

The Novel Triterpenoid CDDO and its Derivatives Induce Apoptosis by Disruption of Intracellular Redox Balance¹

Takashi Ikeda, Michael Sporn, Tadashi Honda, Gordon W. Gribble, and Donald Kufe²

Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 [T. I., D. K.], and Department of Pharmacology, Dartmouth Medical School [M. S.] and Department of Chemistry [T. H., G. W. G.], Dartmouth College, Hanover, New Hampshire 03755

ABSTRACT

The novel oleanane triterpenoid 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO) induces apoptosis of human leukemia cells by activation of the extrinsic caspase-8 pathway. The mechanisms responsible for the proapoptotic effects of CDDO are unknown. The present studies demonstrate that CDDO activates the c-Jun NH₂-terminal kinase and p38 mitogen-activated protein kinase in U-937 leukemia cells. The results also show that CDDO activates stress kinases by increasing levels of reactive oxygen species and decreasing intracellular glutathione (GSH) concentrations. Similar findings were obtained with the C-28 methyl ester (CDDO-Me) and C-28 imidazolide ester (CDDO-Im) derivatives. The results also demonstrate that CDDO-induced: (a) stimulation of Jun NH₂-terminal kinase; (b) activation of caspase-8; (c) loss of mitochondrial transmembrane potential; (d) release of cytochrome *c*; and (e) cleavage of caspase-3 are blocked by pretreatment with the antioxidant N-acetyl-L-cysteine and GSH but not with cysteine. In concert with these results, CDDO-induced apoptosis is also abrogated by N-acetyl-L-cysteine and GSH. These findings demonstrate that CDDO and its derivatives disrupt intracellular redox balance and thereby induce apoptosis.

INTRODUCTION

The synthetic CDDO³ induces differentiation of human myeloid leukemia cells and mouse 3T3-L1 fibroblasts and enhances nerve growth factor-induced neuronal differentiation of rat PC12 cells (1). CDDO inhibits the proliferation of human myeloid leukemia and carcinoma cell lines (1). CDDO also inhibits cytokine-mediated induction of nitric oxide synthase and cyclooxygenase-2 in macrophages, microglia, and fibroblasts (1). Other studies have demonstrated that CDDO induces apoptosis of human myeloid leukemia (2–4), osteosarcoma (5), lung cancer (6), and CLL (7) cells. The mechanism(s) responsible for the multiple effects of CDDO are unclear. CDDO functions as a ligand for the PPAR γ , whereas esterification of the C-28 COOH function with a methyl group (CDDO-Me) results in an antagonist of PPAR γ (8). The finding that both CDDO and CDDO-Me induce apoptosis of human tumor cells has thus indicated that PPAR γ activation is not responsible for this response.

Apoptosis is induced by intrinsic and extrinsic pathways that activate the caspase family of cysteine proteases. The intrinsic apoptotic pathway is activated by release of mitochondrial cytochrome *c* into the cytosol (9–11). Cytochrome *c* forms a complex with Apaf-1 and thereby activates caspase-9 (12, 13). In turn, caspase-9 cleaves and activates caspase-3 (12). In the extrinsic apoptotic pathway, stimula-

tion of the TNF family of death receptors results in the activation of caspase-8 (14, 15). Caspase-8 can directly activate caspase-3 (16). Caspase-8 also cleaves Bid, a proapoptotic member of the Bcl-2 family, and thereby amplifies the apoptotic response by inducing the release of cytochrome *c* (17, 18). Many anticancer drugs induce apoptosis by activation of the intrinsic pathway (19, 20). Moreover, resistance to cytotoxic agents used in the treatment of cancer is often associated with defects in the intrinsic pathway (21, 22). The available evidence indicates that CDDO induces apoptosis, at least in large part, by activation of the extrinsic caspase-8 pathway (2, 4, 5, 7). The mechanistic basis for this CDDO-induced response is, however, unknown.

The present studies demonstrate that CDDO disrupts intracellular oxidant levels. The results also demonstrate that CDDO-induced redox-mediated signals are responsible for activation of caspase-8 and caspase-3, loss of mitochondrial transmembrane potential, and induction of apoptosis.

MATERIALS AND METHODS

Cell Culture. Human U-937 myeloid leukemia cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. U-937/Bcl-x_L (21) and U-937/CrmA (23) were maintained in medium containing 500 μ g/ml geneticin sulfate and then grown in the absence of this agent for 24 h before each experiment. Cells (3×10^5) were treated with 5 μ M CDDO, 0.5 μ M CDDO-Me, or 2 μ M CDDO-Im (IC₅₀s for each agent). In certain experiments, cells were preincubated with 15 mM NAC (Sigma, St. Louis, MO) for 2 h, 15 mM GSH (Sigma) for 2 h, 1 mM cysteine (oxidized cysteine; Sigma) for 2 h, 5 μ g/ml oligomycin (Sigma) for 15 min, or 20 μ M SP600125 (JNK1 inhibitor; Biomol, Plymouth Meeting, PA) for 15 min.

Analysis of JNK Activation. Immunoprecipitates with anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA) were assayed for phosphorylation of GST-c-Jun (2–100) as described (24).

Immunoblot Analysis. Cell lysates and cytoplasmic fractions were prepared as described (24). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with anti-JNK1 (Santa Cruz Biotechnology), anti-phospho-p38 (pTpY; Biosource, Camarillo, CA), anti-caspase-8 (BD Biosciences PharMingen, San Diego, CA), anti-caspase-3 (Santa Cruz Biotechnology), anti-cytochrome *c* (Santa Cruz Biotechnology), anti-Bid (kindly provided by Dr. S. Korsmeyer), or anti- β -actin (Santa Cruz Biotechnology).

Measurement of ROS Levels. Cells were incubated with 10 μ M c-H₂DCFDA (Molecular Probes, Eugene, OR) for 30 min at 37°C to assess ROS-mediated oxidation to the fluorescent compound c-H₂DCF (25). Fluorescence of oxidized c-H₂DCF was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ).

Determination of GSH Levels. To assess GSH levels by flow cytometry, cells were incubated with 200 μ M mBCI (Molecular Probes) for 30 min at 37°C and analyzed by flow cytometry as described (26). mBCI is a thiol-selective derivative of monobromobimane that is essentially nonfluorescent until conjugated with GSH (27, 28). Intracellular GSH concentrations were measured using the Bioxytech GSH-4000 kit (OXIS International, Portland, OR).

Analysis of Mitochondrial Transmembrane Potential. Cells were incubated with 0.5 nM DiOC₆[3] (Molecular Probes) for 30 min at 37°C and analyzed by flow cytometry as described (29).

Received 2/11/03; revised 5/14/03; accepted 5/23/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by Grants CA42802 and CA78814 from the National Cancer Institute and by the National Foundation for Cancer Research. M. B. S. is the Oscar M. Cohen Professor.

² To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Phone: (617) 632-3141; Fax: (617) 632-2934; E-mail: donald_kufe@dfci.harvard.edu.

³ The abbreviations used are: CDDO, 2-cyano-3, 12-dioxolean-1, 9-dien-28-oic acid; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; DiOC₆[3], 3,3-dihexyloxycarbocyanine iodide; mBCI, monochlorobimane; ROS, reactive oxygen species; GSH, glutathione; NAC, N-acetyl-L-cysteine; TNF, tumor necrosis factor; CLL, chronic lymphocytic leukemia; PPAR, peroxisome proliferator-activated receptor; c-H₂DCFDA, 5-(and 6-) COOH-2',7'-dichlorodihydrofluorescein diacetate.

