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Synthetic Triterpenoids Enhance Transforming Growth Factor β/Smad Signaling[1]

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ABSTRACT

We have studied the effects of two new synthetic triterpenoids, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its derivative, 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole (CDDO-Im), on transforming growth factor (TGF)-β/Smad signaling. These agents, at nanomolar concentrations, increase the expression of TGF-β-dependent genes, such as those for plasminogen activator inhibitor 1 and the type II TGF-β receptor, and they synergize with TGF-β in this regard. They prolong the activation of Smad2 induced by TGF-β and markedly enhance the ability of Smad3 to activate a Smad binding element, CAGA-luciferase. In transfection assays, they reverse the inhibitory effects of Smad7. CDDO and CDDO-Im also enhance Smad signaling in the pathways of two other members of the TGF-β superfamily, namely, activin and bone morphogenetic protein. Finally, these triterpenoids induce expression of the transcriptional coactivator p300-CBP-associated factor and synergize with TGF-β in this regard. These are the first studies to report enhancement of Smad signaling by synthetic triterpenoids and should further their optimal use for applications in prevention or treatment of diseases in which there is aberrant function of TGF-β.

INTRODUCTION

Aberrant function of the TGF-β[3]/Smad signaling pathway is a frequent occurrence in human disease (1–4). In this context, it is important to develop new agents to modulate this pathway and to restore normal function (4, 5). At present, there is a paucity of molecules for this purpose. We report here, for the first time, the potent ability of some new synthetic triterpenoids to enhance TGF-β/Smad signaling and to increase the effects of TGF-β in cells of both epithelial and hematopoietic origin.

The TGF-β superfamily consists of more than 40 members, including TGF-βs, activins, and BMPs; the molecules in this set are multifunctional cytokines that affect inflammatory and immune responses, cell growth and differentiation, apoptosis, and morphogenesis (6). They all signal through heteromeric complexes of type II and type I transmembrane Ser/Thr kinase receptors, which then initiate phosphorylation cascades involving receptor-regulated Smads, a co-Smad, and inhibitory Smads (6–8).

In the present studies, we have used synthetic triterpenoids, whose actions often mimic those of TGF-β (9), to modulate the TGF-β/Smad signaling pathway. We have recently developed many new synthetic triterpenoids to be more potent than the naturally occurring parent structure, oleanolic acid, as anti-inflammatory, antiproliferative, and cell-differentiating agents (9–13). These molecules inhibit the expression of genes for inflammatory mediators, such as iNOS and inducible cyclooxygenase (cyclooxygenase 2), inhibit proliferation of many cancer cells, and induce monocytic differentiation of leukemia cells and adipogenic differentiation of fibroblasts (9, 14). It is well known that TGF-β also has potent effects on all of the above functions (6). Therefore, we have investigated interactions of triterpenoids with TGF-β in the Smad pathway.

In the present study, we have used a new synthetic triterpenoid, CDDO-Im, as well as its parent molecule, CDDO, to study effects on TGF-β/Smad signaling. We have first studied the effects of CDDO and CDDO-Im on expression of genes responsive to TGF-β, such as those for PAI-1 and the TGF-β type II receptor (15, 16), and then evaluated transcriptional effects on a Smad binding element, using the TGF-β/Smad-dependent (CAGA)12-Luc reporter (17).

Furthermore, we have explored the effects of triterpenoids on signaling by other members of the TGF-β superfamily, such as activin and BMP2, because these cytokines share common intracellular/nuclear proteins and similar mechanisms of regulation and degradation, although the specific Smads involved may be different (8, 18). A final possible overlap between triterpenoids and TGF-β is their use of common nuclear coactivators, such as the histone acetyl transferases, p/CAF, and p300/cAMP-responsive element-binding protein (CREB)-binding protein, which are known to be involved in transactivation of TGF-β-dependent genes (19–22). Therefore, we have also studied whether CDDO or CDDO-Im could modulate the synthesis of p/CAF. Our findings in all of the above studies show for the first time that synthetic oleanane triterpenoids such as CDDO-Im and CDDO have profound effects on TGF-β/Smad signaling. Because of these effects, triterpenoids may provide a therapeutically useful way to modulate the TGF-β pathway.

