The novel oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and the C-28 methyl ester (CDDO-Me) induce apoptosis of human tumor cells by disruption of redox balance and are currently in clinical trials. The present studies show that CDDO and CDDO-Me block tumor necrosis factor α-induced targeting of NF-κB p65 to the nucleus. CDDO-Me also blocked tumor necrosis factor α-induced phosphorylation of IκBα. In concert with these results, we found that CDDO-Me inhibits IκBα kinase β (IKKβ) activity in cells. In support of a direct mechanism, CDDO-Me inhibited recombinant IKKβ activity in vitro. The results also demonstrate that (i) CDDO and CDDO-Me form adducts with IKKβ, but not IKKβ with mutation of Cys-179 to Ala, and (ii) CDDO-Me inhibits IKKβ by a mechanism dependent on oxidation of Cys-179. These findings indicate that CDDO and CDDO-Me directly block IKKβ activity and thereby the NF-κB pathway by interacting with Cys-179 in the IKKβ activation loop.

The synthetic triterpenoid CDDO induces differentiation of human myeloid leukemia cells and mouse 3T3-Li fibroblasts (1). CDDO also inhibits cytokine-mediated induction of nitric oxide synthase and functions as a ligand for peroxisome proliferator-activated receptor γ (1, 2). Other studies have demonstrated that CDDO and its derivatives at the C-28 position induce apoptosis of human myeloid leukemia (3–7), osteosarcoma (8), multiple myeloma (9), lung cancer (10, 11), breast cancer (12, 13), and pancreatic cancer (14) cells. CDDO, the C-28 methyl ester (CDDO-Me), and the C-28 imidazolide ester induce apoptosis by increasing reactive oxygen species and decreasing intracellular glutathione (6, 9, 14, 15). How the CDDO triterpenoids disrupt redox balance is not known. However, the A-ring of these triterpenoids contains an α,β-unsaturated carbonyl moiety that can form reversible adducts with reactive thiol groups in dithiothreitol (DTT) (16) or with specific cysteine-rich protein targets (17). These findings have indicated that the CDDO triterpenoids increase reactive oxygen species and induce apoptosis by oxidizing critical cysteines in proteins that regulate redox balance and survival.

NF-κB activates the transcription of diverse genes that regulate cell proliferation and survival (18). In the absence of stimulation, the NF-κB proteins (RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB1/p52) localize to the cytoplasm in complexes with members of the IκB family of inhibitor proteins (19). Phosphorylation of IκBα induces ubiquitination and degradation of IκBα and release of NF-κB p65 to the nucleus. In the classical NF-κB pathway, the IκB kinase β (IKKβ) in a complex with the regulatory IKKγ subunit is the major kinase responsible for phosphorylation of IκBα (20). Previous work indicated that CDDO inhibits activation of the NF-κB pathway by a mechanism after translocation of NF-κB to the nucleus (5). The present results demonstrate that CDDO and CDDO-Me block the NF-κB pathway by inhibiting IKKβ. The results also indicate that CDDO and CDDO-Me directly inhibit IKKβ by interacting with Cys-179 in the IKKβ activation loop.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human U-937 myeloid leukemia cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. 293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were treated with CDDO or CDDO-Me (provided by Reata Pharmaceuticals, Inc., human TNF-α (20 ng/ml; BD Biosciences), the proteasome inhibitor MG-132 (25 μM; Calbiochem), or DTT (300 μM; Sigma).

**Subcellular Fractionation**—Nuclear and cytosolic fractions were prepared as described (21).

**Immunoprecipitation and Immunoblot Analysis**—Lysates from subconfluent cells were prepared as described (22). Soluble proteins were incubated with anti-IKKβ (Cell Signaling Technology) or anti-FLAG (Sigma) and precipitated with protein A/G beads. Immune complexes or cell lysates were subjected to immunoblotting with anti-NF-κB p65 (Santa Cruz Biotechnology), anti-lamin B (Calbiochem), anti-IκBα (Santa Cruz Biotechnology), anti-α-tubulin (Santa Cruz Biotechnology), anti-phospho-IκBα (Cell Signaling Technology), anti-β-actin (Sigma), anti-IKKβ, anti-phospho-IKKβ (Cell Signaling Technology), anti-Bcl-2, anti-Bcl-xL (Santa Cruz Biotechnology), or anti-FLAG (Sigma). The immune complexes were detected with horseradish peroxidase-conjugated second antibodies and enhanced chemiluminescence (ECL; Amersham Biosciences).
CDDO-Me Inhibits IKKβ

Binding of CDDO-Me-biotin and CDDO-biotin to IKKβ—CDDO and CDDO-Me were biotinylated as described (23). For in vitro binding studies, (i) anti-FLAG precipitates from 293 cells expressing FLAG-IKKβ or FLAG-IKKβ(C179A) were incubated with 5 μM CDDO-biotin, and (ii) recombinant IKKβ or IKKβ(C179A) was incubated with 1 μM CDDO-Me-biotin or 5 μM CDDO-biotin. In vivo studies, 293 cells expressing FLAG-IKKβ or FLAG-IKKβ(C179A) were cultured with 5 μM CDDO-biotin. Lysates were then precipitated with anti-FLAG. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After washing, the membranes were incubated with streptavidin horseradish peroxidase (Amersham Biosciences) and developed with enhanced chemiluminescence (ECL; Amersham Biosciences).