Apoptosis Assays. Sub-G₁ DNA content was assessed by staining ethanol-fixed and citrate buffer-permeabilized cells with propidium iodide and monitoring by FACScan.

RESULTS

Activation of JNK by CDDO. The apoptotic response of cells to diverse types of stress is associated with JNK activation (20, 30). To determine whether CDDO activates JNK in U-937 cells, anti-JNK immunoprecipitates were analyzed for phosphorylation of GST-c-Jun (2–100). An increase in JNK activity was detectable at 30 min and maximal at 3 h of CDDO treatment (Fig. 1A). Similar findings were obtained when cells were treated with the CDDO derivatives, CDDO-Me or CDDO-Im (Ref. 31; Fig. 1A). To determine whether CDDO selectively activates JNK, lysates were subjected to immunoblotting with an anti-phospho-p38 mitogen-activated protein kinase antibody. An increase in p38 activation was detectable at 30 min and through 6 h of CDDO treatment (Fig. 1B). Activation of p38 was also found when cells were treated with CDDO-Me and CDDO-Im (Fig. 1B). These findings indicate that CDDO and its derivatives activate the JNK and p38 stress kinases.

CDDO Increases Intracellular ROS Levels. Although JNK is activated in the cellular response to oxidative stress (30, 32), we asked if CDDO treatment is associated with changes in intracellular ROS levels. Using oxidation of c-H₂DCFDA as a measure of ROS, the results demonstrate an increase in DCF fluorescence at 5 min of CDDO exposure (Fig. 2A). Additional increases in ROS levels were found at 1 h of CDDO treatment (Fig. 2A). Similar results were obtained with CDDO-Me, whereas more pronounced increases in ROS levels were obtained in cells treated with CDDO-Im for 1 h (Fig. 2A). For comparison, exposure to H₂O₂ was associated with a marked increase in ROS levels at 5 min that decreased at 1 h (Fig. 2A). To extend these findings, cells were pretreated with the antioxidant, NAC, for 2 h before exposure to CDDO. The results show that NAC

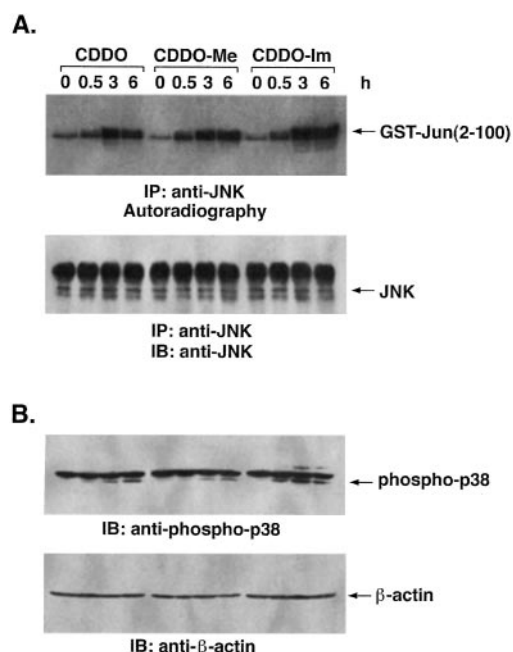


Fig. 1. CDDO induces JNK and p38 mitogen-activated protein kinase activation. U-937 cells were treated with CDDO, CDDO-Me, or CDDO-Im for the indicated times. A, anti-JNK immunoprecipitates were analyzed for phosphorylation of GST-c-Jun (2–100; top panel) and by immunoblotting with anti-JNK (bottom panel). Similar results were obtained in three independent experiments. In B, cell lysates were subjected to immunoblotting with anti-phospho-p38 (top panel) and anti-β-actin (bottom panel). The results are representative of three separate experiments.

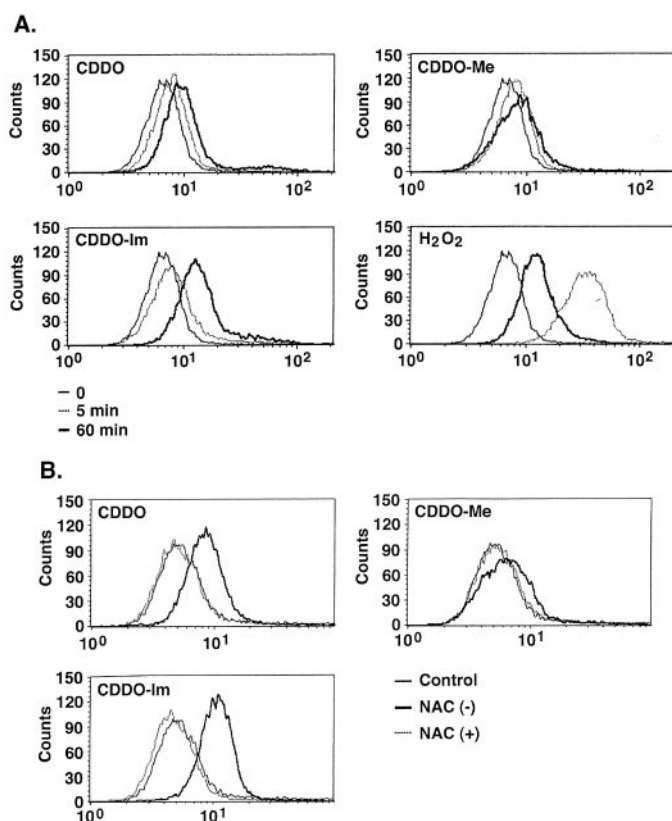


Fig. 2. CDDO increases intracellular ROS levels. In A, U-937 cells were treated with CDDO, CDDO-Me, or CDDO-Im for 5 and 60 min. Fluorescence of oxidized C-H₂DCF was determined by flow cytometry. The data are representative of four independent experiments. In B, cells were preincubated with NAC for 2 h and then treated with CDDO or one of the derivatives for 1 h. C-H₂DCF fluorescence was determined by flow cytometry. The results are representative of three independent experiments.

completely blocks CDDO-induced increases in ROS levels (Fig. 2B). NAC also blocked the pro-oxidant effects of CDDO-Me and CDDO-Im (Fig. 2B).

CDDO Decreases Intracellular GSH Levels. Alterations in cellular redox status, particularly intracellular GSH levels, have been associated with cell death (26, 33, 34). To assess the effects of CDDO on GSH levels, cells were incubated with mBCI and then analyzed by flow cytometry. The results show that treatment with CDDO for 1–6 h is associated with progressive decreases in GSH levels (Fig. 3A). More pronounced decreases in GSH levels were found in cells treated with CDDO-Me, whereas the effects of CDDO-Im were similar to those obtained with CDDO (Fig. 3A). In concert with these results, direct measurement of intracellular GSH concentrations demonstrated that exposure to CDDO and CDDO-Im results in a decrease of ~20% at 6 h (Fig. 3B). By contrast, CDDO-Me treatment for 6 h was associated with a more profound decrease of ~80% (Fig. 3B). Pretreatment of the cells with NAC inhibited the CDDO-induced decreases in GSH levels (Fig. 3C). Similar results were obtained when cells were pretreated with NAC and then exposed to CDDO-Me or CDDO-Im (Fig. 3C). These findings and those with c-H₂DCFDA demonstrate that CDDO both increases ROS and decreases GSH levels and that these effects vary with modifications at the C-28 position.

