EXTENDED REPORT

A synthetic PPAR-γ agonist triterpenoid ameliorates experimental fibrosis: PPAR-γ-independent suppression of fibrotic responses

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ABSTRACT

Background Persistent fibroblast activation initiated by transforming growth factor β (TGF-β) is a fundamental event in the pathogenesis of systemic sclerosis, and its pharmacological inhibition represents a potential therapeutic strategy. The nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR-γ), exerts potent fibrotic activity. The synthetic oleanane triterpenoid, 2-cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO), is a PPAR-γ agonist with potential effects on TGF-β signalling and dermal fibrosis.

Objective To examine the modulation of fibrogenesis by CDDO in explanted fibroblasts, skin organ cultures and murine models of scleroderma.

Material and methods The effects of CDDO on experimental fibrosis induced by bleomycin injection or by overexpression of constitutively active type I TGF-β receptor (TgfbR1ca) were evaluated. Modulation of fibrotic gene expression was examined in human skin organ cultures. To delineate the mechanisms underlying the antifibrotic effects of CDDO, explanted skin fibroblasts cultured in two-dimensional monolayers or in three-dimensional full-thickness human skin equivalents were studied.

Results CDDO significantly ameliorated dermal fibrosis in two complementary mouse models of scleroderma, as well as in human skin organ cultures and in three-dimensional human skin equivalents. In two-dimensional monolayer cultures of explanted normal fibroblasts, CDDO abrogated fibrogenic responses induced by TGF-β. These CDDO effects occurred via disruption of Smad-dependent transcription and were associated with inhibition of Akt activation. In scleroderma fibroblasts, CDDO attenuated the elevated synthesis of collagen. Remarkably, the in vitro antifibrotic effects of CDDO were independent of PPAR-γ.

Conclusions The PPAR-γ agonist triterpenoid CDDO attenuates fibrogenesis by antagonistically targeting canonical TGF-β/Smad and Akt signalling in a PPAR-γ-independent manner. These findings identify this synthetic triterpenoid as a potential new therapy for the control of fibrosis.

INTRODUCTION

Systemic sclerosis (SSc) or scleroderma is characterised by the characteristic triad, inflammation, obliterative microvasculopathy and fibrosis.[1] Fibrosis, the distinguishing pathological hallmark of late-stage SSc, is caused by overproduction of collagen and other extracellular matrix (ECM) components by activated fibroblasts and α-smooth muscle actin (α-SMA)-positive myofibroblasts.[2] Fibroblast activation and myofibroblast transformation result from a complex series of events initiated and orchestrated by the multifunctional cytokine, transforming growth factor β (TGF-β).[3] Microarray-based expression profiling of SSc skin biopsy samples has identified a distinct subset with a 'TGF-β-activated gene signature' that is characterised by extensive skin involvement.[4 5] However, the clinical benefits of targeting TGF-β for SSc therapy remain to be demonstrated.

Multiple physiological mechanisms exist to regulate fibroblast activation in order to prevent excessive tissue remodelling and fibrosis. Recent studies have identified the nuclear receptor, peroxisome proliferator-activated receptor-γ (PPAR-γ), as an important endogenous antifibrotic defence mechanism.[6] Widely expressed in mammalian tissues, PPAR-γ regulates the expression of genes involved in lipid uptake and synthesis, lipolysis, glucose metabolism, cell differentiation, survival and proliferation, as well as immune and inflammatory responses.[7 8] Recent evidence suggests an important novel role for PPAR-γ in the negative regulation of connective tissue biosynthesis during both physiological and pathological matrix remodelling.[9] We showed that natural and synthetic PPAR-γ ligands effectively blocked fibrotic responses elicited by TGF-β in explanted fibroblasts, and attenuated bleomycin-induced skin fibrosis in vivo.[10 11] Subsequent studies in a variety of cell types and model systems confirmed and expanded these findings.[12–14] Moreover, treatment of mice with synthetic PPAR-γ ligands has been shown to attenuate experimentally induced hepatic,[15] cardiac[16] and kidney[17] fibrosis. At the same time, genetic deletion of PPAR-γ in fibroblasts was associated with exaggerated fibrosis in mice injected with bleomycin.[18] Importantly, lesional tissue expression of PPAR-γ is markedly diminished in various rodent models of fibrosis and scleroderma,[11 19 20] as well as in fibrotic skin from patients with cicatrical (scarring) alopecia[21] and SSc.[22] These observations suggest that restoring normal PPAR-γ activity or function in these fibrotic conditions might represent a viable approach to preventing or attenuating persistent fibroblast activation.

Traditional PPAR-γ agonists such as thiazolidinediones have been widely used in type 2 diabetes because of their potent glucose-lowering activity. Recent concerns regarding the safety of this class of drugs have prompted renewed interest in selective PPAR-γ modulators.[23 24] The synthetic pentacycline oleanane triterpenoid, 2-cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO), was originally developed to inhibit nitric oxide synthase.[25] Subsequent studies showed that CDDO binds to and stimulates the activity of PPAR-γ.[26] Further investigation of CDDO has uncovered a broad range of biological activities, including antiangiogenic, antioxidant, anti-inflammatory and antiproliferative effects, which make this compound highly attractive as a potential therapeutic agent for multiple chronic diseases.[27–29] Moreover, recent studies have shown that CDDO inhibited the activation and differentiation of lung and corneal fibroblasts, although the underlying mechanisms remain unknown.[30 31] In light of the myriad salutary biological properties associated with CDDO, the present studies sought to explore its effects in experimental models of scleroderma in vivo and in fibroblasts in vitro.

