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Oleanane triterpenoid CDDO-Me inhibits growth and induces apoptosis in prostate cancer cells through a ROS-dependent mechanism

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ABSTRACT

CDDO-Me, a synthetic triterpenoid derived from oleanolic acid, is a promising anticancer agent that has shown strong activity against a wide variety of cancer types in vitro and in vivo. We have previously shown that CDDO-Me induces apoptosis in prostate cancer cells irrespective of their hormonal status. To further understand the proapoptotic mechanism of CDDO-Me, we investigated the role of reactive oxygen species (ROS) in mediating the apoptosis inducing activity of CDDO-Me in LNCaP and PC-3 prostate cancer cell lines. Here, we show that CDDO-Me induces ROS generation from both nonmitochondrial and mitochondrial sources, which is associated with the induction of apoptosis as characterized by increased annexin V-binding, cleavage of PARP-1 and procaspases-3, -8, -9, loss of mitochondrial membrane potential and release of cytochrome *c*. In addition, CDDO-Me inhibited cell survival Akt, NF-κB and mTOR signaling proteins. The inhibition of ROS generation by N-acetylcysteine (NAC) or by overexpression of antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase-1 (SOD-1) prevented CDDO-Me-induced apoptosis. Pretreatment with NAC blocked annexin V-binding, cleavage of PARP-1 and procaspases-3, -8, -9, loss of mitochondrial membrane potential and release of cytochrome *c* by CDDO-Me. NAC also prevented the inhibition of constitutively active Akt, NF-κB and mTOR by CDDO-Me. Together, these data indicate that ROS plays an essential role in the induction of apoptosis by CDDO-Me in prostate cancer cells.

1. Introduction

Carcinoma of the prostate (CaP) is the most commonly diagnosed cancer and the second leading cause of cancer related mortality in men in the United States. Current therapies, such as radical prostatectomy, local radiotherapy or brachytherapy have only limited efficacy against the metastatic disease [1,2] and effective chemotherapeutic agents are unavailable at present. High fat diet has been linked to the increased risk of metastatic prostate cancer [3]. In contrast, consumption of low fat diet along with high intake of dark green leafy vegetables, fruits, and soy products is considered to significantly reduce the incidence of prostate cancer. The cancer-preventing effects of plant-derived foods have been attributed to the presence of polyphenolic phytochemicals with strong antioxidant activity [4]. Indeed, there has been an increasing use of dietary supplements to prevent and/or treat prostate cancer. Thus, clinical development of plant-derived flavonoids and phenolic/polyphenolic antioxidants as chemopreventive agents could significantly reduce disease-related morbidity and mortality and improve prognosis.

Triterpenes or triterpenoids are members of a larger family of structurally related compounds known as cyclosqualenoids that are widely distributed in the plant kingdom [5]. Oleanolic acid and ursolic acid are naturally occurring triterpenoids that have been used in traditional medicine for centuries as antibacterial, antifungal, anticancer, and anti-inflammatory agents [6–8].

Abbreviations: CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CDDO-Me, C-28 methyl ester derivative of CDDO; H_2DCFDA, 6-carboxy-2,7-dichlorodihydrofluroscein diacetate; DHE, dihydroethidium bromide; ROS, reactive oxygen species; PARP-1, poly-ADP-ribose polymerase-1; NF-κB, nuclear factor kappa B; mTOR, mammalian target of rapamycin; NAC, N-acetylcystein; SOD-1, superoxide dismutase-1; GPx, glutathione peroxidase.

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Synthetic derivatives of oleanolic acid such as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its C-28 methyl ester derivative methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) exhibit greater anti-inflammatory and anticancer activity than the natural oleanolic acid [9–11]. Synthetic CDDOs are able to inhibit the proliferation of diverse types of tumor cell lines [12–14]. Although the mechanisms of the anticancer effects of CDDOs are not fully understood, cancer cell differentiation and activation of caspase-dependent and independent apoptosis contribute to the antitumor activity of CDDOs [15–17]. CDDOs were shown to modulate MAPK (Erk1/2), NF-κB, TGF-β/Smad, and PPARγ signaling [18–20]. CDDOs have also exhibited the antitumor activity in vivo in several mouse model systems [21–23]. We have previously shown that CDDO-Me inhibits the growth of hormone-sensitive and hormone-refractory human prostate cancer cell lines in vitro and in vivo by inducing apoptosis [24,25]. Furthermore, induction of apoptosis was associated with the inhibition of prosurvival Akt, NF-κB and mammalian target of rapamycin (mTOR) signaling proteins [24]. Since most anticancer agents act, at least in part, by inducing reactive oxygen species (ROS) [26–28], we investigated whether apoptosis induction in prostate cancer cells by CDDO-Me is also mediated through ROS generation. Our data demonstrate that CDDO-Me induces intracellular ROS production in prostate cancer cells and inhibition of ROS generation reverses apoptosis and prevents down-regulation of prosurvival Akt, NF-κB and mTOR signaling proteins.

2. Materials and methods

2.1. Materials

CDDO-Me was obtained from the National Cancer Institute, Bethesda, MD through the Rapid Access to Intervention Development Program. A 100 mM stock solution of CDDO-Me was prepared in DMSO, which was subsequently diluted in tissue culture medium to obtain the working concentrations. Antibodies against p-Akt (ser^{473}), NF-κB (p65), p-mTOR (ser^{2448}), procaspases-3, -8, -9, PARP-1, cytochrome *c*, SOD-1, and GPx were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Glutathione (GSH) assay kit was purchased from Cayman Chemical (Ann Arbor, MI). H_2DCFDA and DHE oxidative fluorescent probes were from Molecular Probes (Eugene, OR). Annexin V-FITC apoptosis detection kit II was obtained from BD Pharmingen (San Diego, CA). Rotenone, diphenylene iodonium (DPI) and SOD mimetic Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) were purchased from Sigma Chemical Inc. (St. Louis, MO).

2.2. Cell lines

LNCaP and PC-3 human prostate cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). PC-3 cells were grown in F-12K nutrient mixture (Gibco BRL, Rockville, MD) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 25 mM HEPES buffer. LNCaP cells were grown in RPMI-1640 supplemented with FBS and penicillin/streptomycin. Both cell lines were cultured at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air, and maintained by subculturing cells twice a week.

2.3. Measurement of ROS

H_2DCFDA and DHE fluorescent probes were used to measure the intracellular generation of hydroperoxide (H_2O_2) and superoxide anions ($O_2^{\bullet-}$), respectively. Briefly, 1×10^6 LNCaP or PC-3 cells were plated in 6-well plates and allowed to attach overnight. Cells were treated or not with CDDO-Me and then reacted with 5 μM of H_2DCFDA or 2 μM of DHE for 30 min at 37 °C. Cells were collected by trypsinization and DCFDA fluorescence was analyzed by flow cytometry. For the analysis of DHE fluorescence, cells were counterstained with Hoechst dye and fluorescence emission was detected by Leica fluorescent microscope (Heerburg, Switzerland).