CDDO-induced Stress Kinase Activation Is Blocked by Antioxidants. To determine whether the pro-oxidant effects of CDDO are responsible for the induction of JNK activity, anti-JNK immunoprecipitates were studied from cells that were pretreated with NAC. The results show that NAC substantially blocks CDDO-induced JNK

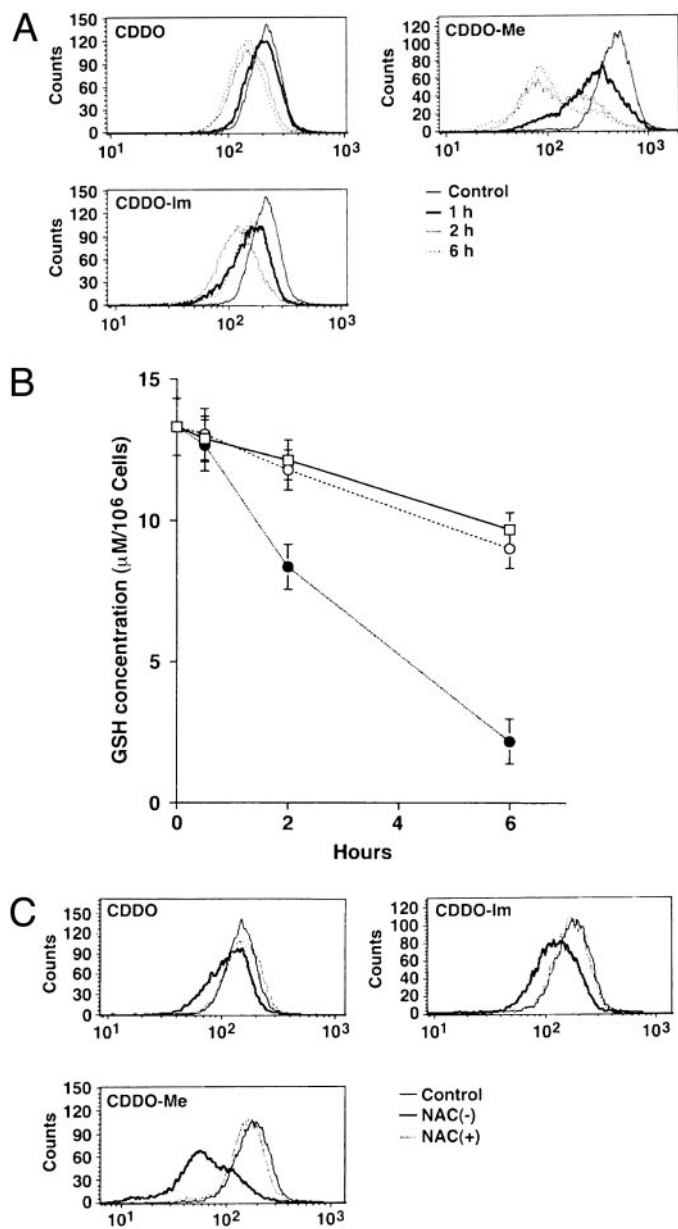


Fig. 3. CDDO decreases intracellular GSH levels. In A, GSH levels were analyzed by flow cytometry after incubation with CDDO, CDDO-Me, or CDDO-Im for the indicated times and staining with mBCI. The data are representative of four independent experiments. In B, intracellular GSH concentrations were determined after treatment with CDDO (□), CDDO-Me (●), or CDDO-Im (○) for the indicated times. The results represent the GSH concentration (mean ± SE) obtained from three independent experiments. In C, cells preincubated with NAC and then treated with CDDO, CDDO-Me, or CDDO-Im for 6 h were analyzed for GSH levels by flow cytometry. Similar results were obtained from three independent experiments.

activation (Fig. 4). As confirmation of these results, pretreatment of cells with GSH also blocked the induction of JNK activity by CDDO (Fig. 4). Similar findings were obtained when cells were pretreated with the antioxidants and then exposed to CDDO-Me or CDDO-Im (Fig. 4). NAC also blocked CDDO-induced activation of p38 (data not shown). These findings demonstrate that CDDO and its derivatives activate stress kinases by a redox-mediated mechanism.

CDDO-induced Caspase Activation Is Mediated by Oxidative Stress. CDDO treatment is associated with caspase-8 activation (2, 4, 5, 7). The present studies demonstrate that treatment of U-937 cells with CDDO-Me also induces caspase-8 cleavage (Fig. 5A). Compared with activation of caspase-8 at 6 h of CDDO treatment, cleavage of

caspase-8 was detectable at 3 h of CDDO-Me treatment (Fig. 5A). Similar results were obtained with CDDO-Im (Fig. 5A). Treatment with CDDO and its derivatives was associated with similar kinetics of caspase-3 activation (Fig. 5A). By contrast, cleavage of Bid was observed at 6–12 h in cells treated with CDDO or CDDO-Me and at 3 h after CDDO-Im exposure (Fig. 5A). To determine whether CDDO-induced caspase activation is related to the prooxidant effects, cells were pretreated with NAC or GSH. The results demonstrate that NAC completely blocks activation of caspase-8 by CDDO and the derivatives (Fig. 5B). GSH also blocked the activation of caspase-8 (Fig. 5B). The results further show that NAC and GSH block CDDO-induced activation of caspase-3 (Fig. 5B). In concert with a ROS-mediated mechanism, treatment with H_2O_2 was associated with caspase-8 and caspase-3 activation but to a somewhat lesser extent than that found with CDDO and its derivatives (Fig. 5C).

Prooxidant Effects of CDDO Induce Mitochondrial Dysfunction and Cytochrome *c* Release. Decreases in the mitochondrial transmembrane potential (ψ_m) are associated with release of cytochrome *c* and induction of apoptosis (35). CDDO-induced changes in ψ_m were analyzed by incubation of cells with DiOC₆[3] and monitoring fluorescence by flow cytometry. A decrease in ψ_m was detectable at 6 h of CDDO treatment and at 3 h for CDDO-Me and CDDO-Im (Fig. 6A). As shown for GSH levels, the effects of CDDO-Me on ψ_m were more pronounced than those found for CDDO and CDDO-Im (Fig. 6A). In concert with a prooxidant effect, the decreases in ψ_m induced by CDDO and its derivatives were blocked by pretreatment with NAC (Fig. 6B) and GSH (data not shown). The kinetics of CDDO-induced cytochrome *c* release were similar to decreases in ψ_m and blocked by NAC and GSH (Fig. 6C). NAC and GSH also blocked cytochrome *c* release in response to CDDO and its derivatives (Fig. 6D). By contrast, treatment of the cells with oligomycin, an inhibitor of the mitochondrial F_0F_1 ATPase (36), had no apparent effect on the decreases in ψ_m induced by CDDO and its derivatives (Fig. 6E and data not shown).