MATERIALS AND METHODS

Cell cultures

Primary cultures of human fibroblasts were established by explantation from neonatal foreskin or from skin biopsy samples from the dorsal forearm of five healthy adults and five patients with diffuse cutaneous SSc.[32] Skin biopsies were performed after informed consent and in compliance with Northwestern University Institutional Review Board for Human Studies regulations. The clinical characteristics of the patients are available upon request. Cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, Maryland, USA), and studied between passages 4 and 8.[33] Mouse 3T3-L1 preadipocytes (American Type Culture Collection (ATCC)) were maintained in maintenance medium (PM-1; Zen-Bio, Research Triangle Park, North Carolina, USA). Adipogenic differentiation of these cells was evaluated by perilipin-1 staining (see online supplementary material). When cells reached early confluence, fresh medium containing the indicated concentrations of CDDO (RAID; NCI, Rockville, Maryland, USA) or human recombinant TGF-β2 (Peprotech, Rocky Hill, New Jersey, USA) was added to the cultures, and incubation was continued for up to 48 h. In selected experiments, cultures were pretreated with rosiglitazone or GW9662 (both from Cayman Chemical, Ann Arbor, Michigan, USA). At the end of the incubation periods, cultures were harvested, and modulation of fibrotic response was examined by real-time quantitative (q)PCR, western blot analysis and luciferase assays. Cytotoxicity was evaluated using lactate dehydrogenase (LDH) cytotoxicity assay kits (Biovision, Mountain View, California, USA) and by trypan blue dye exclusion.

Human skin organ cultures and three-dimensional organotypic raft skin cultures

Foreskin biopsy samples were cut into 0.5 cm × 0.5 cm sections and placed in six-well plates. Explants were maintained in an air/medium interface with the epidermal layer exposed to air, and culture medium was replaced every other day. The cultures were incubated in medium with 10 ng/ml TGF-β2 for 6 days, and CDDO (5 μM) was added 15 min before or 48 h after the addition of TGF-β. In organotypic raft skin cultures, normal human dermal fibroblasts (3×10^5 cells) were resuspended in 1.5 ml reconstitution buffer with rat tail type I collagen (4 mg/ml; BD Biosciences, San Jose, California, USA), seeded in 12-well plates and incubated at 37°C for 48 h to solidify the collagen plug.[34] Keratinocytes isolated from foreskin (6×10^5 cells) were suspended in E medium supplemented with 5 ng/ml epidermal growth factor and seeded on the collagen plug.[35] After 48 h incubation, the three-dimensional organotypic cultures were placed on a metal grid (McMaster-Carr, Atlanta, Georgia, USA) and maintained at an air/medium interface, with fresh E medium replacement every other day for 5 days. Organotypic cultures were then incubated with CDDO (5 μM) for 24 h, followed by TGF-β (5 ng/ml) for a further 6 days. Cultures were then harvested for RNA analysis, or fixed in formalin and processed for histology. Paraffin-embedded sections (4 μm) were examined by Sirius Red staining or Masson's trichrome staining.

Mouse models of scleroderma

Animal protocols were institutionally approved by the Animal Care and Use Committee of Northwestern University or the University of Erlangen-Nuremberg. Skin fibrosis was induced by two distinct approaches: daily subcutaneous injections of bleomycin or intracutaneous injections of an adenovirus expressing a constitutively active mutant of the type I TGF-β receptor ($T\beta RI^{ca}$). In the first protocol, 6–8-week-old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were given daily subcutaneous injections of bleomycin (10 mg/kg) for 14 consecutive days and killed on day 28. In the second protocol, replication-deficient type 5 adenovirus encoding $T\beta RI^{ca}$ (Ad-$T\beta RI^{ca}$) or LacZ (Ad-LacZ) (6.7×10^7 plaque-forming units/mouse) was injected intracutaneously on two occasions separated by 4 weeks, and mice were killed 4 weeks after the second injection.[36] Lesional skin was harvested and analysed as previously described[11] (see online supplementary material). In both protocols, CDDO (5 mg/kg, dissolved in 80% phosphate-buffered saline (PBS), 10% dimethyl sulfoxide and 10% Cremophor-EL (Sigma, St Louis, Missouri, USA)) was injected intraperitoneally daily. In other experiments, the effects of CDDO on modulating established fibrosis were investigated. For this purpose, scleroderma was induced by bleomycin injections as above, and CDDO (5 mg/kg/day) was injected intraperitoneally daily starting at day 15.

Statistical analysis

Data are presented as means±SD. The significance of differences between experimental and control groups was determined by Student t test. In the animal studies, differences between the groups were evaluated using the non-parametric Mann–Whitney U test. $p<0.05$ was considered significant.

RESULTS

CDDO induces PPAR-γ activity and stimulates adipogenesis

In initial studies, we sought to examine the effect of CDDO on PPAR-γ signalling in mesenchymal cells. For this purpose, multipotent mouse 3T3-L1 preadipocytes were incubated with CDDO, or the synthetic PPAR-γ ligand, rosiglitazone, in parallel, for up to 7 days. Phase-contrast microscopy showed a substantial time-dependent accumulation of cytosolic oil droplets induced by both rosiglitazone and CDDO in these cells (figure 1A). Both ligands enhanced the expression of perilipin, a lipid droplet-associated adipocyte marker that is a target of PPAR-γ.[37] Moreover, CDDO stimulated the expression of adipogenic markers, PPAR-γ2 and FABP4 mRNA, in a dose-dependent manner, with a maximal 5–6-fold increase at day 7 (figure 1B). To directly evaluate the effect of CDDO on PPAR-γ activity, we incubated confluent human skin fibroblasts transiently transfected with PPRE-luc with CDDO. After a 24 h incubation,