MATERIALS AND METHODS

Reagents. Details of the synthesis of CDDO and CDDO-Im have been published (11, 12). Sources of other materials were as follows: recombinant mouse IFN-γ (lipopolysaccharide content, <10 pg/ml), TNF-α, and TGF-β were from R&D Systems (Minneapolis, MN); polyclonal iNOS IgG and peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody to phospho-Smad2 was from Upstate Biotechnology (Lake Placid, NY); antibody to Smad2 was from Zymed Laboratories (South San Francisco, CA); FuGene 6 was from Roche Diagnostics (Indianapolis, IN); and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. BMP2 was a gift from Tony Celeste (Wyeth Genetics Institute, Cambridge, MA). Triterpenoids were dissolved in DMSO before addition to cell cultures or enzyme assays; final concentrations of DMSO were 0.1% or less. Controls with DMSO alone were run in all cases.

Cell Culture, Nitric Oxide Production in Primary Mouse Macrophages, SDS-PAGE and Western Blot Analyses, and Northern Blot Analyses. These procedures have been described previously (9, 10).

Plasmids and Transfection Assays. Plasmids have all been reported previously as follows: FLAG-tagged Smad2, Smad3, and Smad4, ALK5, and

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[3] The abbreviations used are: TGF, transforming growth factor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CDDO-Im, 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole; iNOS, inducible nitric oxide synthase; co-Smad, common partner Smad; PAI, plasminogen activator inhibitor; TNF, tumor necrosis factor; Luc, luciferase; β-gal, β-galactosidase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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Smad7 were reported in Refs. 23–25; (CAGA)12-Luc was reported in Ref. 17; and BMP response element-Luc was reported in Ref. 26. cDNAs for p300, CREB-binding protein, and p/CAF were kindly provided by Tony Kouzarides (University of Cambridge, Cambridge, UK). For transient transfection assays, Mv1Lu cells were maintained in DMEM containing nonessential amino acids and 10% fetal bovine serum. Cells were transiently transfected with a total of 350 ng of DNA, using FuGene 6, in serum-free media for 6 h. This was then replaced with fresh medium (0.1% BSA/DMEM) with test compounds. Twenty-four h later, cells were washed with PBS and lysed by 100 μl of 1× reporter lysis buffer (Promega, Madison, WI). Luc activity was measured and normalized to β-gal activity.

PAI-1 Luc Assay. Mink lung epithelial cells (clone 32), stably transfected with the human PAI-1 gene fused to the firefly Luc reporter gene, were generously provided by Dan Rifkin (New York University, NY). Conditions for the transfection and assay have been published previously (27).

RESULTS

CDDO and CDDO-Im Are Potent Inhibitors of the Expression of iNOS in Primary Mouse Macrophages. A classical overlap between triterpenoids and TGF-β is their suppression of the induction of the inflammatory mediator iNOS (9, 28). When primary macrophages are treated with both TNF-α and IFN-γ for 16 h, these two cytokines synergize strongly to induce iNOS, as shown in Fig. 1. Using this system, we have measured the ability of CDDO and CDDO-Im to inhibit the *de novo* expression of iNOS protein. The final product of iNOS, nitric oxide, was also measured by the Griess reaction. When macrophages were treated simultaneously with triterpenoids, TNF-α, and IFN-γ, accumulation of iNOS and nitric oxide was significantly reduced by nanomolar concentrations of either CDDO or CDDO-Im (Fig. 1*A*). CDDO-Im (IC_{50}, 10 nM) is markedly more potent than CDDO (IC_{50}, 100 nM) in this assay, which uses two cytokines (both at 10 ng/ml) to induce iNOS. If IFN-γ alone (50 ng/ml) is used as inducer, the assay is even more sensitive to inhibition, and IC_{50}s are even lower (Fig. 1*A*; see also Ref. 12). Furthermore, as shown in Fig. 1*B*, an even lower dose of CDDO-Im (1 nM) can synergize strongly with TGF-β1 (100 pg/ml) to suppress the induction of iNOS protein; at these low doses, both CDDO-Im and TGF-β1, individually, are relatively weak suppressors of the combined activity of TNF-α and IFN-γ.