CDDO-induced Apoptosis Is Blocked by Antioxidants. As shown previously (2), treatment of U-937 cells with CDDO was associated with the induction of sub-G₁ DNA (Fig. 7A). Treatment with CDDO-Me and CDDO-Im also resulted in the induction of apoptosis (Fig. 7A). At 12 h, the percentage of apoptotic cells obtained with CDDO-Me was significantly higher than that found with CDDO and CDDO-Im (Fig. 7B). However, at 24 h, the induction of apoptosis was similar for all three agents (Fig. 7B). Importantly, pretreatment

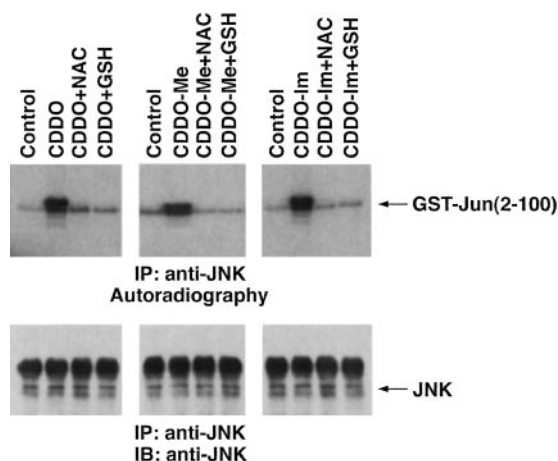


Fig. 4. Antioxidants block CDDO-induced JNK activation. Cells were pretreated with NAC or GSH for 2 h and then exposed to CDDO, CDDO-Me, or CDDO-Im for 3 h. Anti-JNK immunoprecipitates were analyzed for phosphorylation of GST-c-Jun (2–100; top panel) and by immunoblotting with anti-JNK (bottom panel).

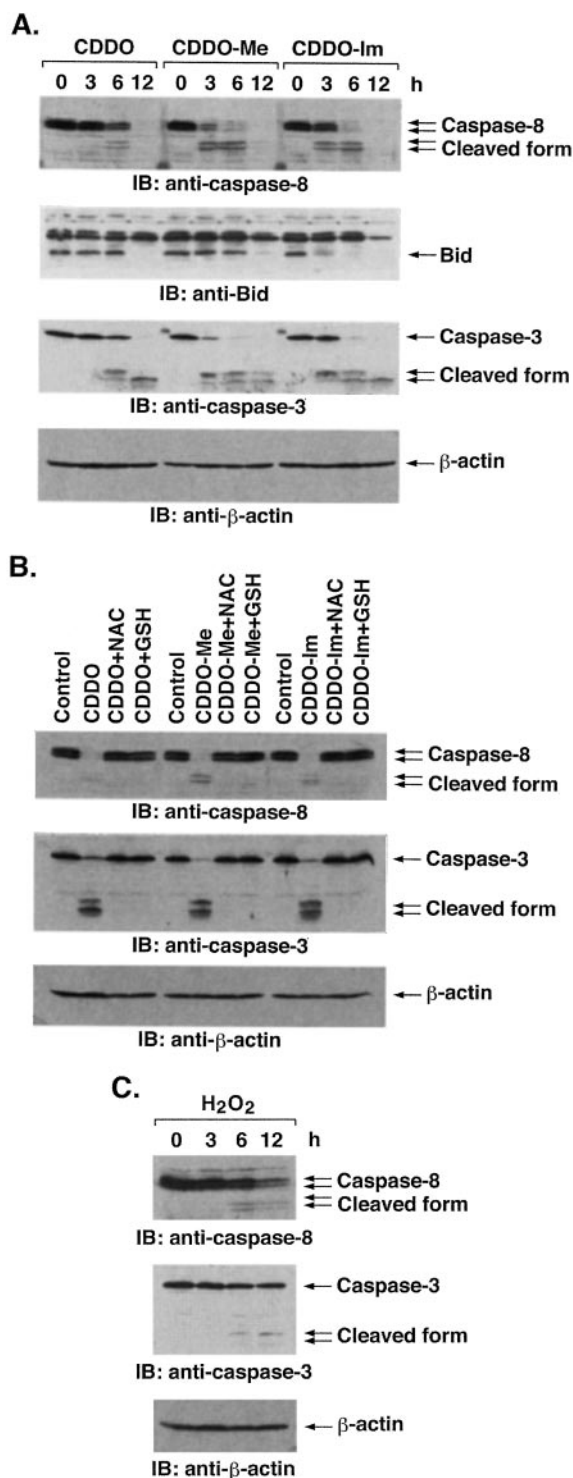


Fig. 5. Antioxidants inhibit CDDO-induced activation of caspase-8 and caspase-3. In A, cells were treated with CDDO, CDDO-Me, or CDDO-Im for the indicated times. In B, cells were pretreated with NAC or GSH and then exposed to CDDO, CDDO-Me, or CDDO-Im for 9 h. In C, cells were treated with 0.5 mM H_2O_2 for the indicated times. Cell lysates were subjected to immunoblot analysis with the indicated antibodies. The results are representative of three independent experiments.

with NAC or GSH blocked CDDO-induced apoptosis (Fig. 7C). As a control, pretreatment with cysteine (oxidized cysteine) had no effect (Fig. 7C). NAC and GSH, but not cysteine, were also effective in blocking CDDO-Me- and CDDO-Im-induced apoptosis (data not shown). Although JNK is activated in the oxidative stress response and JNK contributes to apoptosis, cells were preincubated with the

JNK1 inhibitor SP600125 and then treated with CDDO or its derivatives. As expected, SP600125 blocked CDDO-, CDDO-Me-, and CDDO-Im-induced JNK activation (Fig. 7D, left panel). SP600125 also partially (~30%) decreased apoptosis induced by CDDO, CDDO-Me, and CDDO-Im (Fig. 7D, right panel). These findings demonstrate that CDDO and its derivatives induce apoptosis by pro-oxidant-mediated mechanisms that include, at least in part, the JNK pathway.

CDDO-induced Loss of $r\psi m$ and Apoptosis Is Blocked by CrmA. CDDO-induced apoptosis is mediated in large part by activation of the extrinsic pathway (2, 4, 5, 7). To determine whether CDDO-Me and CDDO-Im induce apoptosis by similar mechanisms, we treated U-937 cells that overexpress Bcl- x_L (inhibitor of the intrinsic pathway) or CrmA (inhibitor of the extrinsic pathway). Treatment with CDDO or its derivatives had no effect on Bcl- x_L or CrmA expression (Fig. 8A). Overexpression of Bcl- x_L blocked CDDO-Me but not CDDO-Im-induced loss of $r\psi m$ (Fig. 8B). Conversely, CrmA inhibited CDDO-Im- but not CDDO-Me-induced decreases in $r\psi m$ (Fig. 8B). As shown previously (2, 4), CDDO-induced apoptosis was attenuated by CrmA and, to a lesser extent, by Bcl- x_L (Fig. 8C). CDDO-Im-induced apoptosis was also blocked by CrmA, whereas Bcl- x_L had little effect (Fig. 8C). Moreover, CDDO-Me-induced apoptosis was unaffected by CrmA (Fig. 8C). These findings demonstrate that CDDO-Me differs from CDDO and CDDO-Im by activating a CrmA-independent proapoptotic pathway.

DISCUSSION

CDDO Increases Intracellular ROS Levels. Previous work has shown that CDDO induces apoptosis of human myeloid leukemia (2, 3), osteosarcoma (5), lung cancer (6), and CLL (7) cells. CDDO is a multifunctional molecule that inhibits induction of nitric oxide synthase and cyclooxygenase-2 and functions as a ligand for PPAR γ (1, 8). The immediate molecular targets responsible for CDDO-induced apoptosis, however, are not known. The present results demonstrate that CDDO treatment is associated with increases in intracellular ROS levels. CDDO-induced increases in ROS were detectable at 5 min and continued to increase for 1 h of treatment. Modification of CDDO with a methyl or imidazole group at C-28 enhanced the potency of CDDO in increasing ROS levels. ROS levels are maintained by a balance between production and scavenging of reactive oxygen intermediates. Production of ROS can be increased by disruption of the mitochondrial respiratory chain or stimulation of oxidoreductases. Alternatively, increases in ROS levels can occur through inhibition of one of several enzymatic or nonenzymatic antioxidant defense systems (37). Additional studies will be needed to determine whether CDDO increases oxidant levels by stimulating ROS production and/or inhibiting their degradation.