2.4. Measurement of cell viability

2×10^4 cells in 100 μl of cell culture medium were seeded into each well of a 96-well plate. After incubation for 24 h, cells were treated with CDDO-Me for additional 72 h. Cell viability was then determined by the colorimetric MTS assay using CellTiter 96 AQueous One Solution Proliferation Assay System from Promega (Madison, WI). After incubation for 2 h at 37 °C, optical density was measured at 490 nm using a microplate reader.

2.5. Annexin V-FITC binding

Induction of apoptosis was assessed by the binding of annexin V to phosphotidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, LNCaP and PC-3 cells treated or not with CDDO-Me were resuspended in the binding buffer provided in the annexin V-FITC apoptosis detection kit II. 5 μl of annexin V-FITC reagent and 5 μl of propidium iodide solution (PI) were added to cell suspension and then incubated for 30 min at room temperature in the dark. Stained cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA).

2.6. Isolation of nuclear proteins

Following treatment with CCDO-Me, cells were washed with PBS and incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% NP40). Cells were vortexed gently for lysis and nuclei were separated from the cytosol by centrifugation at $12{,}000 \times g$ for 1 min. Nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at $12{,}000 \times g$ and protein concentration measured by Bradford assay (Bio-Rad, Richmond, CA). NF-κB in nuclear extracts was detected by Western blotting as described below.

2.7. Measurement of mitochondrial depolarization and cytochrome c release

The loss of mitochondrial potential by treatment with CDDO-Me was determined using mitochondrial potential sensor JC-1 (Molecular Probes, Invitrogen, San Diego, CA). Control (untreated) or treated cells were loaded with mitochondrial sensor by treating 1×10^6 cells in 1 ml culture medium with JC-1 dye (10 μg/mL) for 10 min at 22 °C. Cells were analyzed by flow cytometry. In normal cells, dye is aggregated in mitochondria, fluoresces red, and is detected in the FL2 channel. In cells with altered mitochondrial potential, the dye fails to accumulate in the mitochondria, remains as monomers in the cytoplasm, fluoresces green, and is detected in the FL1 channel.

For CDDO-Me-induced release of cytochrome *c* from mitochondria, LNCaP and PC-3 cells were treated with CDDO-Me (1.25–5 μM) for 24 h and mitochondrial and cytosolic fractions were prepared using ApoAlert Cell Fractionation Kit (Clontech, Laboratories Inc., CA). Mitochondrial or cytosolic protein (10 μg) was separated on a 14% SDS-PAGE gel and after transfer of proteins, membrane was probed with cytochrome *c* antibody.

2.8. Western blotting

Cellular lysates were prepared by detergent lysis (1% Triton-X 100 (v/v), 10 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 5 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatinin, and 10 μg/mL 4-2-aminoethyl-benzenesulfinyl fluoride). Lysates were clarified by centrifugation at 14,000 × *g* for 10 min at 4 °C, and protein concentrations were determined by Bradford assay. Samples (50 μg) were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% Bromophenol Blue, 125 mM Tris–HCl (pH 7.5), and 640 mM 2-mercaptoethanol) and separated on SDS-polyacrylamide gels. Proteins resolved on the gels were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in 10 mM Tris–HCl (pH 8.0), 150 mM NaCl with 0.05% Tween 20 (TPBS) and probed using protein specific antibodies to p-Akt (ser^{473}), p-mTOR (ser^{2448}), NF-κB (p65), procaspases, cytochrome *c*, SOD, GPx or β-actin (loading control) and HRP-conjugated secondary antibody. Immune complexes were visualized with enhanced chemiluminescence (ECL) detection system.

2.9. DNA transfection

For overexpression of SOD-1 or GPx, semi-confluent cultures of PC-3 cells in 60 mm^2 cell culture dishes were transfected with 10 μg of empty or expression plasmid DNA (pcDNA3-SOD or pcDNA-GPx plasmids) using LipofectAMINE Plus reagent. After incubation for 16 h, the medium was replaced with fresh culture medium and cells incubated again for additional 24 h. The expression level of SOD-1 and GPx was confirmed by immunoblotting.

2.10. Statistical analysis

Variance analysis between groups was performed by one-way ANOVA and significance of difference between control and treatment groups was analyzed using Dunnett multiple comparison test. The differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. CDDO-Me induces ROS generation in prostate cancer cells

We investigated whether generation of intracellular ROS is part of the mechanism by which CDDO-Me induces apoptosis in prostate cancer. The generation of ROS by CDDO-Me was assessed by using fluorescent probes H_2DCFDA and DHE that detect H_2O_2 and $O_2^{\bullet -}$, respectively. Fig. 1A shows flow cytometric analysis of LNCaP and PC-3 cells treated with CDDO-Me in the presence or absence of antioxidant NAC and stained with H_2DCFDA. Treatment with CDDO-Me resulted in 2.9-fold (LNCaP) to 0.9-fold (PC-3) increase in DCF fluorescence intensity compared to vehicle-treated control cells. Pretreatment of cells with antioxidant NAC completely blocked the