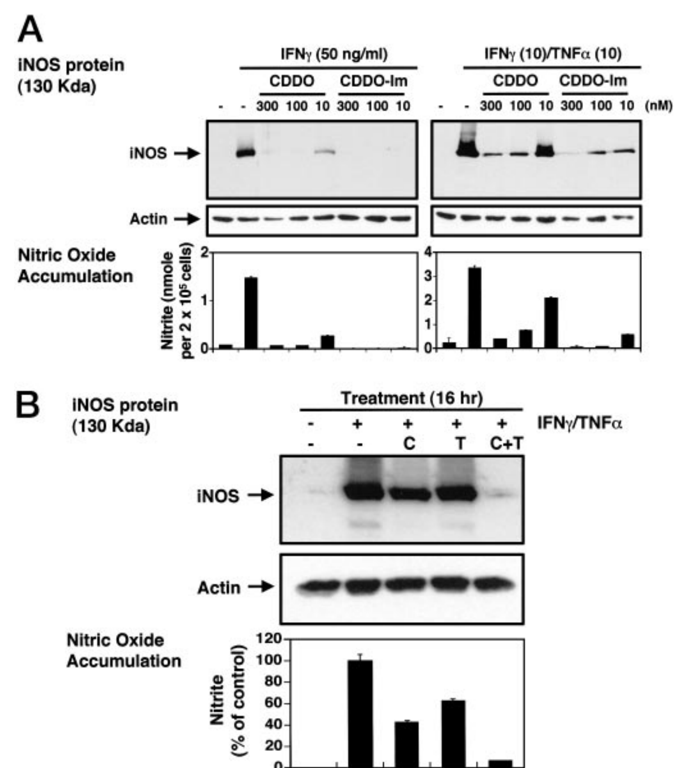

Fig. 1. CDDO and CDDO-Im inhibit the induction of iNOS protein in primary mouse macrophages. Cells were incubated with compounds together with IFN-γ and/or TNF-α as indicated for 16 h. *A*, total proteins were isolated and electrophoresed. Western blots for iNOS are shown. The accumulation of nitric oxide in the supernatant was measured by Griess reaction with sodium nitrite as a standard. *B*, low-dose combinations of CDDO-Im (*C*; 1 nM) and TGF-β1 (*T*; 100 pg/ml) suppress the induction of iNOS protein completely when primary macrophages were stimulated with IFN-γ and TNF-α (10 ng/ml each) for 16 h. *Error bars* show SDs.

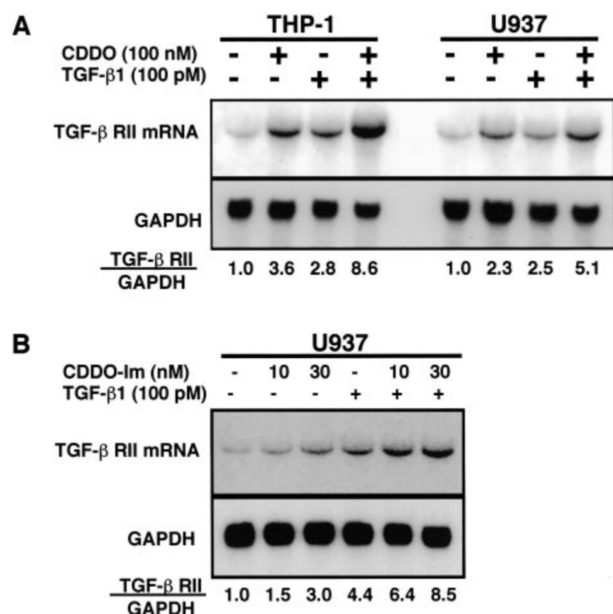

Fig. 2. *A*, CDDO and TGF-β1 synergize induction of mRNA for type II TGF-β receptor in THP-1 and U937 promonocytic leukemia. Cells were incubated for 2 days with CDDO (100 nM) alone or in combination with TGF-β1 (100 pM). Total RNAs were isolated and electrophoresed. Northern blots for TGF-β type II receptor (*TGF-β RII*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) are shown. *B*, same as *A*, except CDDO-Im was used.