CDDO Decreases Intracellular GSH Levels. The present studies also demonstrate that CDDO decreases intracellular GSH levels. In cells, GSH is maintained in the reduced form by GSH reductase and NADPH (38). GSH levels, however, decrease in response to oxidative stress. Depletion of GSH can be an early event that contributes to the induction of apoptosis (26, 39–41). Our results show that CDDO-Me is particularly effective in decreasing intracellular GSH levels as compared with CDDO and CDDO-Im. The finding that CDDO-Im is, conversely, more effective than CDDO and CDDO-Me in increasing intracellular ROS indicates that decreases in GSH concentrations are not solely responsible for the CDDO-induced changes in ROS levels. In addition, the induction of apoptosis is not solely attributable to

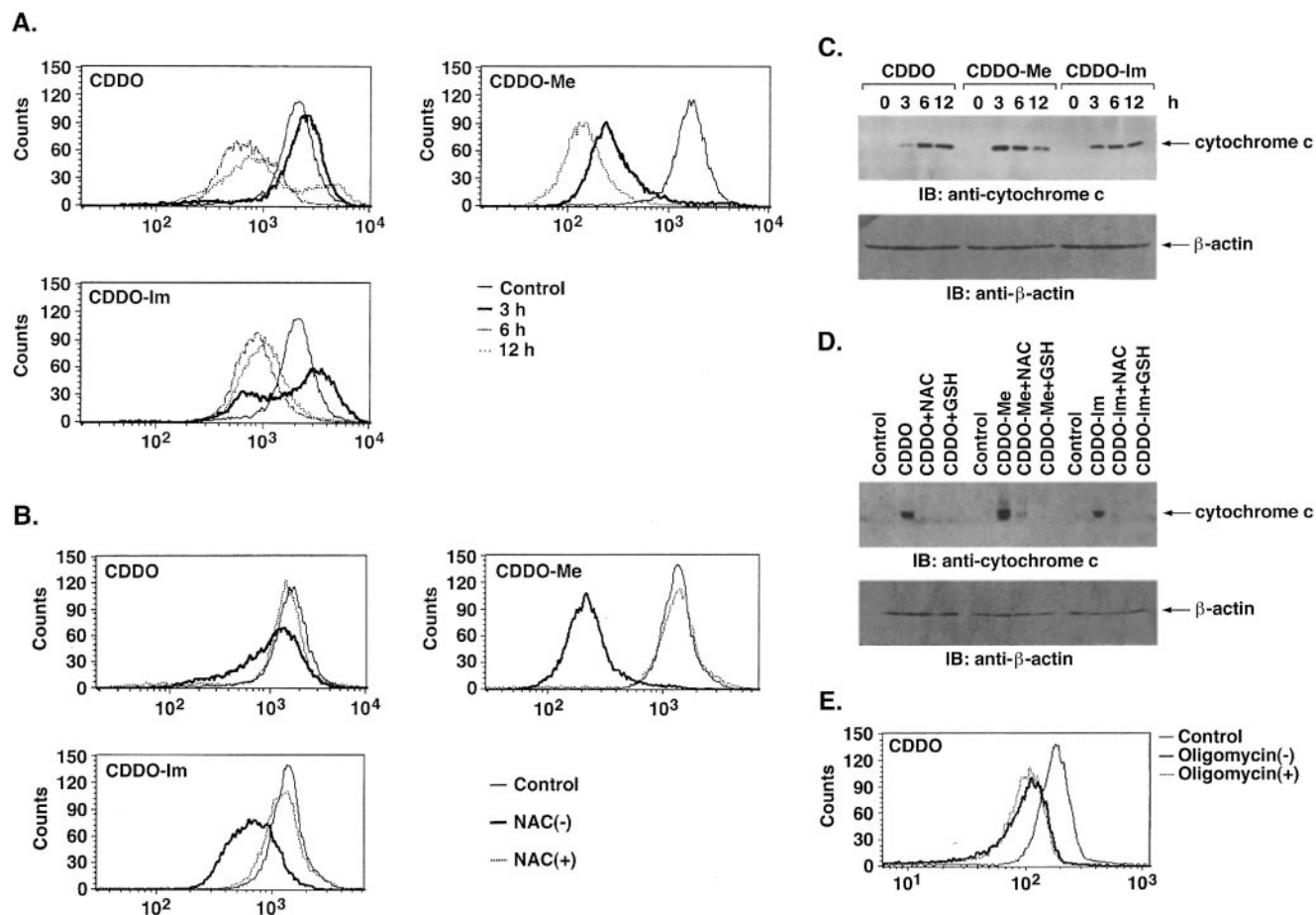


Fig. 6. CDDO-induced loss of *r/m* and cytochrome *c* release is blocked by antioxidants. In A, cells were treated with CDDO, CDDO-Me, or CDDO-Im for the indicated times and then incubated with DiOC₆[3]. The fluorescence of DiOC₆[3] was measured by flow cytometry. The results are representative of three independent experiments. In B, cells were pretreated with NAC for 2 h and then incubated with CDDO for 12 h, CDDO-Me for 6 h, or CDDO-Im for 6 h. The results are representative of three independent experiments. In C, cells were exposed to CDDO or one of the derivatives for the indicated times. Cytosolic fractions were subjected to immunoblotting with anti-cytochrome *c* and anti- β -actin. Similar results were obtained in three independent experiments. In D, cells were pretreated with NAC or GSH for 2 h and then exposed to CDDO or one of the derivatives for 6 h. Cytosolic fractions were subjected to immunoblotting with anti-cytochrome *c*. The results are representative of three independent experiments. In E, cells were pretreated with 5 μ g/ml oligomycin for 15 min, exposed to CDDO for 9 h, and then incubated with DiOC₆[3]. The results are representative of two independent experiments.

decreases in GSH because CDDO, CDDO-Me, and CDDO-Im were equally effective in inducing an apoptotic response. These findings collectively suggest that CDDO and its derivatives regulate redox balance by more than one mechanism.

Prooxidant Effects of CDDO Induce JNK Activation. Treatment of ML-1 myeloid leukemia cells with CDDO is associated with induction of JNK activity and apoptosis (4). The present work demonstrates that U-937 cells also respond to CDDO, CDDO-Me, and CDDO-Im with JNK activation. JNK is activated by exposure of cells to cytokines (*e.g.*, TNF) and by exposure to diverse forms of environmental stress (*e.g.*, osmotic, genotoxic, and oxidative; Ref. 30). We therefore reasoned that CDDO-induced JNK activation might be induced by stimulation of TNF production or of a stress signaling pathway. Analysis of TNF levels in culture supernatants of CDDO-treated cells was uninformative (data not shown). Moreover, treatment of cells with neutralizing anti-TNF antibodies failed to abrogate CDDO-induced JNK activation (data not shown). These findings suggested that CDDO activates JNK by stimulating a stress response. The finding that CDDO increases ROS levels and, more importantly, that antioxidants block CDDO-induced JNK activation provided support for a mechanism mediated by oxidative stress. The finding that DTT interferes with binding of CDDO to PPAR γ has suggested that CDDO may form

reversible, noncovalent interactions with thiol groups (8). Antioxidants, such as GSH and NAC, may therefore attenuate the effects of CDDO by reversible interactions and/or scavenging of ROS. The available evidence indicates that H₂O₂, as a source of ROS, activates protein kinase C δ (42) and thereby the *c*-Abl tyrosine kinase (43, 44). Activated forms of Abl function as upstream effectors of the JNK pathway (45). The apoptosis signal-regulating kinase 1 also functions upstream to JNK and is activated in the response of cells to H₂O₂ (46, 47). Additional studies will be needed to determine whether CDDO activates JNK through these ROS-induced signaling cascades.