RESULTS AND DISCUSSION

CDDO-Me Inhibits NF-κB p65 Activation by Blocking IκBα Phosphorylation—To assess the effects of CDDO-Me on regulation of the NF-κB pathway, we stimulated human U-937 myeloid leukemia cells with TNF-α to induce translocation of NF-κB p65 to the nucleus (Fig. 1A). Treatment of the TNF-α-stimulated cells with CDDO-Me was associated with a concentration-dependent decrease in nuclear translocation of p65 (Fig. 1A). Equal loading and purity of the nuclear lysates was confirmed by immunoblotting with antibodies against nuclear lamin B, cytosolic IκBα, and cytosolic α-tubulin. In concert with these findings, when the TNF-α-stimulated cells were treated with the parent compound, CDDO (data not shown), indicating that this effect is not selective for the methyl ester. NF-κB p65 is released from cytosolic IκBα and targeted to the nucleus in response to phosphorylation and ubiquitination of IκBα (24). To determine whether CDDO-Me affects IκBα phosphorylation, cytosolic lysates from TNF-α-stimulated cells were immunoblotted with anti-phospho-IκBα, anti-IκBα, and anti-β-actin. In concert with these results, CDDO and CDDO-Me also inhibited TNF-α-induced phosphorylation of IκBα (Fig. 1C). In concert with these results, CDDO and CDDO-Me act upstream to IκBα in the NF-κB pathway.

Luciferase Assays—Cells were transfected with pNF-κB-Luc (Stratagene) and SV-40-Renilla-Luc (Promega) in the presence of Lipofectamine 2000 (Invitrogen). After 24 h, lysates prepared in passive lysis buffer were analyzed using the dual luciferase assay kit (Promega).

IKK Kinase Assays—Anti-IKKβ precipitates or recombinant His-IKKβ (Upstate Cell Signaling Solutions) were incubated in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, 0.1 mM sodium vanadate, 10 μM ATP, and 1 mM DTT) with GST-IκBα and [γ-32P]ATP (PerkinElmer Life Sciences) for 30 min at 30 °C. DTT was omitted from the reactions where indicated. The reaction products were analyzed by SDS-PAGE and autoradiography.

Generation of IKKβ(C179A) Mutant—Mutation of IKKβ Cys-179 to Ala was generated by site-directed mutagenesis (Stratagene) using pGEX-IKKβ as the template and confirmed by DNA sequencing. IKKβ and IKKβ(C179A) were purified after cleavage with thrombin to remove the GST moiety.

FIGURE 1. CDDO-Me inhibits NF-κB activation by attenuating IκBα phosphorylation. A, U-937 cells were pretreated with 0.25, 0.5, or 1.0 μM CDDO-Me for 6 h and then stimulated with TNF-α for 15 min. Nuclear lysates were immunoblotted with anti-NF-κB p65 and, as controls for equal loading and purity, with antibodies against nuclear lamin B, cytosolic IκBα, and cytosolic α-tubulin. B, cells were treated with 1.0 μM CDDO-Me for 2 h, and then TNF-α was added for the indicated times. Whole cell lysates were immunoblotted with antibodies against IκBα, Bcl-2, and Bcl-2, and β-actin. C, cells were treated with 0.25, 0.5, or 1.0 μM CDDO-Me for 6 h and with 25 μM MG-132 for 1 h to inhibit the proteasome. The cells were then stimulated with TNF-α for 15 min. Cytosolic lysates were immunoblotted with anti-phospho-IκBα, anti-IκBα, and anti-β-actin.
CDDO-Me Inhibits IKKβ

**FIGURE 2. CDDO-Me inhibits IKKβ kinase activity.** A and B, cells were pretreated with 0.25 and 0.5 μM CDDO-Me for 6 h and then stimulated with TNF-α for 15 min. A, anti-IKKβ precipitates were incubated in kinase reactions with GST-IκBα and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. The precipitates were also immunoblotted with anti-IKKβ. B, lysates were immunoblotted with anti-phospho-IKKβ-Ser-181 and anti-IKKβ. C, anti-IKKβ precipitates from control and TNF-α-treated cells were incubated with 0.25 and 0.5 μM CDDO-Me in kinase buffer (without and with 1 mM DTT) containing GST-IκBα and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. The precipitates were also immunoblotted with anti-IKKβ. D, kinase-active His-IKKβ was incubated with 0.25 and 0.5 μM CDDO-Me for 10 min at 30 °C. IKKβ activity was then assayed in kinase buffer without (left) and with 1 mM DTT (right) containing GST-IκBα and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. Equal loading of His-IKKβ and GST-IκBα was determined by immunoblotting.