CDDO and TGF-β1 Synergize to Induce mRNA for Type II TGF-β Receptor in Leukemia Cells. We have previously reported that CDDO and TGF-β1 synergize to induce monocytic differentiation of leukemia cells (9). For further study of these synergistic effects, we first investigated whether the combination of CDDO and TGF-β1 increased expression of mRNA for TGF-β receptors, as well as for TGF-β itself. Promonocytic human leukemia cells (THP-1 and U937) were incubated for 2 days with CDDO (100 nM) alone or in combination with TGF-β1 (100 pM). As shown in Fig. 2*A,* induction of expression of mRNA for the TGF-β type II receptor by CDDO in both cell lines was significantly enhanced by cotreatment with TGF-β. At even lower doses (10 and 30 nM), CDDO-Im induced the TGF-β type II receptor, again synergistically with TGF-β1 (Fig. 2*B*). Levels of mRNA for TGF-β1, as well as for the type I receptor, did not change with treatment with triterpenoids (data not shown).

CDDO and CDDO-Im Induce PAI-1 Promoter Activity in Mink Lung Epithelial Cells. To determine the effects of CDDO and CDDO-Im on expression of yet another gene that responds to TGF-β, we used a cell line stably transfected with the PAI-1 promoter linked to Luc (27). The activity of PAI-1 is tightly regulated at the transcriptional level, and TGF-β is a major regulator of PAI-1 expression (17, 29). We treated Mv1Lu cells with either CDDO or CDDO-Im for 24 h and then determined PAI-1 Luc activity. As shown in Fig. 3*A,* CDDO and CDDO-Im (300 nM each) induced PAI-1 Luc activity 2- and 8-fold, respectively. Furthermore, CDDO-Im (200 nM) synergized strongly with TGF-β1 (20 pg/ml) to increase this activity (Fig. 3*B*).

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Activation of Smad2 by TGF-β Is Prolonged by CDDO in Leukemia Cells. Because TGF-β signal transduction is mediated in part by Smad proteins, it was important to evaluate effects of CDDO on Smad signaling. Receptor-regulated Smads, such as Smad2 or Smad3, are phosphorylated by the TGF-β type I receptor kinase domain (23, 30–32). These activated receptor-regulated Smads can form a heteromeric complex with the co-Smad, Smad4, which is then translocated into the nucleus to induce transcriptional activation (23, 33). In our first experiments on Smad signaling, we treated U937 cells with TGF-β and/or CDDO for either 20 min or 2 h before harvest. Activation of Smad2 was measured with an antibody to phospho-Smad2; this reagent recognizes phosphorylated sites at serines 465 and 467, which are direct targets of the TGF-β type I receptor kinase. As shown in Fig. 4, within 20 min of treatment, TGF-β1 (200 pM) induced phosphorylation of Smad2, but at this time, CDDO (either 0.2 or 1.0 μM) had no effect, either alone or in combination with TGF-β, on levels of phospho-Smad2. However, within 2 h after treating the cells with TGF-β1 alone, the level of activated Smad2 had declined markedly (compared with the level seen at 20 min); in contrast, the combination of CDDO and TGF-β markedly prolonged the phosphorylation of Smad2. The amount of total Smad2 did not change in the cells.

CDDO-Im and CDDO Enhance Smad Signaling and Reverse Inhibitory Effects of Smad7 in Mink Lung Epithelial Cells. Next, we studied functional effects of CDDO-Im and CDDO on transacti-

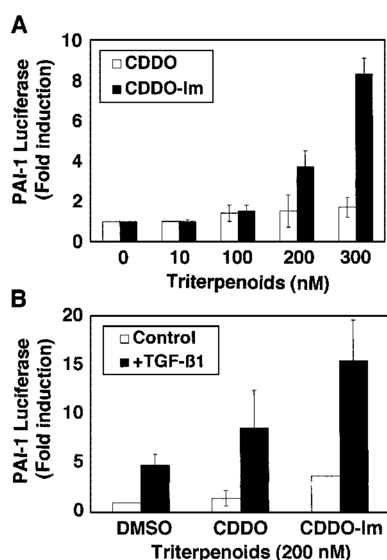

Fig. 3. Triterpenoids induce PAI-1 promoter activity in mink lung epithelial cells stably transfected with the PAI-1 promoter linked to Luc and grown in 10% fetal bovine serum/DMEM. Cells were plated in 0.1% BSA/DMEM in 24- or 96-well plates and treated for 24 h with triterpenoids with or without TGF-β1 (20 pg/ml). Luc activity was measured and normalized to the amount of total protein. *Error bars* show SDs.