CDDO induces caspase activation by a prooxidant mechanism. CDDO treatment is associated with activation of caspase-8 (2, 4, 5, 7). Moreover, inhibition of caspase-8 with CrmA or zIETD-fmk substantially blocks CDDO-induced cytochrome *c* release and caspase-3 activation (2, 4, 5, 7). Caspase-8-mediated cleavage of Bid has been proposed as one mechanism responsible for CDDO-induced cytochrome *c* release (2, 4, 5). Activation of Bax may also contribute to CDDO-induced release of cytochrome *c* (3, 4). These findings have collectively supported a model in which CDDO activates the extrinsic pathway and thereby circumvents resistance associated with defects in the intrinsic apoptotic pathway. The studies' results demonstrate that pretreatment of cells with anti-

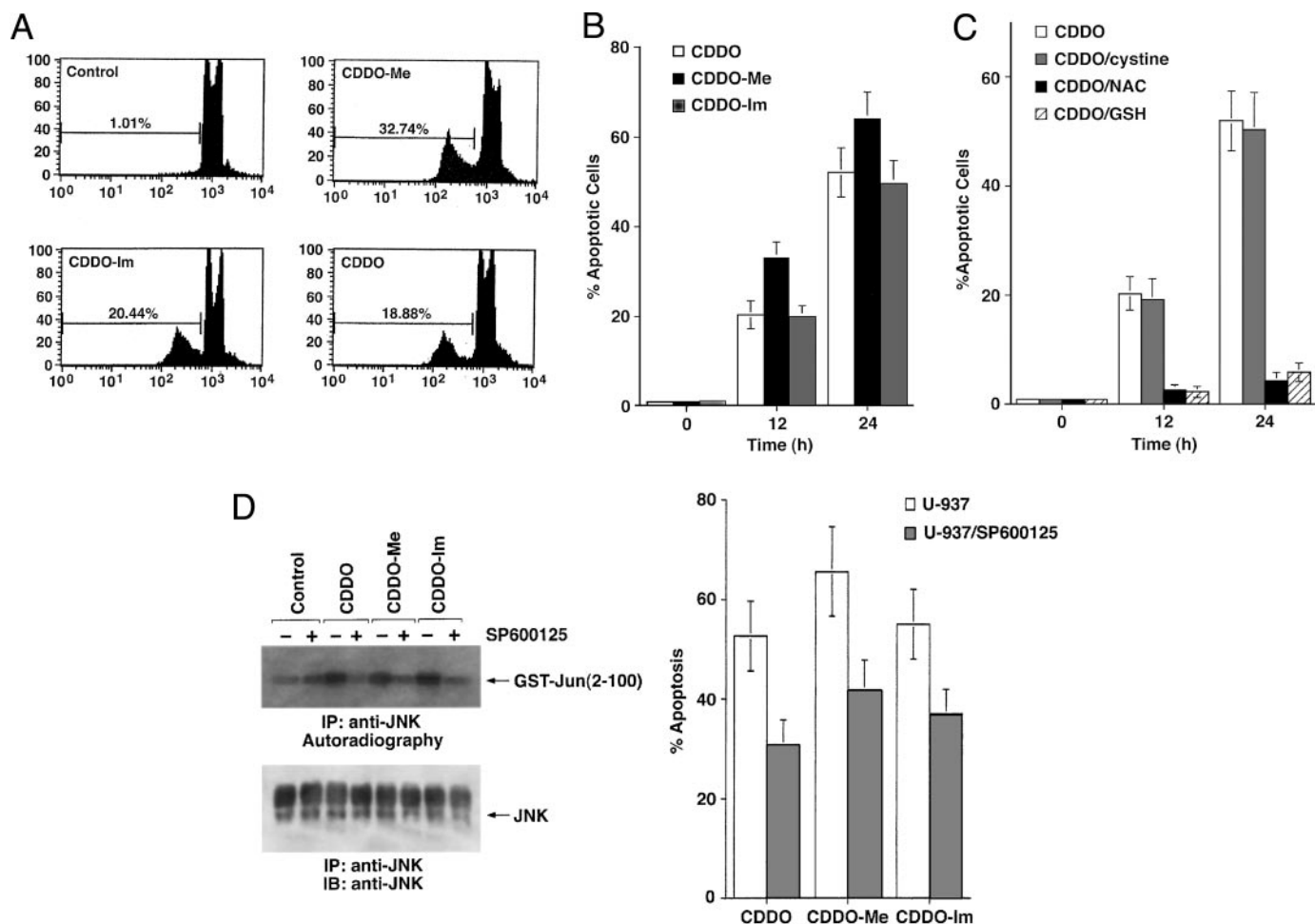


Fig. 7. Antioxidants block CDDO-induced apoptosis. In A, cells were treated with CDDO, CDDO-Me, or CDDO-Im for 12 h. DNA content was analyzed by flow cytometry. In B, cells were treated with CDDO (open bars), CDDO-Me (solid bars), or CDDO-Im (shaded bars) for the indicated times. In C, cells were pretreated with NAC, GSH, or cysteine and then exposed to CDDO for 24 h. In D, cells were pretreated with SP600125 for 15 min and then incubated with CDDO or its derivatives for 3 h. Anti-JNK immunoprecipitates were analyzed for phosphorylation of GST-c-Jun (2–100; left top panel) and by immunoblotting with anti-JNK (left bottom panel). Similar results were obtained in three independent experiments. DNA content was analyzed by flow cytometry, and the results shown as the percentage of the cells with sub-G₁ DNA (mean \pm SD) of three independent experiments (right panel).

