A Synthetic Triterpenoid, 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid (CDDO), Is a Ligand for the Peroxisome Proliferator-Activated Receptor γ


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A novel synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), previously reported to have potent differentiating, antiproliferative, and antiinflammatory activities, has been identified as a ligand for the peroxisome proliferator-activated receptor γ (PPARγ). CDDO induces adipocytic differentiation in 3T3-L1 cells, although it is not as potent as the full agonist of PPARγ, rosiglitazone. Binding studies of CDDO to PPARγ using a scintillation proximity assay give a Ki between 10^-2 to 10^-7 M. In transactivation assays, CDDO is a partial agonist for PPARγ. The methyl ester of CDDO, CDDO-Me, binds to PPARγ with similar affinity, but is an antagonist. Like other PPARγ ligands, CDDO synergizes with a retinoid X receptor (RXR)-specific ligand to induce 3T3-L1 differentiation, while CDDO-Me is an antagonist in this assay. The partial agonism of CDDO and the antagonism of CDDO-Me reflect the differences in their capacity to recruit or displace cofactors of transcriptional regulation; CDDO and rosiglitazone both release the nuclear receptor corepressor, NCoR, from PPARγ, while CDDO-Me does not. The differences between CDDO and rosiglitazone as either partial or full agonists, respectively, are seen in the weaker ability of CDDO to recruit the coactivator CREB-binding protein, CBP, to PPARγ. Our results establish the triterpenoid CDDO as a member of a new class of PPARγ ligands. (Molecular Endocrinology 14: 1550–1556, 2000)

INTRODUCTION

Triterpenoids are a large family of structures synthesized in plants through the cyclization of squalene and have been used in traditional Asian medicine for centuries (1). Naturally occurring triterpenoids like oleanolic acid (OA) and ursolic acid (UA) are known to have relatively weak antiinflammatory and anticarcinogenic activities (2, 3). To increase their usefulness, we have synthesized a series of novel derivatives of OA and UA and have shown that some derivatives of OA are much more potent than the parent compound in suppressing the induction of the enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (4–6). The most active of these synthetic derivatives, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) (Fig. 1), is not only antiinflammatory, but also has potent antiproliferative and differentiating activities (7, 8). One of the effects of CDDO on differentiation can be easily measured by its ability to convert 3T3-L1 fibroblasts into mature adipocytes (8). These fibroblasts un-
dergo dramatic morphological and biochemical changes upon induction of differentiation and accumulate triglyceride (9). The classic inducers for this process have been a combination of 1-methyl-3-isobutyl xanthine, dexamethasone, and insulin (MDI), although more recently, ligands for the peroxisome proliferator-activated receptor γ (PPARγ) such as the thiazolidinedione, rosiglitazone, have also been identified as potent inducers of adipogenic differentiation (10–12).

PPARγ is a member of the nuclear receptor superfamily of transcription factors. It forms heterodimers with the retinoid X receptor (RXR) to activate gene transcription (13–15). This cooperation is reflected in the ability of PPARγ and RXR ligands to synergize in the induction of adipocyte differentiation (16). Furthermore, binding of ligands to nuclear receptors such as PPARγ results in the recruitment or displacement of different cofactors that either enhance or suppress transcription (17). In particular, the binding of an agonist to nuclear receptors results in the recruitment of coactivators such as NCoA/SRC-1 (nuclear receptor coactivator/steroid receptor coactivator-1) and p300/CBP (CREB binding protein) and leads to activation of transcription (18, 19). In contrast, corepressors such as NCoR (nuclear receptor corepressor) or SMRT (silencing mediator for retinoid and thyroid hormone receptors) can suppress transcription by binding to receptors either in the absence of their ligands or when an antagonist is bound (20, 21).

Here we demonstrate that the adipogenic effect of CDDO is due to its binding to PPARγ. It not only induces differentiation as a single agent, but also acts synergistically with an RXR-specific ligand. Binding and transactivation studies indicate that CDDO is a partial agonist for PPARγ. We also report that the C-28 methyl ester of CDDO, CDDO-Me (6, 7), is a PPARγ antagonist, and that these opposite activities of CDDO and CDDO-Me can be explained by their differential effects on the interactions of cofactors with PPARγ.

RESULTS

CDDO Induces Differentiation in 3T3-L1 Cells

To induce adipocytic differentiation, 3T3-L1 fibroblasts were treated with MDI mix (Fig. 2B), or rosiglitazone.
tazone at 100 nM (Fig. 2E) or 1 μM (Fig. 2F) for 2 days. Accumulation of triglyceride droplets was evident on the sixth day, as shown by positive staining with Oil Red O. Treatment with CDDO (100 nM), however, induced differentiation more slowly and less effectively (Fig. 2C); the percentage of differentiated cells was approximately 30% by day 6 (Fig. 2C) and peaked at 50% by day 8 (not shown). Interestingly, unlike rosiglitazone at 1 μM (Fig. 2F), a higher dose of CDDO (1 μM) was not effective (Fig. 2D), even when evaluated at day 10. In fact, this higher dose was inhibitory to differentiation induced by MDI or rosiglitazone (data not shown).