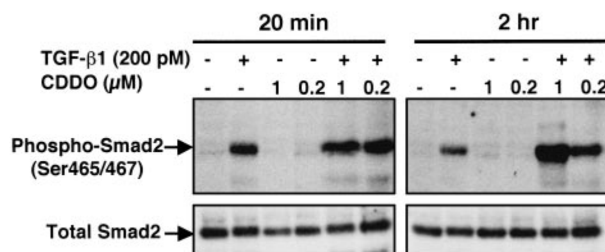

Fig. 4. Activation of Smad2 by TGF-β is prolonged by CDDO in U937 cells. Cells were treated with TGF-β and/or CDDO for 20 min or 2 h before harvest. Total proteins were obtained and subjected to electrophoresis. Gels were transferred to a membrane and probed with antibody to Smad2 or phospho-Smad2.

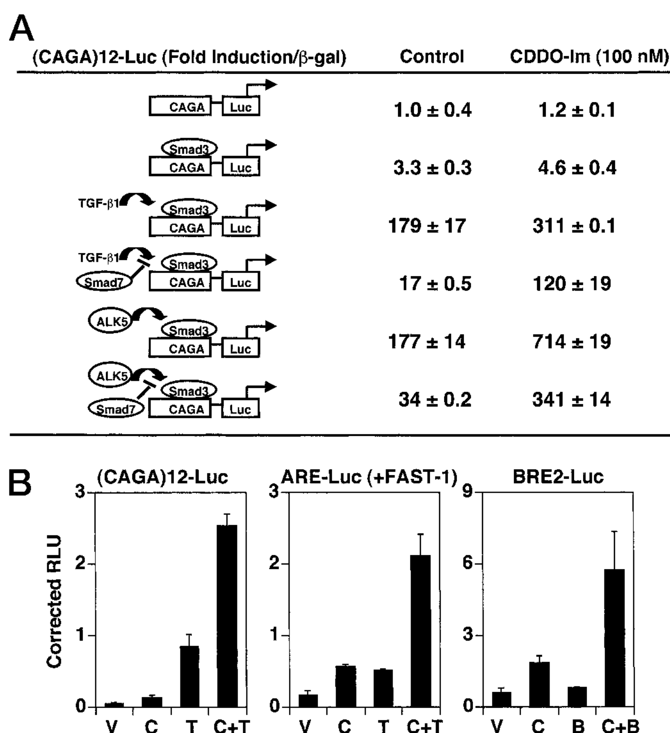

Fig. 5. *A,* CDDO-Im enhances Smad signaling and reverses the inhibitory effects of Smad7 in mink lung epithelial cells (Mv1Lu). All cells were transfected with (CAGA)12-Luc (20 ng) and pCMV-β-gal (20 ng). Some cells were also transfected with Smad3 (100 ng), Smad7 (100 ng), or ALK5 (20 ng) for a total of 350 ng DNA/well, in 24-well plates. Six h later, cells were treated with control buffer, TGF-β1 (10 pg/ml), and/or CDDO-Im (100 nM) and incubated for an additional 24 h before harvest and Luc assay. Fold induction of (CAGA)12-Luc activity was normalized to β-gal. Shown here is one representative experiment; similar data have been obtained at least three times. SDs are shown. *B,* CDDO-Im enhances all three TGF-β-related pathways: (CAGA)12-Luc (pathway induced by TGF-β and activin); ARE-Luc (pathway induced by activin and TGF-β); and BRE-Luc (pathway induced by BMP2). Each response was further enhanced by the addition of CDDO-Im (100 nM). Mv1Lu cells were treated with compounds for 24 h before harvesting for Luc assay [*V,* vector control; *C,* CDDO-Im (100 nM); *T,* TGF-β1 (1 ng/ml); *B,* BMP2 (100 ng/ml)]. *Error bars* show SDs.