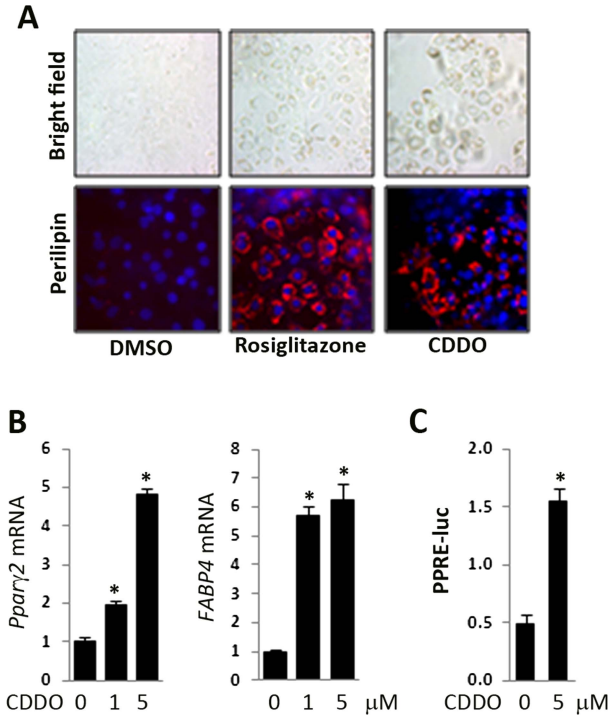

Figure 1 2-Cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO) induces peroxisome proliferator-activated receptor γ (PPAR-γ) activation and promotes adipogenesis. (A and B) Confluent 3T3-L1 preadipocytes were incubated with CDDO (5 μM or indicated concentration) or rosiglitazone (10 μM) for 7 days before harvesting. (A) Cultures were immunostained with antibody to perilipin-1. Upper panels, phase-contrast microscopy; lower panels, immunofluorescence. Representative images. Original magnification ×200. DMSO, dimethyl sulfoxide. (B) RNA was examined by real-time quantitative PCR. The results, normalised with *36B4*, represent the means±SD of triplicate determinations from a representative experiment. (C) Human skin fibroblasts transiently transfected with PPRE-luc were incubated in medium with or without CDDO for 24 h. Cell lysates were assayed for luciferase activity. The results, normalised with *Renilla* luciferase, represent the means±SD of three transfection assays in triplicate. *p<0.05.

whole cell lysates were assayed for luciferase activity. The results showed that CDDO caused a more than threefold increase in PPRE promoter activity in transfected fibroblasts (figure 1C). The concentration of CDDO used in these experiments was not toxic to 3T3-L1 cells or fibroblasts (data not shown). Taken together, these results demonstrate that CDDO can function as a potent PPAR-γ agonist that induces PPAR-γ-dependent adipogenic responses in both preadipocytes and skin fibroblasts.

CDDO ameliorates dermal fibrosis in the mouse

In light of the antifibrotic activity ascribed to conventional PPAR-γ ligands,[10 11] we evaluated the effects of CDDO on fibrosis using two complementary mouse models of scleroderma. In the first approach, skin fibrosis was induced in C57BL/6J mice by daily subcutaneous injections with bleomycin for 14 consecutive days. Mice were given concurrent CDDO (5 mg/kg) or vehicle by intraperitoneal injections from day 1 through day 27. They were killed after 28 days, and lesional skin was harvested and analysed. Injection of CDDO was well tolerated, and no significant weight loss or other signs of toxicity were noted. After 28 days, increased thickness of the dermis was noted in all mice injected with bleomycin compared with PBS (322±72 μm vs 136 ±34 μm, five mice/group, p<0.001) (figure 2A). Both excessive collagen deposition and dermal thickness were markedly ameliorated (by 53%, p=0.01) when CDDO was administered concomitantly with bleomycin. Moreover, the dramatic loss of the subcutaneous adipose layer accompanying dermal fibrosis in mice injected with bleomycin alone was substantially attenuated in mice treated with CDDO. Furthermore, upregulated expression of multiple fibrotic marker genes in lesional skin was attenuated in CDDO-treated mice (figure 2B).

In a complementary experimental approach, C57BL/6J mice were given two intradermal injections of Ad-TβRI^{ca} or Ad-LacZ separated by 4 weeks, along with daily intraperitoneal injections of CDDO or vehicle as above. They were killed 4 weeks after the second adenovirus injection, and lesional skin was harvested for analysis. Ectopic expression of TβRI^{ca} induced local TGF-β signalling, which was associated with significant dermal thickening, increased collagen deposition, and myofibroblast accumulation (figure 2C–E, six to eight mice/group). Concomitant treatment of the mice with CDDO almost completely abrogated these fibrotic responses, and attenuated the induction of TGF-β target genes (data not shown).

To evaluate the effects of CDDO on established fibrosis, treatment was started on day 15 of daily bleomycin injections. In this model of fibrosis regression, CDDO resulted in significant reduction of dermal thickness in lesional skin compared with mice injected with bleomycin plus vehicle (figure 2F, n=5). Moreover, CDDO markedly attenuated the increased levels of α-SMA expression (figure 2G). The results from these complementary mouse models of scleroderma indicate that CDDO antagonised fibrotic responses triggered by bleomycin, or by activation of TGF-β signalling in the skin.