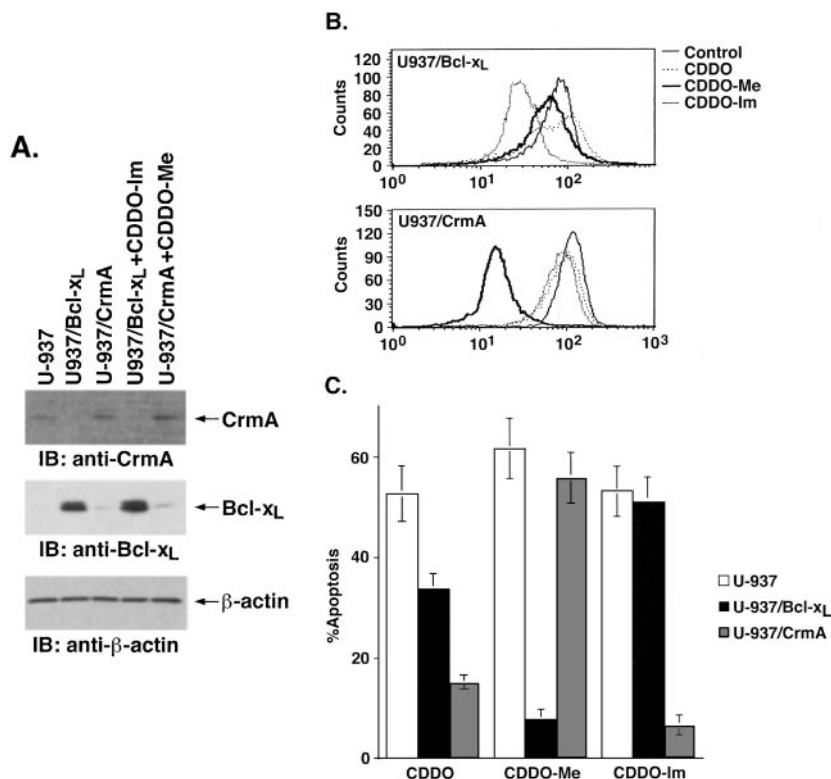
oxidants blocks CDDO-induced activation of caspase-8 and caspase-3. Although these results indicate that caspase-8 is activated by a ROS-mediated mechanism, the signals responsible for this effect are not known. Studies in CLL cells have shown that CDDO decreases expression of c-FLIP, an endogenous inhibitor of caspase-8 (7). The finding, however, that down-regulation of c-FLIP expression with antisense oligonucleotides is insufficient to induce apoptosis indicates that CDDO has other targets (7). In our studies, immunoblot analysis of CDDO-treated U-937 cells demonstrated little if any effect on c-FLIP expression (data not shown). Thus, ROS-induced activation of caspase-8 in CDDO-treated U-937 cells appears to be mediated by a mechanism independent of c-FLIP down-regulation.

CDDO-induced Apoptosis Is Abrogated by Antioxidants. The present results demonstrate that CDDO-induced loss of $r\ell/m$ is also mediated by a ROS-dependent mechanism. Increases in intracellular ROS have been shown to induce loss of $r\ell/m$ (37). Moreover, $r\ell/m$ is regulated by intracellular GSH levels (33). Thus, both CDDO-induced increases in ROS and decreases in GSH levels are likely to be responsible for loss of $r\ell/m$. In concert with these results and the effects of antioxidants on caspase-8 activation, our results further demonstrate that CDDO-induced apoptosis is blocked by NAC and GSH. These findings collectively support a model in which CDDO

and its derivatives disrupt redox balance and thereby induce caspase-8 activation, mitochondrial dysfunction, and apoptosis. Activation of caspase-8 with CDDO or one of the derivatives thus may be useful in the treatment of tumors with defects in the intrinsic apoptotic pathway.

CDDO-Me Induces Apoptosis by a CrmA-insensitive Mechanism. CDDO-induced apoptosis is mediated predominantly by a CrmA-sensitive mechanism (2, 4). Similar results were obtained in the present work with CDDO-Im. The finding that CDDO-Me-induced apoptosis is not attenuated by CrmA, however, indicates that this derivative activates a caspase-8-independent pro-apoptotic pathway. The delay in Bid cleavage in cells treated with CDDO-Me may also reflect the activation of a distinct pathway. In this context, our results show that CDDO-Me is substantially more effective than CDDO or CDDO-Im in decreasing intracellular GSH levels. CDDO-Me was also more effective than CDDO or CDDO-Im in inducing loss of $r\ell/m$. Decreases in GSH levels are associated with mitochondrial dysfunction through loss of $r\ell/m$ and thereby induction of apoptosis (26, 39–41). The results thus indicate that modification of CDDO at position 28 with a methyl ester, but not with an imidazolide function, results in more potent depletion of intracellular GSH levels, loss of $r\ell/m$, and induction of apoptosis by a caspase-8-independent mechanism. Taken together,

Fig. 8. Modification of CDDO at C-28 defines the apoptotic response. U-937 (open bars), U-937/Bcl-x_L (solid bars), and U-937/CrmA (shaded bars) cells were treated with the indicated agents for 24 h. In A, cell lysates were subjected to immunoblotting with anti-Bcl-x_L and anti-CrmA. In B, cells were treated with CDDO, CDDO-Me, or CDDO-Im for 6 h and then incubated with DiOC₆[3]. C, apoptosis. The results are presented as the percentage apoptosis (mean ± SD) of three independent experiments.



our findings show that CDDO and its derivatives disrupt intracellular oxidant levels and that the mechanisms responsible for alterations in redox balance and induction of apoptosis can vary depending on modifications at position 28.

ACKNOWLEDGMENTS

We thank Kamal Chauhan for excellent technical support.

REFERENCES

- Suh, N., Wang, Y., Honda, T., Gribble, G. W., Dmitrovsky, E., Hickey, W. F., Maue, R. A., Place, A. E., Porter, D. M., Spinella, M. J., Williams, C. R., Wu, G., Dannenberg, A. J., Flanders, K. C., Letterio, J. J., Mangelsdorf, D. J., Nathan, C. F., Nguyen, L., Porter, W. W., Ren, R. F., Roche, N. S., Subbaramaiah, K., and Sporn, M. B. A novel synthetic oleanane triterpenoid, 2-cyno-3, 12-dioxoolean-1, 9-dien-28-oic acid, with potent differentiating, antiproliferative and anti-inflammatory activity. *Cancer Res.*, 59: 336–341, 1999.
- Ito, Y., Pandey, P., Place, A., Sporn, M., Gribble, G., Honda, T., Kharbanda, S., and Kufe, D. The novel triterpenoid CDDO induces apoptosis of human myeloid leukemia cells by a caspase-8 dependent mechanism. *Cell Growth Differ.*, 11: 261–267, 2000.
- Konopleva, M., Tsao, T., Ruvolo, P., Stiouf, I., Estrov, Z., Leysath, C. E., Zhao, S., Harris, D., Chang, S., Jackson, C. E., Munsell, M., Suh, N., Gribble, G., Honda, T., May, W. S., Sporn, M. B., and Andreeff, M. Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood*, 99: 326–335, 2002.
- Stadheim, T. A., Suh, N., Ganju, N., Sporn, M. B., and Eastman, A. The novel triterpenoid 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J. Biol. Chem.*, 277: 16448–16455, 2002.
- Ito, Y., Pandey, P., Sporn, M., Datta, R., Kharbanda, S., and Kufe, D. The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol. Pharmacol.*, 59: 1094–1099, 2001.
- Kim, K., Lotan, R., Yue, P., Sporn, M., Suh, N., Gribble, G., Honda, T., Wu, G., Hong, W., and Sun, S. Identification of a novel synthetic triterpenoid, methyl-2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells. *Mol. Cancer Ther.*, 1: 177–184, 2002.
- Pedersen, I., Kitada, S., Schimmer, A., Kim, Y., Zapata, J., Charneau, L., Rassenti, L., Andreeff, M., Bennett, F., Sporn, M., Liotta, L., Kipps, T., and Reed, J. The triterpenoid CDDO induces apoptosis in refractory CLLB cells. *Blood*, 100: 2965–2972, 2002.
- Wang, Y., Porter, W. W., Suh, N., Honda, T., Gribble, G. W., Leesnorton, L. M., Plunkett, K. D., Mangelsdorf, D. J., Blanchard, S. G., Willson, T. M., and Sporn, M. B. A synthetic triterpenoid, 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma. *Mol. Endocrinol.*, 14: 1550–1556, 2000.
- Liu, X., Kim, C., Yang, J., Jemmerson, R., and Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, 86: 147–157, 1996.
- Yang, J., Liu, X., Bhalla, K., Kaekyung Kim, C., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science (Wash. DC)*, 275: 1129–1132, 1997.
- Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science (Wash. DC)*, 275: 1132–1136, 1997.
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, 91: 479–489, 1997.
- Srinivasula, S., Ahmad, M., Alnemri, T., and Alnemri, E. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol. Cell*, 1: 949–957, 1998.
- Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Kramer, P. H., Peter, M. E., and Dixit, V. M. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell*, 85: 817–827, 1996.
- Boldin, M., Goncharov, T., Goltsev, Y., and Wallach, D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell-death. *Cell*, 85: 803–815, 1996.
- Stennicke, H., Jurgenmeier, J., Shin, H., Deveraux, B., Wolf, X., Yang, X., Zhou, Q., Ellerby, H., Ellerby, L., Bredesen, D., Green, D., Reed, J., Froelich, C., and Salvesen, G. Pro-caspase-3 is a major physiologic target of caspase-8. *J. Biol. Chem.*, 273: 27084–27090, 1998.
- Luo, X., Budiharjo, H., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, 94: 481–490, 1998.
- Li, H., Zhu, H., Xu, C.-j., and Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the fas pathway of apoptosis. *Cell*, 94: 491–501, 1998.
- Kroemer, G., and Reed, J. C. Mitochondrial control of cell death. *Nat. Med.*, 6: 513–519, 2000.
- Herr, I., and Debatin, K. M. Cellular stress response and apoptosis in cancer therapy. *Blood*, 98: 2603–2614, 2001.
- Datta, R., Manome, Y., Taneja, N., Boise, L. H., Wechselbaum, R. R., Thompson, C. B., Slapak, C. A., and Kufe, D. W. Overexpression of Bcl-x_L by cytotoxic drug exposure confers resistance to ionizing radiation-induced internucleosomal DNA fragmentation. *Cell Growth Differ.*, 6: 363–370, 1995.
- Bunz, F. Cell death and cancer therapy. *Curr. Opin. Pharmacol.*, 1: 337–341, 2001.
- Datta, R., Kojima, H., Banach, D., Bump, N. J., Talanian, R. V., Alnemri, E. S., Wechselbaum, R. R., Wong, W. W., and Kufe, D. W. Activation of a CrmA-insensitive, p35-sensitive, pathway in ionizing radiation-induced apoptosis. *J. Biol. Chem.*, 272: 1965–1969, 1997.