vation of a Smad-dependent reporter gene construct, using transfection assays with a Smad binding element linked to Luc. This construct [SBE-Luc, (CAGA)12-Luc] contains multiple CAGA sites and is specific for response to TGF-β and activins, compared with BMPs (17). Mv1Lu cells were transiently transfected with (CAGA)12-Luc and pCMV-β-gal. To determine the roles of different Smads, including receptor-regulated Smads, co-Smads, and inhibitory Smads, we used multiple combinations of their individual DNA expression vectors in separate experimental treatments. In the experiments shown in Fig. 5*A*, cells were transfected with various combinations of Smad3, Smad7, as well as the constitutively active type I TGF-β receptor, ALK5. Cells were then treated with either control buffer, TGF-β1 (10 pg/ml), and/or CDDO-Im (100 nM) and incubated further before assay for Luc activity. CDDO-Im had no significant effect on (CAGA)12-Luc in the absence of Smads. When the Smad3 expression vector was cotransfected with (CAGA)12-Luc, basal activation of the reporter was enhanced 3-fold, although CDDO-Im did not significantly increase this further. However, when cells in which CAGA-Luc activity had been stimulated either by adding exogenous TGF-β1 or by cotransfection with ALK5, we found a marked further stimulatory effect of CDDO-Im. In these experiments, transfection with Smad3, followed by treatment with TGF-β1 or transfection with ALK5, caused almost identical levels of increased activity of the (CAGA)12 reporter (179- and 177-fold over control, respectively), in the absence of any triterpenoid. In these situations, in which TGF-β signaling was

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already activated, CDDO-Im then further enhanced the (CAGA)12-Luc response in a striking manner, up to 700-fold over control. When an inhibitory Smad, Smad7, was cotransfected into this system, (CAGA)12-Luc activation induced by either TGF-β or ALK5 was markedly decreased. Most notably, CDDO-Im was able to reverse the effects of Smad7 in both of the above cases, restoring (CAGA)12-Luc response (shown in Fig. 5*A*). Results similar to those obtained with CDDO-Im have also been obtained with CDDO, but the potency of CDDO is significantly weaker in these transfection assays.

CDDO-Im Enhances Reporter Activity for Activins and BMPs. The TGF-β superfamily consists of more than 40 members, including TGF-βs, activins, and numerous BMPs. Because we found remarkable synergy of CDDO and CDDO-Im with TGF-β itself, we wished to know whether these synergistic effects were specific for TGF-β alone, or whether other members of the superfamily can synergize with triterpenoids. We used three different response elements, namely, (CAGA)12-Luc (specific for TGF-β and activins but does not respond to BMPs), activin response element-Luc (responds to activins and TGF-β but not to BMPs), and BMP response element-Luc (specific for BMPs), to evaluate the activity of CDDO-Im (17, 26). After transfection with vectors bearing the respective response elements, Mv1Lu cells were treated with TGF-β1 or BMP2, and/or CDDO-Im. As shown in Fig. 5*B*, each response was greatly enhanced by the addition of CDDO-Im; stimulation was 64-fold over control on the (CAGA)12 response, 13-fold over control on the ARE (+FAST-1) response, and 10-fold over control on the BRE response. The response of the BRE reporter, on an absolute basis, is even greater than the (CAGA)12-Luc response. Taken together, these data suggest that the ability of CDDO-Im to amplify response of TGF-β superfamily members is not restricted to TGF-β itself because BRE-Luc does not respond to TGF-β or Smad3.