CDDO inhibits fibrotic responses induced by TGF-β

To explore the mechanism underlying the antifibrotic and anti-TGF-β effects of CDDO noted in two complementary mouse models of scleroderma, we carried out in vitro studies using two-dimensional cultures of explanted normal human skin fibroblasts. While TGF-β markedly enhanced type I collagen and α-SMA accumulation and mRNA expression in these cells, preincubation with CDDO abrogated each of the TGF-β-induced fibrotic responses in a dose-dependent manner (figure 3A–C). Importantly, CDDO was capable of reversing the stimulation of type I collagen and α-SMA even when it was added to the cultures up to 24 h after TGF-β (figure 3D). To further investigate the effects of CDDO on collagen gene regulation, we preincubated fibroblasts transiently transfected with Col1A2-luc with CDDO, followed by TGF-β for 24 h. Stimulation of Col1A2 promoter activity in TGF-β-stimulated fibroblasts was significantly attenuated by CDDO (data not shown).

To evaluate the effects of CDDO on fibroblast activity and function in a context morphologically relevant for the skin, we used an organotypic human skin raft model comprising epidermal keratinocytes and dermal fibroblasts. In these skin rafts, fibroblasts embedded in a collagen-rich three-dimensional matrix are, in comparison with two-dimensional monolayer cultures grown on plastic dishes, subjected to substantially lower levels of mechanical stress, approximating those of normal dermis (unpublished results). As the topography and biomechanical properties of the organotypic skin raft mimic those of human skin, the three-dimensional skin equivalent serves as an attractive tractable model system for the study of tissue fibrosis.[38 39] Incubation of the skin rafts in medium with TGF-β for 5 days resulted in a marked increase in the expression of *COL1A1*, *COL1A2* and *α-SMA* in fibroblasts within the dermal compartment (figure 3E and data not shown). Treatment of the

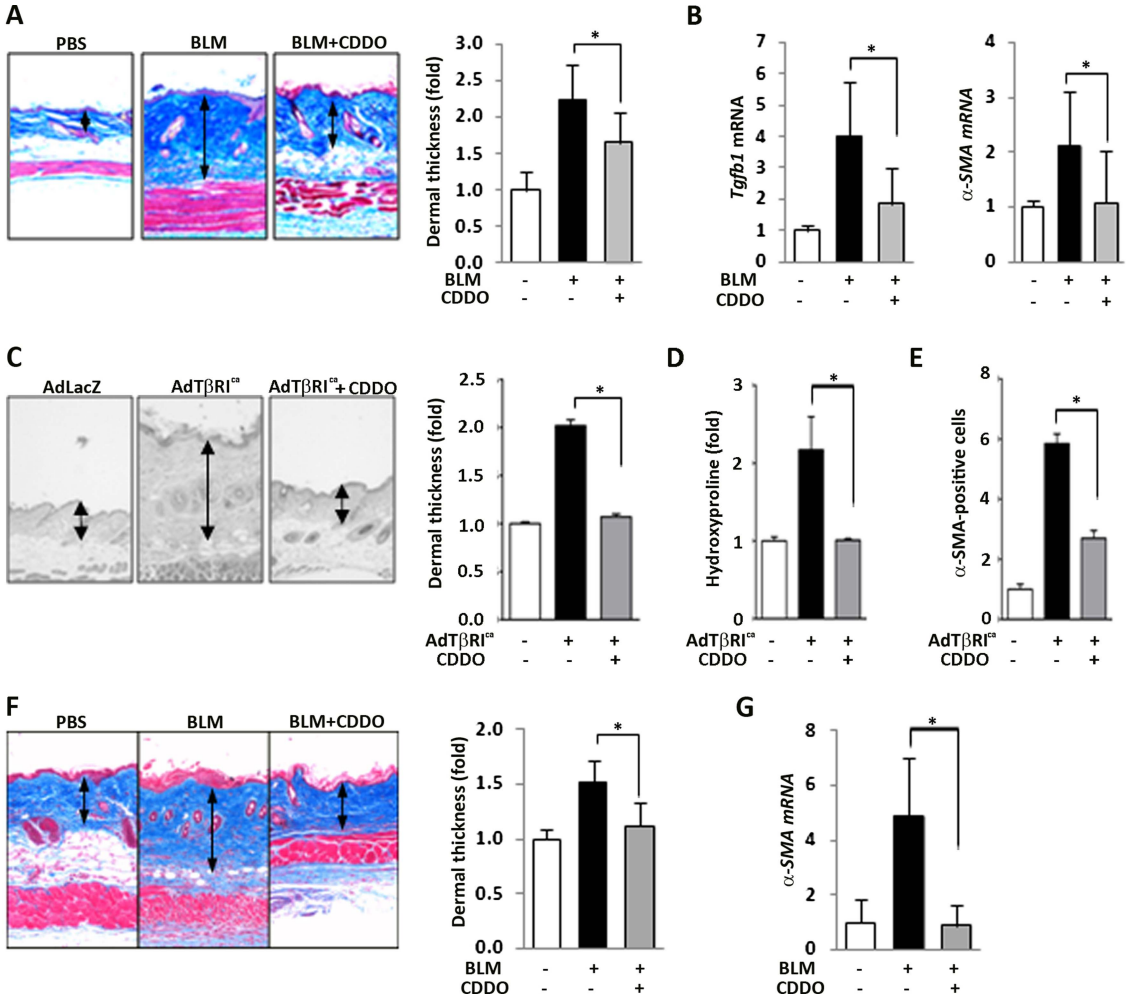

Figure 2 2-Cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO) ameliorates dermal fibrosis in mouse models of scleroderma. Mice were given subcutaneous injections of bleomycin (BLM) daily for 14 days (A, B, F and G), or Ad-TβRI^{ca} or control adenovirus twice separated by 4 weeks (C–E) along with daily intraperitoneal injections of CDDO from day 1 to 27 (A–E) or from day 15 to 27 (F and G). At the end of the experiments, lesional skin was harvested for analysis. (A and F) Left panels, Trichrome (original magnification ×200). Arrows delineate the dermis. Right panel, quantification of dermal thickness. The results, representing the means±SD of five determinations/high-power field from five mice/group, are shown as fold change compared with control (phosphate-buffered saline (PBS)-treated) mice. (B and G) RNA was isolated and examined by real-time quantitative PCR. The results, normalised with *36b4* or β-actin, represent the means±SD of triplicate determinations from three to five mice/group. *p<0.05. (C) Left panels, haematoxylin and eosin (original magnification ×200). Right panel, quantification of dermal thickness. The results, representing the means±SEM of six to eight mice/group, are shown as fold change compared with control (Ad-LacZ). (D) Hydroxyproline assays. The results, representing the means±SEM of triplicate determinations from six to eight mice/group, are shown as fold change compared with control (Ad-LacZ). (E). Immunohistochemistry. α-Smooth muscle actin (α-SMA)-positive cells were quantified as described in Materials and Methods. Results are the means±SEM. *p<0.05.