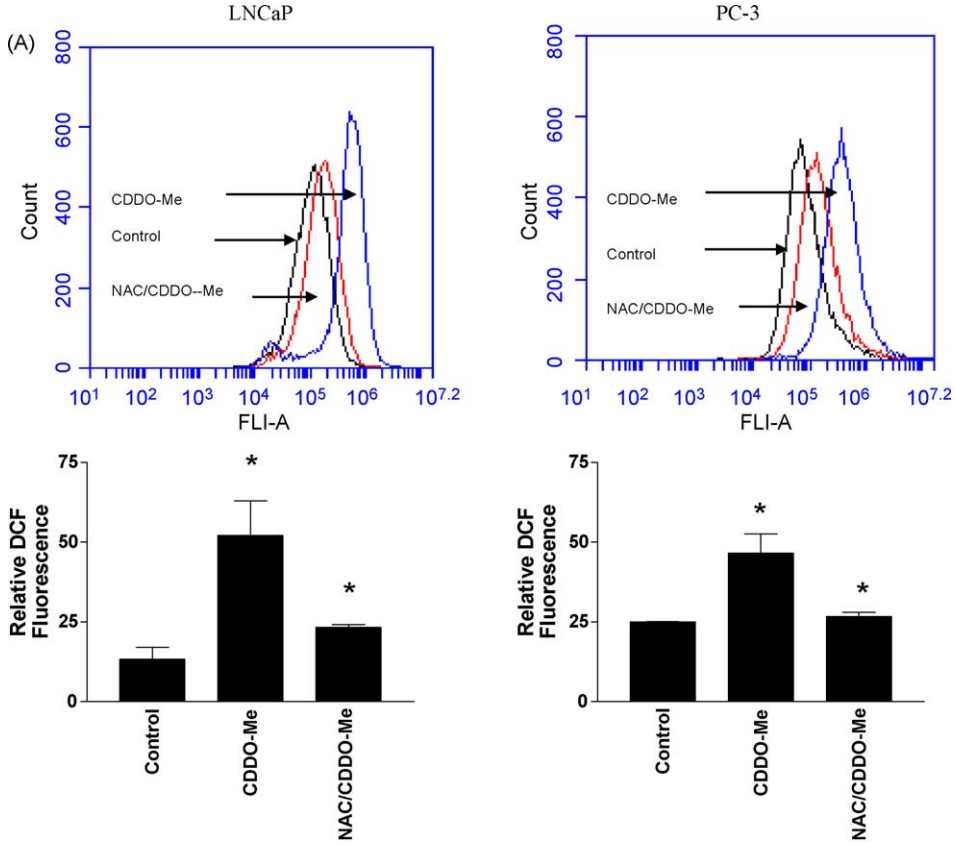

Fig. 1. CDDO-Me induces ROS generation and depletes glutathione in prostate cancer cells. (A). Subconfluent LNCaP and PC-3 cells were treated with CDDO-Me (2.5 μM) for 2 h with or without pretreatment with NAC (200 mM) for 2 h. Cells were reacted with 5 μM H_2DCFDA for 30 min at 37 °C. Cells were collected and DCF fluorescence was measured by flow cytometry. Flow cytographs and bar graphs show significant increase in mean fluorescence intensity in both cell lines treated with CDDO-Me ($p < 0.01$), which was significantly blocked by NAC ($p < 0.01$). (B) LNCaP and PC-3 cells were treated with CDDO-Me as described above and reacted with fluorescent probe DHE for 30 min. Cells were counter stained with Hoechst dye. The staining of cells was analyzed by fluorescent microscope. Fluorescent micrographs show bright DHE fluorescence of cells treated with CDDO-Me, which is blocked by NAC (200× magnification). Bar graphs show significant increase in percent of LNCaP and PC-3 cells with DHE fluorescence after treatment with CDDO-Me compared to control cells ($p < 0.01$). (C) LNCaP and PC-3 cells were treated with CDDO-Me (2.5 μM) for 20 h and glutathione levels were measured using a commercially available GSH assay kit (Cayman Chemical, Ann Arbor, MI). GSP was significantly depleted in both cell lines after treatment with CDDO-Me ($p < 0.01$).

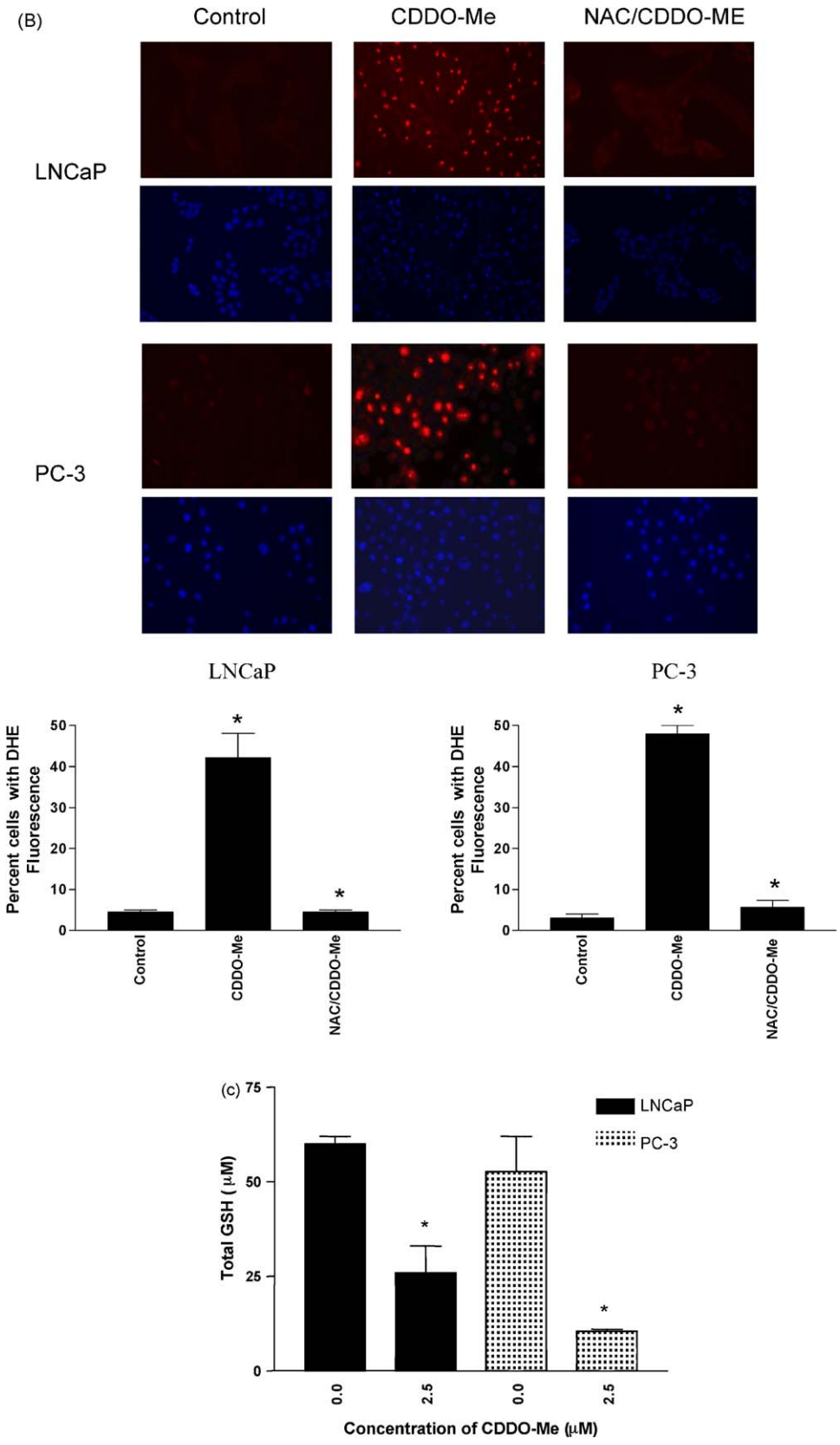

Fig. 1. (*Continued*).