CDDO Induces Coactivator p/CAF mRNA Expression in Leukemia Cells. Transcriptional activation can be regulated at multiple levels, such as *de novo* synthesis of a transcription factor, translocation of the transcription factor from cytosol to nucleus, or posttranslational modification. In the case of Smad-mediated signaling, both changes in localization and phosphorylation play a role in their ability to transactivate downstream genes during TGF-β treatment (7, 34, 35). Because TGF-β activates Smad2 and Smad3, and this in turn regulates interaction with coactivators, such as p300/CBP or p/CAF (19–22), we have investigated the effects of CDDO on such nuclear coactivators. First, we have investigated whether CDDO might increase the expression of coactivators because they are limiting factors for transcriptional activation of many genes (36–39). Therefore, we have looked at the levels of p300, CBP, and p/CAF in U937 cells. It is interesting to note that phorbol ester (TPA), which we have found can potentiate the ability of oleanane triterpenoids to induce leukemia cell differentiation,[4] can induce p/CAF (40). As shown in Fig. 6*A*, CDDO (100 nM) induces p/CAF mRNA in U937 cells with maximum induction at 12–20 h, whereas TPA increases p/CAF mRNA at a different time (6–12 h). The expression levels of both p300 and CBP were not significantly increased by CDDO (data not shown). Interestingly, we have found that CDDO-Im (10 nM) synergizes strongly with TGF-β1 (100 pM) to induce p/CAF in these cells (Fig. 6*B*).

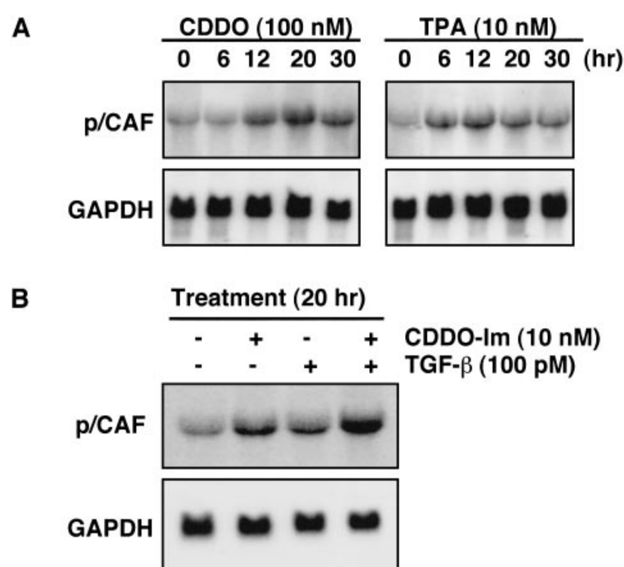

Fig. 6. CDDO induces p/CAF mRNA expression in U937 cells. *A*, CDDO (100 nM) induces p/CAF mRNA with maximum induction at 12–20 h, whereas maximal induction with TPA occurs at 6–12 h. *B*, CDDO-Im (10 nM) synergizes strongly with TGF-β1 (100 pM) to induce p/CAF mRNA. Total RNA was harvested and subjected to Northern blot analysis.

DISCUSSION

We have previously shown that triterpenoids inhibit the growth of many cancer cells, inhibit expression of inflammatory mediators, such as iNOS and cyclooxygenase 2, and induce apoptosis (9, 10, 13, 41–43). Because triterpenoids synergize with TGF-β in many ways, we have explored the effects of triterpenoids on TGF-β/Smad signaling. In combination with TGF-β, CDDO and CDDO-Im not only increase the expression of TGF-β-dependent genes, such as those for PAI-1 and the type II TGF-β receptor, but these triterpenoids also markedly enhance Smad signaling. This is the first report to indicate that the multifunctional action of synthetic oleanane triterpenoids is directly linked to the TGF-β pathway.

We have also shown that CDDO and CDDO-Im prolong the activation of a receptor-activated Smad, such as Smad2, and markedly enhance the ability of Smad3 to activate a Smad binding element (CAGA-Luc), which is known to be a specific determinant for gene transcription mediated by TGF-β. Most interestingly, CDDO and CDDO-Im also reverse inhibitory effects of Smad7, which is a key regulator of TGF-β signaling (3, 24, 44). Indeed, recent reports have even implicated Smad7 as an important regulator of TGF-β activity in human disease because aberrant expression of Smad7 may contribute to inflammatory bowel disease and scleroderma (1, 3, 45). Because expression of Smad7 can be induced by inflammatory mediators such as lipopolysaccharide, interleukins, IFN-γ, and TNF-α (46, 47), Smad7 can act as a mediator to regulate some of the effects of TGF-β on the inflammatory process (48, 49). When we examined the level of expression of Smad7 mRNA, we found that this was not altered by CDDO or CDDO-Im in cells that were stimulated with either IFN-γ, TNF-α, or TGF-β (data not shown). Therefore, we next looked at the effect of CDDO and CDDO-Im on an important functional aspect of Smad7, namely, inhibition of the capacity of TGF-β to activate gene transcription, as measured in the CAGA-Luc reporter assay. In this case, we found strong ability of the triterpenoids to reverse the inhibitory action of Smad7.