rafts with CDDO significantly attenuated the upregulation of each of these genes. Picrosirius Red staining of 4 μm-thick sections showed that TGF-β induced a notably increased red birefringence, indicating the accumulation of highly cross-linked collagen, in the dermal compartment (figure 3F). Pretreatment of the rafts with CDDO prevented collagen fibre maturation, with a predominance of green-coloured collagen fibres representing attenuated cross-linking (figure 3F).[40]

To further characterise the modulation of cutaneous fibrotic responses by CDDO, we performed experiments using human skin organ cultures. Incubation of the organ cultures with TGF-β resulted in increased collagen accumulation, and preincubation with CDDO markedly attenuated this response (figure 3G). Similar results were seen even when CDDO was added to the cultures 48 h after TGF-β. The stimulation of *COL1A1* and *α-SMA* mRNA expression by TGF-β was also significantly suppressed by CDDO (figure 3H).

Epithelial–mesenchymal transition has been considered to play an important role in fibrosis.[1] CDDO markedly attenuated TGF-β-induced epithelial–mesenchymal transition in human A540 epithelial cells (see online supplementary figure S1).

CDDO abrogates TGF-β/Smad and Akt signalling

To delineate the TGF-β signalling pathways that are targeted by CDDO, we transiently transfected fibroblasts in two-dimensional monolayer cultures with the Smad-responsive $(SBE)_4$-luc followed by TGF-β in the presence or absence of CDDO. The results of transient transfection assays showed that stimulation of $(SBE)_4$-luc activity by TGF-β was completely abrogated in the presence of CDDO (figure 4A). Surprisingly, however, there was no change in TGF-β-induced Smad2 phosphorylation or nuclear translocation in CDDO-treated fibroblasts (figure 4B). These results indicate that CDDO blocked