CDDO-Me-induced increase in DCF fluorescence in both cell lines. The ethidium bromide fluorescence (DHE staining) was assessed by fluorescent microscopy. As can be seen in Fig. 1B, very few control LNCaP (4.5%) or PC-3 (3%) cells stained with DHE compared to 42% (LNCaP) and 47% (PC-3) cells that exhibited bright HE fluorescence following treatment with CDDO-Me. In contrast, pretreatment with NAC reduced CDDO-Me-induced HE staining of cells to the background levels in both cell lines.

3.2. CDDO-Me causes decrease in intracellular glutathione levels

Glutathione is an intracellular antioxidant that scavenges ROS directly and indirectly. Whether CDDO-Me exacerbates oxidative stress by causing depletion of intracellular glutathione was investigated. As shown in Fig. 1C, treatment with CDDO-Me significantly reduced the levels of glutathione in both cell lines (LNCaP = 59% reduction; PC-3 = 80% reduction, $p < 0.01$). Collectively, the oxidation of H_2DCFDA, DHE and depletion of glutathione suggested that ROS generation might play a role in killing of prostate cancer cells by CDDO-Me.

3.3. Mitochondria as sources of ROS generated by CDDO-Me

Whether mitochondrial ROS is generated by CDDO-Me was investigated next. For this purpose, cells were treated with CDDO-Me in the presence or absence of DPI, a specific inhibitor of NADPH oxidase or rotenone, a specific inhibitor of the respiratory chain complex 1 and ROS generation was measured by oxidation of H_2DCFDA by flow cytometry [29]. DPI slightly reduced the DCF fluorescence (~25%) in untreated cells; but it completely inhibited the CDDO-Me-mediated oxidation of H_2DCFDA in both cell lines (Fig. 2A). Treatment with rotenone also only partially reduced

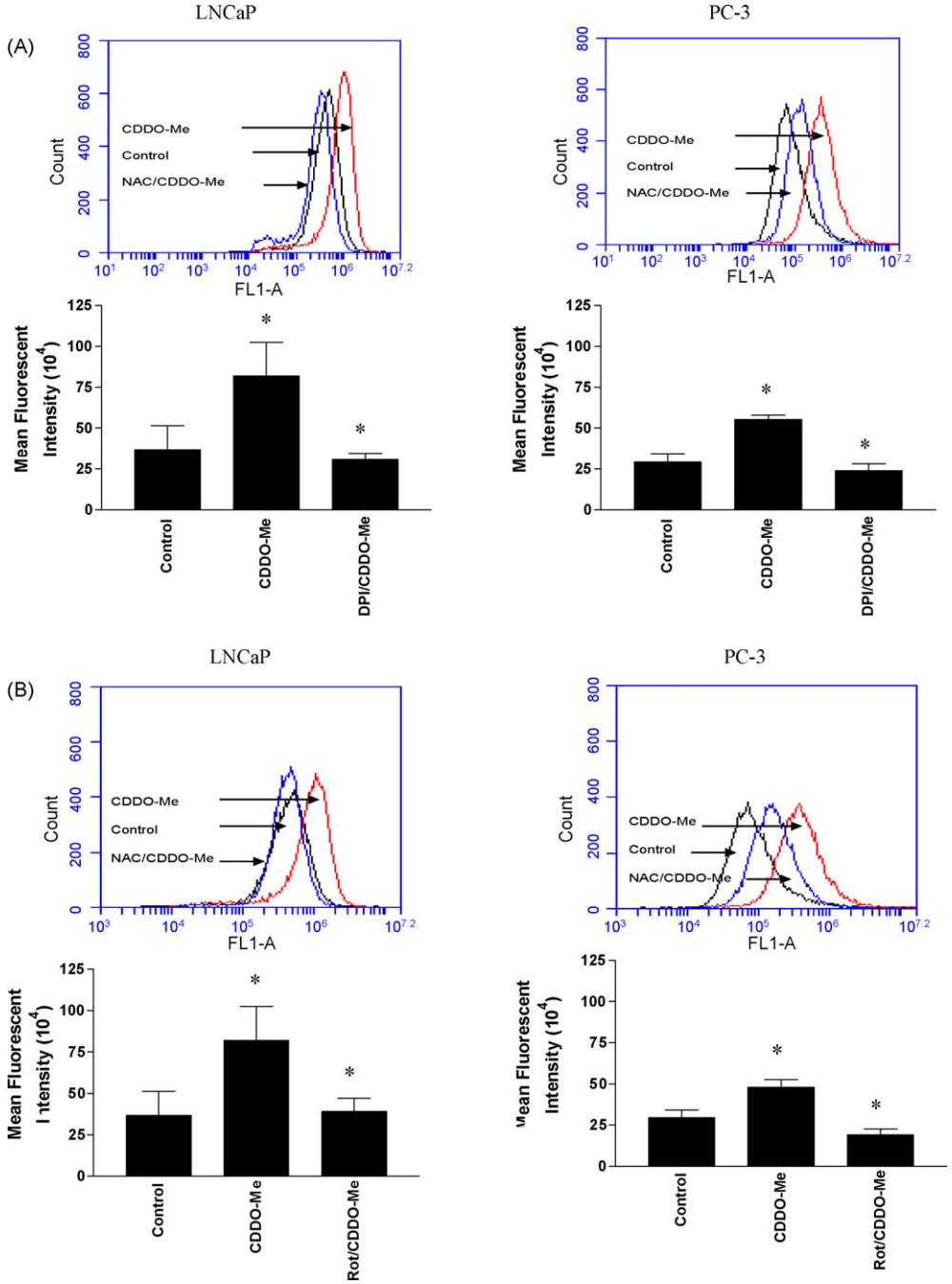

Fig. 2. CDDO-Me causes mitochondrial ROS generation. The effect of respiratory chain inhibitors DPI (NADPH oxidase inhibitor) and rotenone (Rot, a mitochondrial respiratory chain 1 inhibitor) on generation of ROS by CDDO-Me. LNCaP and PC-3 cells were pretreated with DPI (10 μM, A) or rotenone (200 nM, B) for 1 h before treating cells with CDDO-Me (2.5 μM) for 1 h. Control cells were untreated or treated with CDDO-Me or inhibitors alone. ROS generation was measured by an increase in DCF fluorescence resulting from oxidation of H_2DCFDA by flow cytometry. DPI and rotenone significantly prevented oxidation of H_2DCFDA in both cell lines ($p < 0.01$).

H_2DCFDA oxidation in untreated cells but it almost completely blocked the CDDO-mediated oxidation of H_2DCFDA in both cell lines. These data showing inhibition of CDDO-Me-induced ROS generation by DPI and rotenone indicated the involvement of mitochondria in the generation of ROS by CDDO-Me.