Our demonstration that triterpenoids are not selective for the TGF-β pathway but also enhance signaling by other members of the TGF-β superfamily, such as BMPs, strongly suggests that the effects of these compounds are unlikely to be targeted selectively to upstream components of the pathways but possibly result from more general effects such as stabilization of the Smad pathway or enhancement of their transcriptional activating activity. Although the specific Smads used by these other family members may be different from those used by TGF-β, their pathways share common intracellular/nuclear pro-

[4] N. Suh, unpublished observations.

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teins and similar mechanisms of regulation and degradation (3, 4, 35, 50). BRE-Luc, which responds to neither TGF-β nor Smad3 (26, 51) but responds to BMP-dependent activation of Smad1, Smad5, or Smad8, is activated by CDDO-Im to an even greater extent than are (CAGA)12-Luc or FAST1/ARE-Luc, which respond to Smad3/TGF-β and Smad2/activin/TGF-β, respectively.

Finally, another possible mechanism for synergy of CDDO and CDDO-Im with TGF-β might be their ability to potentiate the action of coactivators, such as p/CAF and/or CBP/p300. Many nuclear transcription factors require p300 and CBP for transcriptional activation (36–38). Because cellular concentrations of p300 and CBP are known to be limiting, nuclear transcription factors will compete for p300 and CBP (36, 39). Likewise, Smad3 also interacts strongly with p300/CBP and competes for this complex (19–21). Moreover, Smad3 also interacts with another coregulator, p/CAF, as a transcriptional activator (22). We have shown here that CDDO-Im induces expression of p/CAF, which, like CBP/p300, may also be limiting. Thus, increased expression of p/CAF may also contribute to the synergism between triterpenoids and Smad signaling.

It is already known that many steroids, as well as deltanoids (vitamin D analogues), retinoids, and phorbol esters, enhance the response to TGF-β by inducing the synthesis of TGF-β itself or its receptors (52–56). Furthermore, there is now an abundant literature on the interaction of steroids and their relatives with Smads (57–62). However, despite its triterpenoid structure, CDDO does not bind to any of the known nuclear steroid receptors or orphans,[5] other than to PPAR-γ (14), which is a relatively promiscuous receptor with an exceptionally large binding pocket. Other than induction of adipogenic differentiation in fibroblasts (14), none of the anti-inflammatory or antiproliferative effects of CDDO and CDDO-Im that we have described here appear to be mediated by PPAR-γ, particularly because we have seen such effects in cells that have been treated with an irreversible PPAR-γ antagonist or in cells that genetically are PPAR-γ null.[6]

In conclusion, CDDO and CDDO-Im have potent anti-inflammatory, antiproliferative, and differentiating activity in many different cell types, and these triterpenoids synergize with multifunctional cytokines of the TGF-β family to enhance Smad signaling. This enhancement of Smad signaling by triterpenoids is undoubtedly an important aspect of their antiproliferative, anti-inflammatory, and differentiating effects. The present study provides information for the first time on the mechanisms whereby triterpenoids interact with the TGF-β/Smad pathway. Although we do not yet know the immediate, proximate molecular target for the binding of either CDDO or CDDO-Im (or one of their metabolites), it is clear that the pathway we have reported here will be of importance in explaining the overall mechanism of action of these intriguing new molecules. Hopefully, these studies of mechanism can further their optimal use for prevention and therapy of disease. Although we have emphasized the potentially beneficial effects of TGF-β/Smad signaling, it is important to recognize that there may also be detrimental effects that result from the ability of this pathway to enhance fibrosis and angiogenesis as well as suppress immune function (2, 4, 63–66).

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[5] D. Mangelsdorf and R. Heyman, unpublished results.
[6] A. Place and N. Suh, unpublished results.

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