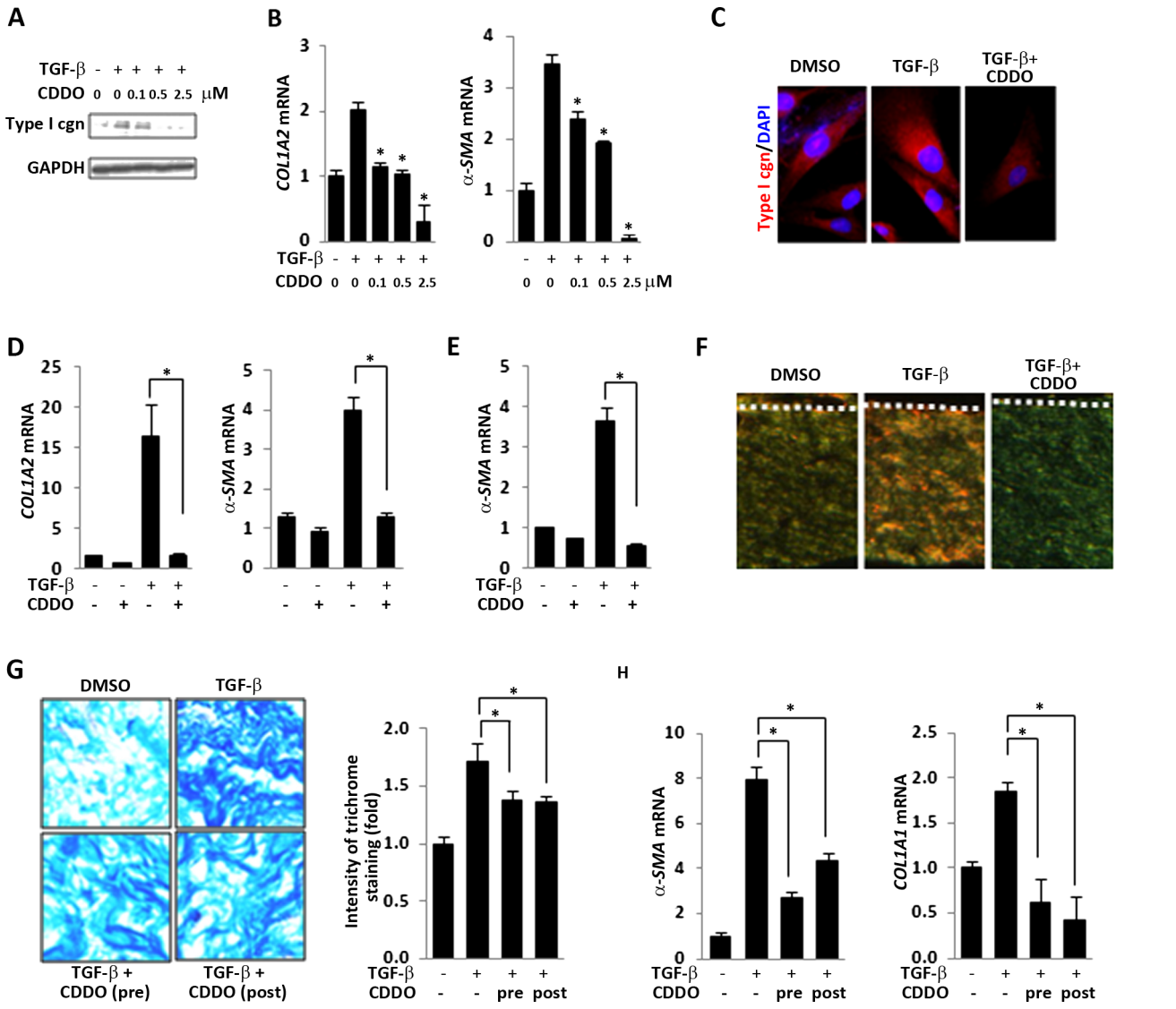

Figure 3 2-Cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO) abrogates stimulation of collagen synthesis and myofibroblast differentiation. (A–D) Confluent skin fibroblasts were incubated with CDDO (2.5 μM unless otherwise indicated) and transforming growth factor β (TGF-β; 10 ng/ml) after (A–C) or before (D) CDDO. (A) Whole cell lysates were examined by western blot analysis. Type I cgn, type I collagen. Representative images. (B and D) RNA was examined by real-time quantitative PCR. The results, normalised with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are the means±SD of triplicate determinations from a representative experiment. *p<0.05. (C) Immunofluorescence. Red, type I collagen. Blue, 4′,6-diamidino-2-phenylindole (DAPI). Representative images. Original magnification ×400. (E and F) Three-dimensional human skin equivalents populated with skin fibroblasts were incubated with CDDO (5 μM) for 24 h, followed by TGF-β (5 ng/ml) for 5 days, and rafts were harvested. (E) Total RNA was isolated and examined by real-time quantitative PCR. The results, normalised with GAPDH, are the means±SD of triplicate determinations from a representative experiment. *p<0.05. (F) 4 μm-thick paraffin-embedded sections were stained with Picrosirius Red. Mature collagen fibres in the dermal compartment appear red when visualised under polarised light, whereas less mature collagen fibres containing fewer cross-links appear yellow/green. Representative images (original magnification ×40). Dashed lines, dermal/epidermal junction. (G and H) Human skin organ cultures were incubated in medium with TGF-β (10 ng/ml) for 6 days. CDDO (5 μM) was added to the cultures 15 min before or 48 h after addition of TGF-β. (G) 4 μm-thick paraffin-embedded sections were stained with Masson's trichrome. Left panels, representative images (original magnification ×400); right panel, relative fold change in staining intensity. (H) Skin tissues were harvested, and total RNA was isolated and examined by real-time quantitative PCR. The results, normalised with GAPDH, are the means±SD of triplicate determinations from a representative experiment. *p<0.05. DMSO, dimethyl sulfoxide; α-SMA, α-smooth muscle actin.

TGF-β signalling by disrupting Smad-dependent transcription but without preventing Smad2/3 activation.

In addition to canonical Smad signalling, TGF-β also induces Smad-independent cellular pathways that are implicated in fibrotic responses. To investigate the modulation of non-canonical TGF-β signalling by CDDO, we focused on the Akt pathway previously shown to be regulated by CDDO in lung fibroblasts.[41] Confluent dermal fibroblasts were incubated with TGF-β for up to 24 h in the presence or absence of CDDO, and whole cell lysates were examined. The results of western blot analysis showed that, while TGF-β induced an approximately twofold increase in phospho-Akt, preincubation of the cultures with CDDO had little effect on Akt activation at 120 min, but completely abrogated the response at 24 h (figure 4C). Together, these results indicate that CDDO was able to disrupt both canonical and non-canonical pathways mediating profibrotic TGF-β responses.