To quantify the degree of differentiation, the enzyme glycerol-3-phosphate dehydrogenase (GPDH), a key enzyme in triglyceride synthesis, was used as a marker (22). GPDH activity correlated well with visual detection of triglyceride droplets under the light microscope. Cells treated with CDDO were assayed for GPDH activity on day 8 while those treated by rosiglitazone or MDI were assayed on day 6, as shown in Fig. 3A, which confirms that CDDO is a weaker inducer than MDI or rosiglitazone, and that it has no adipogenic activity at 1 μM. The C-28 methyl ester of CDDO, CDDO-Me, did not induce differentiation in 3T3-L1 cells at all concentrations tested on day 8 (Fig. 3B). Furthermore, it acted in a dose-dependent manner as an antagonist and inhibited differentiation induced by 100 nM rosiglitazone (Fig. 3B). Even though CDDO also inhibits differentiation at 1 μM, at concentrations where it acted as a differentiating agent (100 nM or lower), it did not inhibit differentiation induced by rosiglitazone and its activity was additive to that of the full agonist (data not shown).

**CDDO Binds to and Transactivates PPARγ**

The adipogenic effect of CDDO suggested that it might be a ligand for PPARγ. Therefore, binding studies were performed using a scintillation proximity assay (SPA), which has been successfully used in the study of PPARs and their ligands (23). Using this assay, CDDO and rosiglitazone were shown to compete for bound 3H-CDDO, with K<sub>i</sub> values of 310 nM and 50 nM, respectively (Fig. 4). Importantly, the presence of dithiothreitol (DTT) in the binding buffer interfered with CDDO binding to PPARγ. We repeated these experiments using 3H-rosiglitazone as the ligand and non-radioactive CDDO or CDDO-Me as competitors. Again, the presence of DTT blocked the ability of either CDDO or CDDO-Me to compete for binding to PPARγ; the K<sub>i</sub> values in this assay were determined to be 12 nM for CDDO and 130 nM for CDDO-Me (Fig. 5 and Table 1). Both triterpenoids were also tested for binding to PPARα, either in the presence or absence of DTT, and neither binds to PPARα (Table 1).

To determine whether bound CDDO can transactivate PPARγ, a Gal4-PPARγ chimeric protein was used...
to drive the expression of secreted placental alkaline phosphatase (SPAP) linked to the DNA binding sequence of Gal4. Figure 6A shows that CDDO transactivates Gal4-PPARγ in a dose-dependent manner, although the maximal level of transactivation achieved by CDDO was only 26% of that obtained with rosiglitazone (1 μM). We also tested the ability of CDDO to transactivate the wild-type PPARγ receptor in the context of a natural PPARγ response element (PPRE) derived from the acyl-CoA oxidase gene promoter. In both panels A and B, reporter activities, normalized against β-gal, are expressed in reference to that found for 1 μM rosiglitazone (100%).

**Table 1.** Ki Values for CDDO and CDDO-Me Competing for Binding to PPARα or PPARγ, Using 3H-GW2331 (35) and 3H-Rosiglitazone as Ligands, Respectively

<table>
<thead>
<tr>
<th>Compounds</th>
<th>hPPARα (Ki, nM)</th>
<th>hPPARγ (Ki, nM)</th>
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<tr>
<td></td>
<td>+ DTT</td>
<td>− DTT</td>
</tr>
<tr>
<td>CDDO</td>
<td>3,000–100,000</td>
<td>7,600</td>
</tr>
<tr>
<td>CDDO-Me</td>
<td>1,500–21,000</td>
<td>12,000</td>
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The assays were performed in the absence or presence of 10 mM DTT.

CDDO Synergizes with an RXR-Specific Ligand to Induce 3T3-L1 Differentiation

The above data demonstrate that CDDO is a partial agonist for PPARγ. Since PPARγ is known to heterodimerize with RXR and activate transcription (13, 26), we determined if CDDO would synergize with the RXR-specific ligand LG100268 (27). Figure 7 shows that although LG100268 alone at 1 μM induced only slight differentiation in 3T3-L1 cells, it greatly potentiates the activity of CDDO. In contrast, not only did CDDO-Me fail to synergize with LG100268 to induce differentiation, it inhibited the differentiation induced by the RXR ligand (Fig. 7). Unlike the GPDH assays for CDDO in Fig. 3A, this experiment was performed on day 6 to minimize the differnetiating effect of CDDO and maximize the level of synergism between CDDO and LG100268.