3.4. Effect of antioxidants on CDDO-Me-induced cell death

To evaluate the role of ROS in CDDO-Me-induced cell death of prostate cancer cells we measured the effect of general antioxidant NAC, overexpression of SOD-1 or GPx and SOD mimetic MnTBAP on cell growth inhibition by CDDO-Me. LNCaP and PC-3 cells were treated with CDDO-Me in the presence or absence of NAC or MnTBAP, whereas cells overexpressing SOD or GPx were treated with CDDO-Me alone for 72 h and cell growth measured by MTS assay. As shown in Fig. 3A, NAC completely protected both LNCaP and PC-3 cells from growth inhibition by CDDO-Me at concentrations ranging from 1.25 to 5 μM ($p < 0.01$). In a similar fashion, SOD mimetic MnTBAP also blocked the growth inhibitory activity of CDDO-Me (Fig. 3B, $p < 0.01$). The anti-growth inhibitory effect of antioxidants was confirmed in cells overexpressing SOD-1 or GPx (Fig. 3C). PC-3 cells transfected with SOD or GPx expression plasmids showed increased levels of SOD-1 and GPx, and they were resistant to CDDO-Me in a concentration dependent manner. Thus, these data demonstrate that antioxidants render prostate cancer cells resistant to CDDO-Me, indicating that ROS generation plays an essential role in the growth inhibitory (cell death) activity of CDDO-Me.

3.5. NAC blocks CDDO-Me-induced apoptosis in prostate cancer cells

Whether ROS plays a role in the induction of apoptosis by CDDO-Me in prostate cancer cells was investigated by measuring the effect of NAC on CDDO-Me-mediated binding of annexin V-FITC, activation of procaspases, loss of mitochondrial potential and cytochrome *c* release from mitochondria.

3.5.1. Effect on annexin V-FITC-binding

LNCaP and PC-3 cells were pretreated with NAC for 2 h before treating them with CDDO-Me for 20 h and staining with annexin V-FITC. As shown in Fig. 4A, a small percentage of untreated LNCaP (4%) and PC-3 (9%) cells bound annexin V-FITC. In contrast, the percentage of annexin V-FITC binding LNCaP cells significantly increased after treatment with CDDO-Me at concentrations of 1.25–5 μM (27–33%, $p < 0.01$). Similarly, the percentage of annexin V-FITC binding PC-3 cells also increased from 28% to 55% at 1.25–5 μM CDDO-Me ($p < 0.01$). In both cell lines, pretreatment with NAC completely blocked the CDDO-Me-induced binding of annexin V-FITC, indicating that ROS plays a role in the induction of apoptosis by CDDO-Me.

3.5.2. Effect on cleavage of PARP-1

Next, we determined the effect of NAC on cleavage of PARP-1 by CDDO-Me at 1.25–5 μM. Fig. 4B clearly demonstrates the cleavage of PARP-1 by CDDO-Me in both cell lines as demonstrated by the presence of 89 kDa cleaved PARP-1 fragment at concentrations of 1.25–5 μM. In contrast, pretreatment with NAC blocked the cleavage of PARP-1 by CDDO-Me, indicating that ROS plays a role in the induction of apoptosis by CDDO-Me.

3.5.3. Effect on activation of procaspases

To further delineate the role of ROS in the induction of apoptosis by CDDO-Me, we measured the effect of NAC on processing of procaspases-3, -8 and -9 by CDDO-Me. Cell lysates of LNCaP and PC-3 cells pretreated or not with NAC followed by 20 h treatment with CDDO-Me were analyzed for the cleavage of procaspases by Western blotting. Cells treated with CDDO-Me showed complete processing of native procaspases-3, -8 and -9 over a dose range of 1.25–5 μM in both cell lines (Fig. 4C). On the other hand, pretreatment with NAC completely protected procaspases from cleavage by CDDO-Me at concentrations of 1.25 and 2.5 μM. At 5 μM CDDO-Me, protection by NAC was less compared to lower concentrations.

3.6. NAC prevents mitochondrial depolarization and release of cytochrome c by CDDO-Me

As a measure of mitochondrial involvement in the induction of apoptosis by CDDO-Me, we evaluated mitochondrial depolarization in cells treated with CDDO-Me using mitochondrial potential JC-1 probe. There was significant change in mitochondrial potential after treatment of both cell lines with CDDO-Me for 20 h. The percentage of LNCaP cells with green fluorescence changed from 3.8 ± 1.2% at 0 μM CDDO-Me to 39 ± 2.6% at 2.5 μM CDDO-Me (Fig. 5A). The loss of membrane potential was effectively blocked by pretreating cells with NAC. Mitochondrial depolarizing effect of CDDO-Me on PC-3 cells was similar to LNCaP cells (e.g., 8 ± 2.8%, 54.5 ± 2.2% and 16 ± 3.3% of cells with green fluorescence at 0, 2.5 μM CDDO-Me, and NAC/2.5 μM CDDO-Me, respectively, $p < 0.01$).

We also measured the effect of NAC on the release of cytochrome *c* from mitochondria in cells treated with CDDO-Me. Western blot analysis of mitochondrial fraction of LNCaP and PC-3 cells treated with CDDO-Me (1.25–5 μM) demonstrated the release of cytochrome *c* from the mitochondria in both cell lines (Fig. 5B). However, pretreatment with NAC completely (LNCaP) to significantly (PC-3) prevented the release of cytochrome *c* from mitochondria

Taken together, the blocking of CDDO-mediated annexin V-FITC-binding, cleavage of PARP-1 and procaspases-3, -8, -9, loss of mitochondrial membrane potential and release of cytochrome *c* from mitochondria by NAC indicate that ROS plays a significant role in the induction of apoptosis in prostate cancer cells by CDDO-Me.

3.7. NAC blocks the inhibition of Akt, mTOR and NF-κB signaling proteins by CDDO-Me

Akt/mTOR and Akt/NF-κB are major antiapoptotic pathways that confer survival advantage and resistance of cancer cells to various forms of anticancer therapies. We have previously shown that CDDO-Me inhibits Akt, NF-κB, and mTOR and their downstream effectors [25]. Here we demonstrate that ROS is involved in the inhibition of Akt, NF-κB and mTOR by CDDO-Me. For this, cellular lysate or nuclear extract of LNCaP and PC-3 cells treated with CDDO-Me (1.25–5 μM) for 20 h with or without pretreatment with NAC was analyzed for p-Akt and p-mTOR (cellular lysate) and NF-κB (nuclear extract), respectively. CDDO-Me significantly to completely abolished p-Akt, p-mTOR and NF-κB at 1.25 μM and almost completely inhibited these signaling proteins at 2.5 μM and above in both cell lines (Fig. 6). Pretreatment with NAC prevented the inhibition of these proteins at all concentrations of CDDO-Me tested (e.g., 1.25–5 μM). This result suggests that ROS may have a role in the inhibition of cell survival signaling proteins by CDDO-Me.