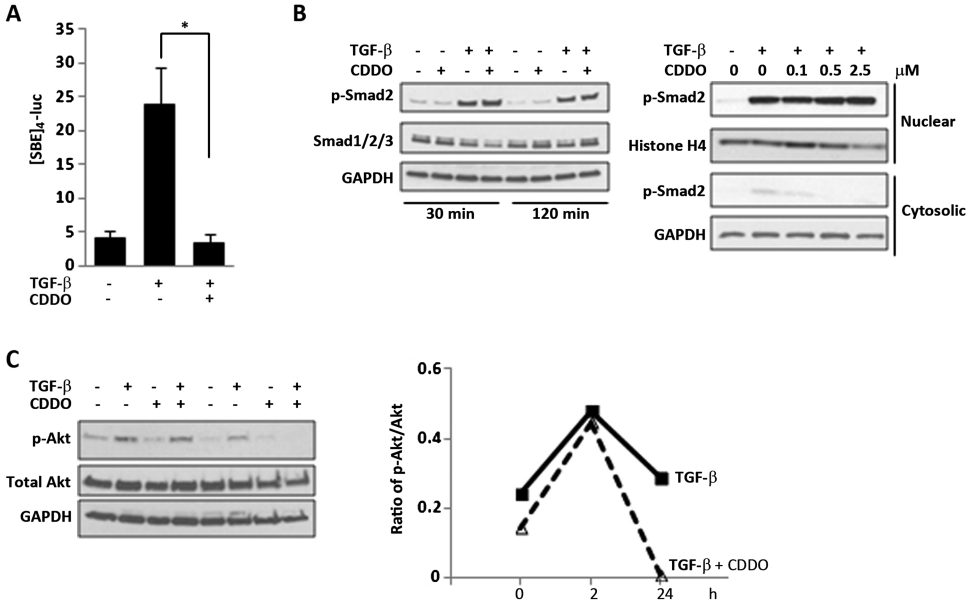

Figure 4 2-Cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO) blocks Smad-dependent transcription and Akt activation. Confluent skin fibroblasts were transiently transfected with $(SBE)_4$-luc (A) or left untransfected (B and C). Cultures were preincubated with CDDO (2.5 μM or indicated concentrations), followed by further incubation with transforming growth factor β (TGF-β; 10 ng/ml) for up to 24 h (A) or indicated time periods (B). (A) Whole cell lysates were assayed for their luciferase activities. The results, normalised with *Renilla* luciferase, represent the means±SD of triplicate experiments. *p<0.05. (B) Whole cell lysates (left panel) or cytosolic and nuclear fractions (right panel) were examined by western blot analysis. Representative images. (C) Whole cell lysates were examined by western blot analysis. Bands were quantified by densitometry. Representative images. Relative phospho-Akt (p-Akt) levels normalised with total Akt are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CDDO ameliorates collagen overproduction in explanted scleroderma fibroblasts

We previously demonstrated that explanted scleroderma fibroblasts maintained their activated phenotype ex vivo with constitutive TGF-β signalling even in the absence of exogenous ligand.[42] Evaluation of the antifibrotic effects of CDDO in fibroblasts explanted from five patients with diffuse scleroderma showed 50–60% reduction in levels of cellular and secreted type I collagen (figure 5A), and >50% reduction in *COL1A1* and *COL1A2* mRNA, in each SSc cell line (figure 5B).

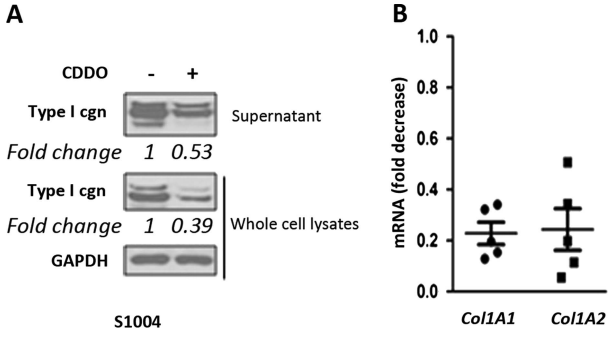

Figure 5 2-Cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO) normalises collagen overproduction in scleroderma fibroblasts. Skin fibroblasts explanted from patients with systemic sclerosis (n=5) were incubated with CDDO (5 μM) for 24 h. (A) Whole cell lysates and culture supernatants were analysed by western blot analysis. Bands were quantified by densitometry. Relative levels normalised with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown below the images. Representative images. (B) RNA was examined by real-time quantitative PCR. Results are expressed as dot plots of mRNA levels relative to untreated cultures. Bars indicate the means±SEM of triplicate determinations from five patients with systemic sclerosis.

Inhibition of profibrotic responses by CDDO is PPAR-γ independent

Since CDDO shows agonist activity on PPAR-γ signalling, we sought to explore the potential role of PPAR-γ in mediating the inhibitory effects of CDDO on TGF-β-dependent fibrotic responses. To this end, GW9662, a PPAR-γ ligand that irreversibly blocks PPAR-γ signalling, was used.[43] Preincubation of confluent normal fibroblasts with GW9662 failed to rescue TGF-β-induced stimulation of collagen synthesis or $(SBE)_4$-luc activity in the presence of inhibitory CDDO, while effectively blocking the inhibitory effects of rosiglitazone (figure 6 and data not shown). We conclude on the basis of these experiments that, in contrast with the traditional PPAR-γ ligand, rosiglitazone, CDDO inhibition of TGF-β-induced fibrotic responses was largely PPAR-γ independent.

DISCUSSION

Since activation of fibroblasts and myofibroblasts by TGF-β underlies the initiation and progression of fibrosis in SSc, disrupting TGF-β activity represents an appealing approach to fibrosis therapy.[44] We showed previously that synthetic or natural ligands of PPAR-γ blocked Smad-dependent TGF-β responses in mesenchymal cells,[10 11 45] suggesting that activation of PPAR-γ may represent a pharmacological strategy for modulating fibrosis. Although the thiazolidinedione class of drug comprises potent PPAR-γ ligands, their clinical use has been associated with diverse side effects, including cardiovascular complications,[23] making the discovery of novel PPAR-γ ligands the focus of intensive research.

The synthetic oleanane triterpenoid, CDDO, has previously been shown to enhance PPAR-γ activity in mouse progenitor cells and human lung fibroblasts.[26 41] Here, we confirmed the ability of

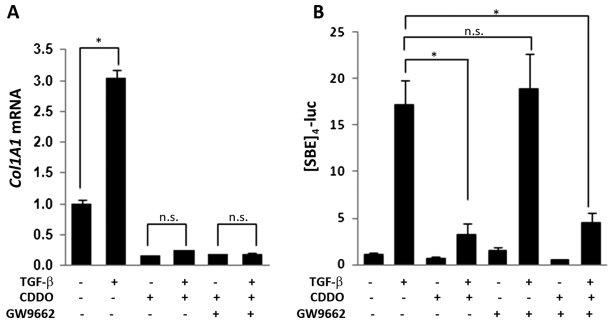

Figure 6 Inhibition of in vitro profibrotic responses is peroxisome proliferator-activated receptor γ (PPAR-γ) independent. Confluent skin fibroblasts, untransfected (A) or transiently transfected with $(SBE)_4$-luc (B), were preincubated with GW9662, followed by 2-cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO; 2.5 μM) and transforming growth factor β (TGF-β; 10 ng/ml) for 24 h. (A) RNA was examined by real-time quantitative PCR. The results, normalised with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), represent the means±SD of triplicate determinations from a representative experiment. *p<0.05. (B) Luciferase activities, normalised with *Renilla* luciferase in each sample, are means±SD of triplicate experiments. *p<0.05.