**FIGURE 3. CDDO-Me inhibits IKKβ by modification of Cys-179.** A, 293 cells were transfected with FLAG-IKKβ or FLAG-IKKβ(C179A). At 48 h after transfection, the cells were treated with 0.25 and 0.5 μM CDDO-Me for 6 h. Anti-FLAG precipitates were incubated in kinase reactions containing GST-IκBα and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. The precipitates were also immunoblotted with anti-IKKβ. B, 293 cells were transfected with FLAG-IKKβ or FLAG-IKKβ(C179A). At 48 h after transfection, lysates were immunoprecipitated with anti-FLAG. The precipitates were incubated with 0.25 and 0.5 μM CDDO-Me in kinase buffer (no DTT) containing GST-IκBα and [γ-32P]ATP. The reaction products and precipitates were analyzed as described in panel A. C, 293 cells were cotransfected with pH-κB-Luc, SV-40-Renilla-Luc, and FLAG-IKKβ or FLAG-IKKβ(C179A). At 24 h after transfection, cells were treated with 0.25 and 0.5 μM CDDO-Me for 6 h and then assayed for luciferase activity. The results are expressed as the fold activation (mean ± S.D. of three separate experiments) relative to that obtained with the FLAG-IKKβ control (lane 1, assigned a value of 1).

**CDDO-Me Directly Inhibits IKKβ**—The IKKβ kinase function is necessary and sufficient for phosphorylation of IκBα (25). To determine whether CDDO-Me inhibits IKKβ activity, anti-IKKβ immunoprecipitates were prepared from cells pretreated with CDDO-Me and then stimulated with TNF-α. Incubation of the precipitates in kinase reactions with GST-IκBα and [γ-32P]ATP demonstrated that CDDO-Me treatment is associated with inhibition of IKKβ activity (Fig. 2A). Consistent with these results, CDDO-Me inhibited TNF-α-induced autophosphorylation of IKKβ on Ser-181 (Fig. 2B). To determine whether CDDO-Me inhibits IKKβ activity in vitro, anti-IKKβ precipitates from TNF-α-stimulated cells were incubated with GST-IκBα in the absence and presence of CDDO-Me. The results show that IKKβ activity is also inhibited by CDDO-Me in vitro (Fig. 2C, left). Notably, addition of DTT to the kinase reactions blocked CDDO-Me-mediated inhibition of IKKβ activity (Fig. 2C, right). In this regard, DTT contains thiol groups that form reversible adducts with the CDDO α,β-unsaturated carbonyl moiety (16). To determine whether the effects of CDDO-Me are direct, we preincubated recombinant kinase-active His-IKKβ with CDDO-Me and then assayed for phosphorylation of GST-IκBα. His-IKKβ activity was inhibited by CDDO-Me (Fig. 2D, left). By contrast, the inhibitory effect of CDDO-Me was blocked in the presence of DTT (Fig. 2D, right). Taken together with the finding that DTT abolishes CDDO-Me-mediated inhibition of IKKβ, these results indicate that CDDO-Me directly inhibits IKKβ activity.

**CDDO-Me Inhibition of IKKβ Is Reversed by Mutation of Cys-179**—IKKβ contains a cysteine at position 179 in its activation loop. To determine whether this cysteine is involved in inhibition by CDDO-Me, we transfected 293 cells to express wild-type FLAG-IKKβ or
with inhibition of wild-type IKKβ (Fig. 3A). By contrast, CDDO-Me had no apparent effect on IKKβ(C179A) activity (Fig. 3A). In concert with these results, CDDO-Me also had little effect on IKKβ(C179A) activity when added directly to in vitro kinase assays (Fig. 3B). Moreover, CDDO-Me-induced inhibition of NF-κB-mediated transcription was substantially attenuated in cells expressing IKKβ(C179A) as compared with that obtained with wild-type IKKβ (Fig. 3C). These findings indicate that CDDO-Me inhibits IKKβ by reacting with Cys-179.