24. Yoshida, K., Weichselbaum, R., Kharbanda, S., and Kufe, D. Role for Lyn tyrosine kinase as a regulator of stress-activated protein kinase activity in response to DNA damage. *Mol. Cell. Biol.*, *20*: 5370–5380, 2000.
25. Karp, D. R., Shimooku, K., and Lipsky, P. E. Expression of gamma-glutamyl transpeptidase protects Ramos B cells from oxidation-induced cell death. *J. Biol. Chem.*, *276*: 3798–3804, 2001.
26. Lizard, G., Gueldry, S., Sordet, O., Monier, S., Athias, A., Miguet, C., Bessedé, G., Lemaire, S., Solary, E., and Gambert, P. Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. *FASEB J.*, *12*: 1651–1663, 1998.
27. Shrieve, D. C., Bump, E. A., and Rice, G. C. Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J. Biol. Chem.*, *263*: 14107–14114, 1988.
28. Hulbert, P. B., and Yakubu, S. I. Monobromobimane: a substrate for the fluorimetric assay of glutathione transferase. *J. Pharm. Pharmacol.*, *35*: 384–386, 1983.
29. Shapiro, H. M. Membrane potential estimation by flow cytometry. *Methods*, *21*: 271–279, 2000.
30. Davis, R. Signal transduction by the JNK group of MAP kinases. *Cell*, *103*: 239–252, 2000.
31. Honda, T., Honda, Y., Favalaro, F. G., Jr., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., and Sporn, M. B. A novel dicyanotriterpenoid, 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-nitrile, active at picomolar concentrations for inhibition of nitric oxide production. *Bio-org. Med. Chem. Lett.*, *12*: 1027–1030, 2002.
32. Chen, Y. R., and Tan, T. H. The c-Jun N-terminal kinase pathway and apoptotic signaling (review). *Int. J. Oncol.*, *16*: 651–662, 2000.
33. Marchetti, P., Hirsch, T., Zamzami, N., Castedo, M., Decaudin, D., Susin, S. A., Masse, B., and Kroemer, G. Mitochondrial permeability transition triggers lymphocyte apoptosis. *J. Immunol.*, *157*: 4830–4836, 1996.
34. Briehl, M. M., Baker, A. F., Siemankowski, L. M., and Morreale, J. Modulation of antioxidant defenses during apoptosis. *Oncol. Res.*, *9*: 281–285, 1997.
35. Matsuyama, S., and Reed, J. C. Mitochondria-dependent apoptosis and cellular pH regulation. *Cell Death Differ.*, *7*: 1155–1165, 2000.
36. Matsuno-Yagi, A., and Hatéfi, Y. Studies on the mechanism of oxidative phosphorylation. ATP synthesis by submitochondrial particles inhibited at F0 by venturicidin and organotin compounds. *J. Biol. Chem.*, *268*: 6168–6173, 1993.
37. Nordberg, J., and Arner, E. Reactive oxygen species, antioxidants, and mammalian thioredoxin system. *Free Radic. Biol. Med.*, *31*: 1287–1312, 2001.
38. Moran, L. K., Gutteridge, J. M., and Quinlan, G. J. Thiols in cellular redox signalling and control. *Curr. Med. Chem.*, *8*: 763–772, 2001.
39. Liu, W., Kato, M., Akhand, A. A., Hayakawa, A., Suzuki, H., Miyata, T., Kurokawa, K., Hotta, Y., Ishikawa, N., and Nakashima, I. 4-hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. *J. Cell Sci.*, *113*: 635–641, 2000.
40. Troyano, A., Fernandez, C., Sancho, P., de Blas, E., and Aller, P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. The role of intracellular oxidation. *J. Biol. Chem.*, *276*: 47107–47115, 2001.
41. Tan, S., Somia, N., Maher, P., and Schubert, D. Regulation of antioxidant metabolism by translation initiation factor 2alpha. *J. Cell Biol.*, *152*: 997–1006, 2001.
42. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. Activation of protein kinase C by tyrosine phosphorylation in response to H₂O₂. *Proc. Natl. Acad. Sci. USA*, *94*: 11223–11237, 1997.
43. Sun, X., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. Interaction between protein kinase C δ and the c-Abl tyrosine kinase in the cellular response to oxidative stress. *J. Biol. Chem.*, *275*: 7470–7473, 2000.
44. Sun, X., Majumder, P., Shioya, H., Wu, F., Kumar, S., Weichselbaum, R., Kharbanda, S., and Kufe, D. Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species. *J. Biol. Chem.*, *275*: 17237–17240, 2000.
45. Kharbanda, S., Ren, R., Pandey, P., Shafman, T. D., Feller, S. M., Weichselbaum, R. R., and Kufe, D. W. Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. *Nature (Lond.)*, *376*: 785–788, 1995.
46. Gotoh, Y., and Cooper, J. Reactive oxygen species and dimerization-induced activation of apoptosis signaling-regulating kinase 1 in tumor necrosis factor- α signaling transduction. *J. Biol. Chem.*, *274*: 17477–17482, 1998.
47. Saitoh, M., Nishitoh, H., Makiko, F., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. Mammalian thioredoxin is direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.*, *17*: 2596–2606, 1998.