15d-PGJ₂ interacts directly with MEK and ERK and that preincubation with FR148083 inhibits binding of 15d-PGJ₂ to MEK (Figure 7D). This confirms that 15d-PGJ₂ binds directly to ATP binding domain of MEK, likely inactivating MEK and preventing downstream ERK phosphorylation. Because biotinylated CDDO was unavailable for this study, we cannot directly prove that CDDO binds the same conserved cysteine in the ATP binding domain, but we note that CDDO also inhibits ERK phosphorylation and has been shown to interact directly with multiple cellular proteins (51), suggesting that MEK and ERK are also targets for CDDO in primary HLFs. Overall, this result combined with the inhibition of ERK phosphorylation, suggests that 15d-PGJ₂ and CDDO bind to the ATP binding domain of MEK, inhibiting its ability to phosphorylate ERK. Because MEK and ERK share a conserved ATP binding domain, it is likely that 15d-PGJ₂ and CDDO also bind to the ERK ATP binding domain to the same conserved cysteine. In this case, the failure of FR148083 to inhibit binding of 15d-PGJ₂ could be due to differences in the relative affinities of the two compounds for ERK. Alternatively, 15d-PGJ₂ could bind to a different cysteine in ERK.

TG2 plays an unrelated role in the pathogenesis of celiac disease, which is characterized by the presence of antiglutin

antibodies. Expression of TG2 is dysregulated in the small intestine in celiac, and TG2 is responsible for generating celiac neoantigens by crosslinking gluten peptides (52). As a result, a number of inhibitors of TG2 activity have been developed as potential therapeutics. However, the first generation of activity inhibitors also have significant activity against clotting factor XIII, a member of the TG family, resulting in significant bleeding complications. Our study demonstrates the therapeutic potential of using small molecules to target the expression of TG2 rather than targeting its enzymatic activity.

Because TG2 participates in multiple profibrotic processes, including crosslinking of ECM and regulating fibronectin expression, it has been proposed as a key effector protein in fibrosis. Here, we report that two small molecule electrophilic compounds with therapeutic potential have similar effects, inhibiting TG2 expression and functioning by targeting the ERK pathway. ERK1/2 are the only known catalytic substrates of MEK1/2. Therefore, binding and inactivating MEK by these electrophilic compounds serve as ideal candidates to target the ERK/MAPK pathway. Furthermore, this opens up the potential of using available preclinical MEK/ERK inhibitors as therapeutic interventions for pulmonary fibrosis. Clinical trials are in progress with CDDO derivatives for the treatment of diabetic kidney disease. In these trials, CDDO has been shown to be orally available and appears to improve kidney function (53, 54). We hope that information gained from this and future studies will lead to clinical trials of small molecules that target TG2 for therapy of fibrotic diseases. ■

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