4. Discussion

Our previous studies have demonstrated that among the three synthetic CDDOs derived from oleanolic acid (e.g., CDDO, CDDO-Im and CDDO-Me), CDDO-Me was most active against prostate cancer cells regardless of their hormonal status [24]. The growth

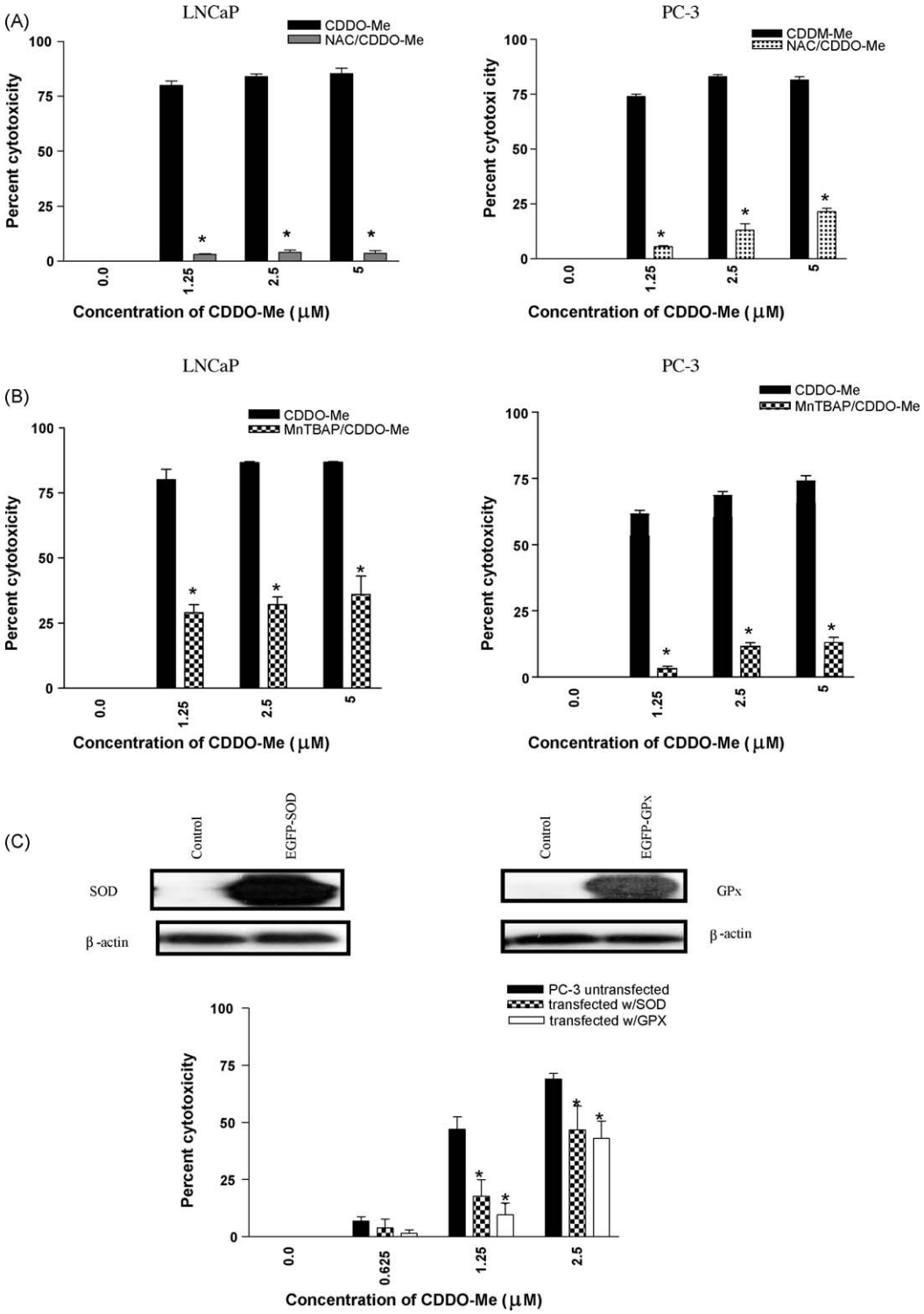

Fig. 3. Antioxidants protect prostate cancer cells from-CDDO-Me-induced growth inhibition. (A) Effect of NAC. 2×10^4 LNCaP or PC-3 cells were seeded in each well of a microtiter plate in 0.1 ml of culture medium. Cells were allowed to adhere for 24 h and pretreated with NAC (200 mM) for 2 h before treating with CDDO-Me at concentrations ranging from 0 to 5 μM for 72 h. Cell viability was measured by MTS assay using CellTiter AQueous assay system from Promega. Data are presented as mean ± SD percent reduction in viability obtained in three independent experiments. NAC significantly prevented the cytotoxicity of CDDO-Me in both cell lines ($p < 0.01$). (B). Effect of SOD mimetic MnTBAP. LNCaP and PC-3 cells were pretreated with MnTBAP (100 μM) for 2 h before treating with CDDO-Me at concentrations ranging from 0 to 5 μM for 72 h. Cell viability was measured by MTS assay as described above. (C) Effect of overexpression of antioxidant enzymes SOD-1 and GPx. PC-3 cells were transfected with SOD-1 or GPx expression plasmids LipofectAMINE Plus reagent for 16 h. Cells were then treated with CDDO-Me at 0.625–2.5 μM for 72 h and growth inhibition measured by MTS assay. *1.25 μM CDDO-Me, SOD-1 and GPx transfected cells vs control, $p < 0.01$; 2.5 μM CDDO-Me, SOD-1 and GPx transfected cells vs control, $p < 0.05$.

inhibition and killing of prostate cancer cells by CDDO-Me were attributed to the induction of apoptosis as demonstrated by an increase in annexin V-binding, cleavage of PARP-1 and activation of caspases. In addition, these studies also identified progrowth/prosurvival signaling proteins such as Akt, mTOR and NF-κB as potential molecular targets of CDDO-Me [25].