CDDO to enhance PPAR-γ signalling and to promote adipogenesis in mesenchymal cell types. We evaluated the effects of CDDO on fibrotic responses in vivo using two experimental approaches to model fibrosis. These studies showed that CDDO attenuated skin fibrosis in a bleomycin-induced model characterised by inflammatory cell infiltration in the dermis that mimics the inflammatory stage of SSc.[46] Since CDDO has anti-inflammatory effects, the attenuation of skin fibrosis observed in the present studies may reflect immunosuppression or inhibition of fibrotic TGF-β signalling, or both. Previous studies have shown that CDDO can either synergise with or antagonise TGF-β in a context-dependent manner.[47 48] To evaluate the effects of CDDO on TGF-β-induced fibrotic responses directly, we used a complementary model of scleroderma induced by constitutive TGF-β signalling that is independent of inflammation. The results showed that CDDO abrogated dermal thickening and myofibroblast accumulation in this inflammation-independent scleroderma model.[36] Moreover, in monolayer cultures of explanted skin fibroblasts, CDDO dramatically attenuated the stimulation of fibrotic responses elicited by TGF-β, indicating that CDDO directly antagonises TGF-β activity in fibroblasts. The antifibrotic properties of CDDO were further investigated using a three-dimensional human skin equivalent culture system, where fibroblasts are embedded in a relatively soft and mechanically unstrained collagen matrix in topographic proximity to a stratified squamous epidermal layer. Incubation of this organotypic skin raft culture with TGF-β results in stimulation of resident fibroblasts with activated Smad2/3 signalling, increased secretion of collagen, and transdifferentiation into α-SMA-positive myofibroblasts. Moreover, over the course of a 7-day incubation period, the dermal compartment develops sclerosis and evidence of collagen fibre maturation accompanied by increased stiffness. Thus, these activated human skin equivalents acquire the biochemical, biomechanical and morphological features of scleroderma. In this ex vivo scleroderma model, CDDO attenuated the upregulation of fibrotic gene expression and alleviated other hallmark features of cutaneous fibrosis. Moreover, CDDO abrogated fibrotic responses induced ex vivo by TGF-β in human skin organ cultures.

To explore the mechanisms underlying the antagonistic effects of CDDO on TGF-β responses, we carried out a series of experiments using explanted dermal fibroblasts. The results showed that CDDO abrogated Smad-dependent transcriptional activity without preventing Smad phosphorylation and nuclear translocation. In contrast, other studies using lung and corneal fibroblasts showed that CDDO had no significant effects on Smad signalling.[30] Since dermal fibroblasts explanted from patients with scleroderma show enhanced Smad2/3 activation that might account for the increased ECM production in vitro, it was not surprising that CDDO reduced collagen gene expression in scleroderma fibroblasts. In agreement with other studies,[41] we found that CDDO attenuated activation of Akt induced by TGF-β. Although CDDO served as a potent stimulus for PPAR-γ activation in fibroblasts, we showed, using the irreversible PPAR-γ antagonist GW9662, that the antifibrotic effects of CDDO were PPAR-γ independent. Synthetic oleanane triterpenoids are known to be the most potent inducers of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is the master regulator of antioxidative and cytoprotective responses in a variety of cell types.[27] Expression of Nrf2 is reduced in lung fibroblasts from patients with idiopathic pulmonary fibrosis.[49] Intriguingly, we found that Nrf2 was stabilised by CDDO treatment in dermal fibroblasts (data not shown). The involvement of the Nrf2 pathway in mediating the antifibrotic effects of CDDO is currently under investigation.

Triterpenoids are derived from isopentenyl pyrophosphate oligomers, which comprise the largest group of natural plant products.[28] Synthetic oleanane triterpenoids such as CDDO have both cytoprotective and anticancer activities and have been shown to inhibit liver, lung and prostate cancer in preclinical models.[50–53] By targeting multiple signalling pathways in addition to Nrf2, PPAR-γ and PI3K/Akt, including JAK/STAT, mTOR and NF-κB, synthetic oleananes regulate cellular stress responses.[54] Several CDDO derivatives are currently in clinical trials. A methyl ester derivative of CDDO, bardoxolone methyl, is currently in phase I clinical trials for the treatment of leukaemia, solid tumours and other non-neoplastic diseases (http://www.clinicaltrials.gov). In a recent phase II randomised, placebo-controlled trial, bardoxolone methyl was shown to increase glomerular filtration rate in diabetic patients with impaired renal function.[55 56] Synthetic derivatives of CDDO may have utility in a variety of chronic inflammatory and neurodegenerative conditions as well.

In summary, we show that the synthetic pentacyclic triterpenoid, CDDO, a PPAR-γ agonist and potent activator of the Nrf2 pathway, ameliorated experimental skin fibrosis in two complementary mouse models of scleroderma, and inhibited TGF-β-induced profibrotic responses by blocking Smad and Akt signalling in a PPAR-γ independent manner. While the precise mechanism of the CDDO–Smad antagonism remains to be elucidated, CDDO is known to directly interact with a large number of cellular targets, with context-dependent biological outcomes determined by the cell types and their differentiation states, as well as dose.[54] Ultimately, the clinical efficacy of CDDO may be determined by its ability to simultaneously modulate entire signalling networks rather than a single target. Together, these observations suggest that CDDO and its related olenane derivatives represent novel orally active agents with excellent safety profiles and plausible clinical potential in the therapy of complex chronic diseases such as SSc.

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A synthetic PPAR-γ agonist triterpenoid ameliorates experimental fibrosis: PPAR-γ-independent suppression of fibrotic responses

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