CDDO and CDDO-Me Differentially Recruit Cofactors to PPARγ

To further explore the mechanisms of action of CDDO and CDDO-Me, a mammalian two-hybrid system was used to examine the ability of CDDO or CDDO-Me to recruit the coactivator, CBP, to PPARγ or to release the corepressor, NCoR, from it; rosiglitazone is known to have both of these activities (28). Figure 8A shows that rosiglitazone recruits CBP to PPARγ in a dose-dependent manner, as expressed by the level of expression of the reporter gene chloramphenicol acetyltransferase (CAT) normalized against β-gal activity.

Fig. 7. CDDO Synergizes with LG100268 to Induce 3T3-L1 Differentiation

3T3-L1 cells were differentiated and GPDH activity was assayed as described in Materials and Methods. Shown are GPDH activities of cells differentiated with CDDO or CDDO-Me (0.01 μM or 0.1 μM), in the absence or presence of 1 μM LG100268, assayed on day 6 (different from those obtained for CDDO in Fig. 3A, which was done on day 8).
CDDO also recruits CBP to PPARγ in a dose-dependent manner, but much less so than rosiglitazone. CDDO-Me is also a weaker recruiter of CBP in the concentrations tested. A maximum of 0.3 µM CDDO-Me was used since 1 µM CDDO-Me was toxic to the COS-1 cells used in the transfection assay. We then tested the ability of PPARγ, when bound with CDDO and CDDO-Me, to interact with the corepressor NCoR. Unlike coactivators, the two-hybrid system indicates that NCoR interacts with PPARγ in the absence of ligands (28). When rosiglitazone was added, however, NCoR was released from PPARγ in a dose-dependent manner (Fig. 8B), leading to a decrease in CAT reporter expression. Interestingly, CDDO, although only a partial agonist, was equally capable of releasing NCoR from PPARγ (Fig. 8B). CDDO-Me, which does not transactivate PPARγ, did not lead to a dissociation of the corepressor (Fig. 8B).

DISCUSSION

Previous studies have shown that CDDO is a multifunctional agent, with marked antiinflammatory, antiproliferative, and differentiating activities, as shown by studies in a wide variety of cells (8). It is therefore important to understand the mechanisms of action of this molecule. Although the present studies do little to elucidate the antiinflammatory and antiproliferative activities of CDDO, they do provide the first data that explain some of its ability to control cell differentiation, at least in the context of the conversion of 3T3-L1 fibroblasts to adipocytes. We have shown that CDDO is an effective agent for adipogenic conversion of 3T3-L1 fibroblasts, although it is less active than a prototypical PPARγ ligand such as rosiglitazone.

Binding competition assays, using labeled CDDO or rosiglitazone, indicate that CDDO is a ligand for PPARγ, and that this binding could transactivate both the Gal4-PPARγ chimeric and wild-type receptor. The functional interaction of CDDO with PPARγ has been further confirmed by the ability of CDDO to synergize with a ligand specific for RXR; RXR and PPARγ are known to form functional heterodimers (13). Further studies on cofactor interactions are consistent with the observation that CDDO is a partial agonist for PPARγ and that its methyl ester is an antagonist.

Two interesting observations in this study warrant further discussion. One is the biphasic dose response of CDDO in the induction of 3T3-L1 differentiation. At 1 µM, CDDO not only failed to induce differentiation (Fig. 3A), but it could also inhibit those induced by all other known inducers tested, including MDI, rosiglitazone, or RXR-specific ligands (data not shown); the mechanism of this inhibition is unknown. However, based on our studies of CDDO in different biological systems (8), CDDO was shown to be a multifunctional molecule and could be interacting with cellular targets other than PPARγ to inhibit the differentiation process. This characteristic is not unique to CDDO. Recent studies of another well known PPARγ ligand, 15-deoxy-A12,14-PGJ2 (15d-PGJ2), indicate the presence of other cellular targets, namely components of the nuclear factor-κB (NF-κB pathway), for this prostaglandin (29, 30). The antiinflammatory activities of 15d-PGJ2, in terms of its ability to suppress reporter expression driven by NF-κB or AP-1 elements, have been shown to be dependent on PPARγ (30).