Current studies were aimed at determining the role of reactive oxygen species in the induction of apoptosis by CDDO-Me in

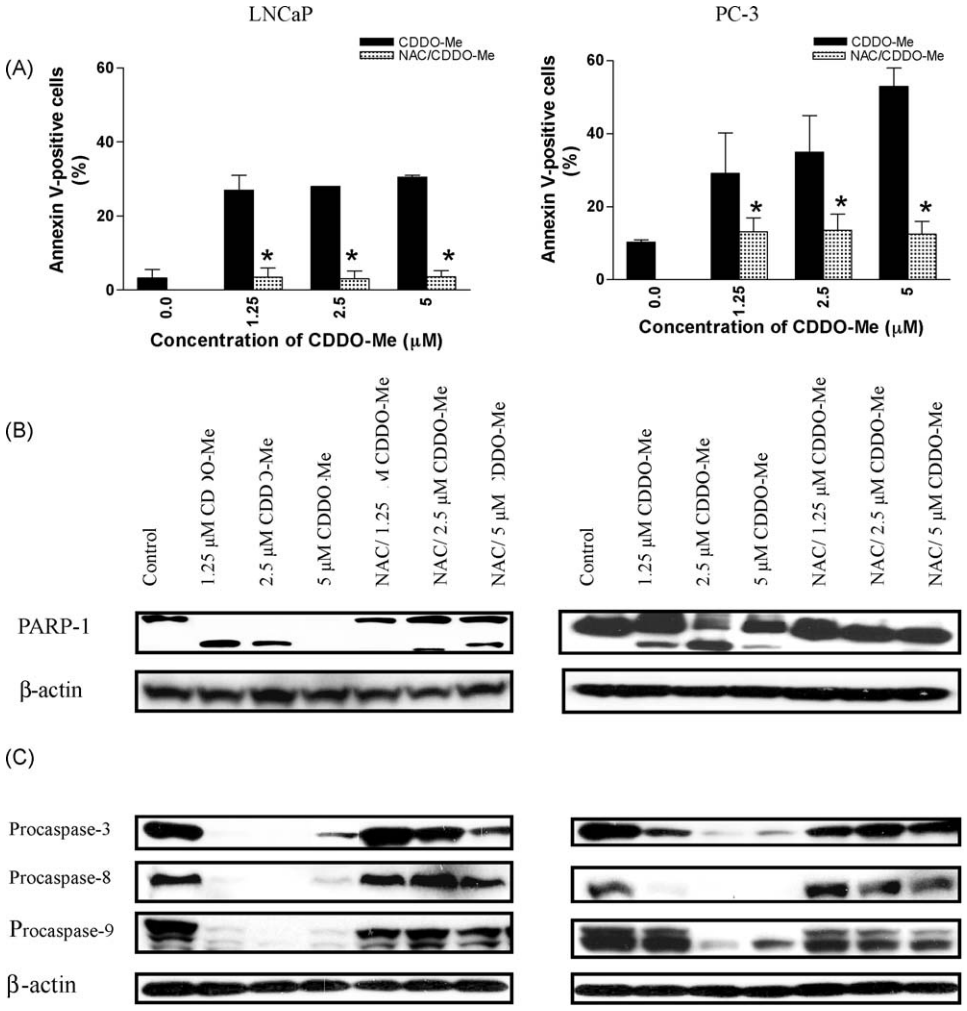

Fig. 4. Pretreatment with NAC blocks CDDO-Me-induced apoptosis in prostate cancer cells. (A) Effect on annexin V-FITC binding. LNCaP and PC-3 cells were pretreated with NAC (200 mM) for 2 h before treating with CDDO-Me at concentrations of 1.25–5 μM for 20 h. Cells were then incubated with 5 μl of annexin V-FITC reagent and 5 μl of PI for 30 min at room temperature. Annexin V-FITC-binding was assessed by flow cytometry. Results are presented as percentage of annexin V-FITC positive cells. Bar graph represents mean ± SD of three separate experiments. NAC significantly reduced the percentage of annexin V-FITC binding cells ($p < 01$). (B) Effect of NAC on CDDO-Me-induced cleavage of PARP-1. LNCaP and PC-3 cells were pretreated with NAC (200 mM) for 2 h before treating with CDDO-Me at concentrations of 1.25–5 μM for 20 h. Cellular lysate prepared from untreated and treated cells was analyzed for the cleavage of native PARP-1 by Western blotting. Immunoblots showing native (116 kDa) and cleaved (89 kDa) PARP-1 protein bands are presented. Similar results were obtained in three independent experiments. (C) NAC prevents CDDO-Me-induced cleavage of procaspases-3, -8 and -9. LNCaP and PC-3 cells were pretreated with NAC (200 mM) for 2 h before treating with CDDO-Me at 1.25–5 μM for 20 h. Cellular lysates (50 μg/lane) prepared from control and treated cells were analyzed by Western blotting using anti-caspase-3, -8 and -9 antibodies. The lane labeling is same as in (B). Data shown are from one of the three experiments.

prostate cancer cells. ROS is generated intracellularly as byproducts of normal aerobic metabolism or as second messengers in various signal transduction pathways or in response to environmental stress [30,31]. Depending upon the concentration, ROS elicits a wide spectrum of biological responses ranging from mitogenic to proliferative effects at low concentration to macromolecular damage and cell death at high concentrations [32]. The generation of ROS is part of the mechanism by which most chemotherapeutic agents or ionizing radiation kill tumor cells [26–28]. Our present findings provide evidence of mitochondrial involvement in the generation of ROS by CDDO-Me. CDDO-Me caused the oxidation of H_2DCFDA and DHE, indicating the production of hydrogen peroxide and superoxide anion, respectively. The generation of ROS in response to CDDO-Me was further supported by the finding that pretreatment with NAC, a general antioxidant blocked the oxidation of both H_2DCFDA and DHE. The mitochondrial involvement in the generation of ROS was confirmed by the result that CDDO-ME-induced ROS generation is markedly inhibited in the presence of mitochondrial chain 1 complex inhibitors DPI and rotenone. In addition, data demonstrating prevention of CDDO-Me-induced cytotoxicity by NAC or SOD mimetic MnTBAP or resistance of cancer cells overexpressing SOD-1 and GPx to CDDO-Me suggest the involvement of H_2O_2 and $O_2^{\bullet -}$ in killing of prostate cancer cells by CDDO-Me. These findings are consistent with the results of previous studies showing participation of ROS in killing of leukemia and pancreatic cancer cells by CDDO-Me [15,33].