CDDO-Me Inhibits IKKβ by Oxidizing Cys-179—CDDO forms reversible adducts with DTT and cysteine-rich protein targets (16, 17). To determine whether CDDO-Me interacts directly with IKKβ in vitro, recombinant IKKβ was incubated with CDDO-Me conjugated to biotin (CDDO-Me-biotin). Analysis of the reaction products demonstrated the formation of IKKβ-CDDO adducts (Fig. 4A). By contrast, the interaction was substantially blocked when recombinant IKKβ(C179A) was incubated with CDDO-Me-biotin (Fig. 4A). Unlabeled CDDO-Me competed with CDDO-Me-biotin for binding to IKKβ, indicating that the interaction with IKKβ is not due to the biotin moiety (Fig. 4B, left). In addition, unlabeled CDDO-Me and CDDO-Me-biotin were similarly effective in inhibiting IKKβ (Fig. 4B, right). Previous studies of certain direct chemical inhibitors of IKKβ have demonstrated the induction of IKKβ dimerization (26, 27). Immunoblot analysis of lysates from cells expressing FLAG-IKKβ demonstrated that CDDO-Me treatment is associated with the induction of a higher molecular mass species that reacts with anti-IKKβ (Fig. 4C). The absence of this high molecular mass species in cells expressing FLAG-IKKβ(C179A) (Fig. 4C) suggests that CDDO-Me may induce the formation of IKKβ dimers by a mechanism dependent on the interaction with Cys-179. Cells were also pretreated with DTT to block the interaction between CDDO-Me and IKKβ. The results demonstrate that DTT reverses CDDO-Me-induced inhibition of 1κBα phosphorylation.

FIGURE 4. CDDO-Me inhibits IKKβ by oxidizing Cys-179. A, recombinant IKKβ or IKKβ(C179A) was incubated with 1 μM CDDO-Me-biotin for 1 h. The reaction products were analyzed by SDS-PAGE, transfer of proteins to a nitrocellulose membrane, and detection with streptavidin horseradish peroxidase. B, left, recombinant IKKβ was incubated with 1 μM CDDO-Me-biotin and 0 (—), 1 (+), or 5 (++) μM unlabeled CDDO-Me. The reaction products were analyzed as described in panel A. Right, kinase-active His-IKKβ was incubated with 1 μM CDDO-Me or 1 μM CDDO-Me-biotin for 10 min at 30 °C. IKKβ activity was then assayed in kinase buffer (without DTT) containing GST-1κBα and [γ-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. Equal loading of His-IKKβ and GST-1κBα was determined by immunoblotting. C, 293 cells expressing IKKβ or IKKβ(C179A) were treated with CDDO-Me for 6 h. Lysates were immunoblotted with anti-IKKβ and anti-β-actin. The asterisk (*) identifies the position of the higher molecular mass species that reacts with anti-IKKβ. D and E, U-937 cells were pretreated with 300 μM DTT for 1 h and/or 1 μM CDDO-Me for an additional 6 h before stimulation with TNF-α for 15 min. Cytosolic lysates (D) were immunoblotted with anti-phospho-1κBα, anti-1κBα, and anti-β-actin. Nuclear lysates (E) were immunoblotted with the indicated antibodies.

FLAG-IKKβ with a C179A mutation. Analysis of anti-FLAG precipitates for phosphorylation of GST-1κBα demonstrated similar levels of activity for FLAG-IKKβ and FLAG-IKKβ(C179A) (Fig. 3A). CDDO-Me treatment was associated
CDDO-Me Inhibits IKKβ

A. In Vitro

Flag-IKKβ

WT C179A

kDa

115

82

CDDO-Biotin

Adducts

Streptavidin Peroxidase

IP: anti-Flag

IB: anti-Flag

B. 293 Cells

Flag-IKKβ

WT C179A

kDa

115

82

CDDO-Biotin

Adducts

IP: anti-Flag

IB: anti-Flag

C. Recombinant-IKKβ

WT C179A

kDa

115

82

CDDO-Biotin

Adducts

Streptavidin Peroxidase

IP: anti-Flag

IB: anti-IKKβ

D. TNF-α

CD20/CDDO-Me

IKKβ

Cys-179

Degradation

NF-κB p65

Nuclear Targeting

FIGURE 5. CDDO also interacts directly with IKKβ. A, anti-FLAG precipitates from 293 cells expressing FLAG-IKKβ or FLAG-IKKβ(C179A) were incubated with 5 μM CDDO-biotin for 1 h. The reaction products were analyzed by SDS-PAGE, transfer of proteins to a nitrocellulose membrane, and detection with streptavidin horseradish peroxidase. B, 293 cells expressing FLAG-IKKβ or FLAG-IKKβ(C179A) were cultured with 5 μM CDDO-biotin for 6 h. Anti-FLAG precipitates were analyzed by SDS-PAGE, transfer to a nitrocellulose membrane, and detection with streptavidin horseradish peroxidase. C, recombinant IKKβ or IKKβ(C179A) was incubated with 5 μM CDDO-biotin for 1 h. The reaction products were analyzed by SDS-PAGE, transfer of proteins to a nitrocellulose membrane, and detection with streptavidin horseradish peroxidase. D, schema depicting CDDO/CDDO-Me inhibition of IKKβ and the NF-κB pathway.

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