The second observation is the different binding conditions CDDO and rosiglitazone require in the in vitro binding studies. Unlike the results obtained with rosiglitazone, the presence of DTT interfered with the binding of CDDO to PPARγ. Due to the presence of an α,β-unsaturated carbonyl function in the A-ring of CDDO, we searched for direct adduct formation between CDDO and DTT but found none. Although we could demonstrate no covalent bond formation between CDDO and DTT, it is still possible that a reversible noncovalent interaction exists. Again, this sensitivity to DTT is not unique to CDDO. 15d-PGJ2 has also been shown to be sensitive to thiol groups found in DTT or cysteine (29, 30), although there is no convincing chemical evidence to support the notion that a covalent adduct is found between 15d-PGJ2 and these agents.

The molecular coordinates of the interaction of CDDO with PPARγ remain to be determined. It would appear that a free –COOH group at C-28 is important for agonistic activity in the 3T3-L1 cells, since the methyl ester of CDDO acts as an antagonist in this system. Thus, in 3T3-L1 cells, we have shown that CDDO-Me can block the differentiating effects of rosiglitazone and the RXR-specific ligand, LG100268, as well as those of CDDO itself (data not shown). Although CDDO-Me binds to PPARγ, it does not transactivate the receptor, which may be the result of its
failure to cause release of a co-repressor such as NCO-R. Given the fact that CDDO is also an inhibitor of differentiation at 1 μM, the mechanisms of the inhibitory actions of CDDO-Me at the same concentration could be attributed to either a direct antagonism of PPARγ, other mechanisms independent of this receptor, or both. It is also important to note that at concentrations higher than 1 μM, CDDO-Me becomes toxic to many cells and thus should not be used at those doses to attribute the activities to the antagonism of PPARγ.

Although the results we described here provide a reasonable explanation for the differentiating effects of CDDO on 3T3-L1 cells, they do not account for other notable activities of CDDO, particularly its ability to suppress the expression of the enzyme iNOS in macrophages. Neither do they explain the ability of CDDO to act as a potent antiproliferative agent on a wide variety of tumor cells or to induce differentiation in leukemia cells. Thus, we have found that while CDDO can suppress iNOS expression in macrophages at doses below 1 nM, a number of PPARγ ligands, including rosiglitazone and 15d-PGJ₂, are inactive in this assay at concentrations below 1 μM (our unpublished data and Refs. 31 and 32). Furthermore, unlike CDDO, thiazolidinediones such as rosiglitazone do not induce differentiation in leukemia cells (our unpublished data). Given the diverse biological activities of CDDO in these systems, we are therefore left with the conclusion that it is likely that another functional receptor system (or systems) beyond PPARγ remain to be identified for CDDO, if we wish to understand the mechanism of action of this agent in cells other than 3T3-L1. The identification of PPARγ as a receptor for CDDO represents the first important step in our understanding of the actions of CDDO, but it is only a beginning in this intriguing problem.

MATERIALS AND METHODS

Reagents and Plasmids

The synthesis of CDDO and its methyl ester have been described previously (7). 3H-CDDO (6 Ci/mmol) was prepared by isobutyl xanthine, dexamethasone, and 0.35 mM insulin were used for 2 days. Cells were then cultured in DMEM/10% FBS/insulin for the rest of the differentiation process. All other treatments are for day 0 to day 2 only, and medium was changed every 2 days. For Oil Red O staining, cells were fixed in 10% formaldehyde for 1 h and stained with Oil Red O for 2 h. The nuclei were counterstained with hematoxylin and photographed. Lysis buffer for GPDH analysis includes 50 mM Tris, pH 8, 100 mM NaCl, 0.5% NP-40, 1 mM DTT and was supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin and aprotinin. GPDH enzyme activity was measured as the consumption of 0.2 mM NADH at 340 nm using 0.2 mM DHAP as the substrate (22).

Transfection Assays

For Gal4-PPARγ transactivation studies, CV-1 cells were transfected as described previously (28). Wild-type PPARγ transfections were performed in HeLa cells using Lipofectamine Plus (Life Technologies, Inc., Gaithersburg, MD) according to manufacturer’s instructions. Percentage of transactivation was normalized against 1 μM rosiglitazone. For mammalian two-hybrid assays, COS-1 cells in 24-well plates were transfected using Lipofectamine Plus. Twenty nanograms of CMX-β-gal, 60 ng pGS-CAT, 60 ng VP16-PPARγ2, and 60 ng Gal4-cofactors were used for each well. Ligands were added 4 h after transfection; CAT and β-gal activities were measured 40 h later.

SPA Binding Assays

The details of SPA assays have been published elsewhere (34). In brief, human PPARγ ligand-binding domain was expressed in Escherichia coli as a polyhistidine-tagged fusion protein. The protein was purified, biotinylated, and immobilized on streptavidin-modified SPA beads. DTT was washed away and binding assays were performed in 50 mM HEPES, pH 7, 50 mM KCl, 5 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate (CHAPS), and 0.1 mg/ml BSA. When DTT was used, its concentration was 10 mM.

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