We also investigated the role of ROS in CDDO-induced apoptosis in prostate cancer cells. CDDO-Me-mediated induction of apoptosis was confirmed by an increase in binding of annexin V, PARP-1 cleavage and activation of procaspases -3, -8 and -9. Activation of the most apical initiator procaspase-8, effector procaspase-3 and activation of caspase-9 and the release of cytochrome *c* in both cell lines indicated that both death receptor (extrinsic) and mitochondrial (intrinsic) pathways of apoptosis participate in the destruction of prostate cancer cells by CDDO-Me. The fact that each of these apoptosis-related processes was blocked by NAC indicated the important role ROS plays in CDDO-Me-induced apoptosis. The activation of both pathways of apoptosis by CDDO-Me in prostate cancer cells is consistent with previous

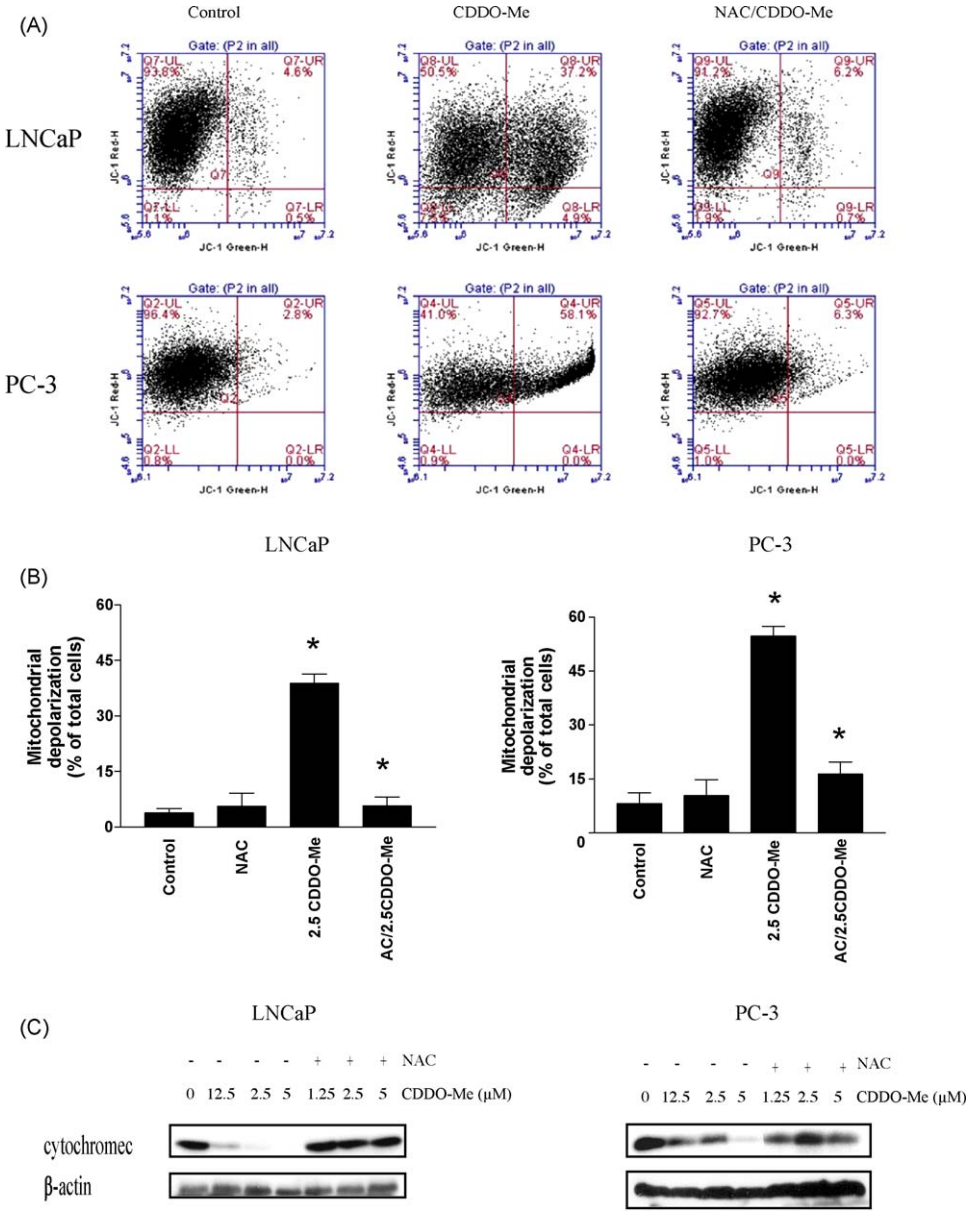

Fig. 5. NAC prevents CDDO-Me-induced mitochondrial depolarization and release of cytochrome *c*. (A) Effect on mitochondrial depolarization. LNCaP and PC-3 cells were pretreated with NAC (200 mM) for 2 h before treating with CDDO-Me at 1.25–5 μM for 20 h. Cells were then loaded with mitochondrial sensor dye JC-1 for 10 min and analyzed by flow cytometry. Data are shown as flow cytographs of cells fluorescing red (FL2 channel) or green (FL1 channel). (B) Histograms show percentage of cells with loss of mitochondrial potential difference. Similar results were obtained in two separate experiments. NAC significantly prevented the loss of mitochondrial potential difference in both cell lines ($p < 0.01$). (C) Effect on cytochrome *c* release. For cytochrome *c* release from mitochondria, mitochondrial fractions were prepared from control and treated cells using ApoAlert Cell Fractionation Kit (Clontech, Laboratories Inc., CA), and cytochrome *c* analyzed by Western blotting. Data shown are from one of the three experiments.

reports showing activation of caspase-dependent and caspase-independent pathways of apoptosis by CDDOs in leukemic and solid tumor cell lines [16,17]. We also found that CDDO-Me causes marked depletion of intracellular levels of GSH. The depletion of glutathione by CDDO-Me is likely to contribute to caspase-dependent apoptosis through perturbations in intracellular redox tone or activation of caspase-3 directly [33,34].

PI3K/Akt/mTOR and Akt/NF-κB are major antiapoptotic/pro-survival pathways [35,36] that are frequently hyperactivated in most cancers [37–39]. p-Akt promotes cell growth and survival by inactivating downstream substrates such as Bad, procaspase-9, and Forkhead transcription factors [40,41]. NF-κB family of transcription factors controls the expression of numerous genes involved in immune and inflammatory responses, cell proliferation, oncogenesis, angiogenesis, and apoptosis [35,37]. mTOR, a serine-threonine kinase, controls cell growth, survival, division and motility [42,43]. Our results demonstrate that antiapoptotic p-Akt, p-mTOR and NF-κB which are constitutively active in LNCaP and PC-3 cells are inhibited by CDDO-Me, suggesting that inhibition of these antiapoptotic proteins may be necessary for the induction of apoptosis by CDDO-Me. The inhibition of these signaling proteins by CDDO-Me was prevented by NAC. The latter implies that ROS may have a role in the inhibition of these prosurvival signaling proteins by CDDO-Me. Although the role of ROS in cell growth regulation is complex, free radicals have been shown to activate prosurvival pathways including MAP kinases and Akt/mTOR/NF-κB signaling pathways [44]. What exactly is the role of ROS in the inhibition of Akt, mTOR and NF-κB by CDDO-Me is unclear. CDDO-Me inhibits NF-κB by preventing phosphorylation and degradation of IκBα, an inhibitor of NF-κB [14]. It could

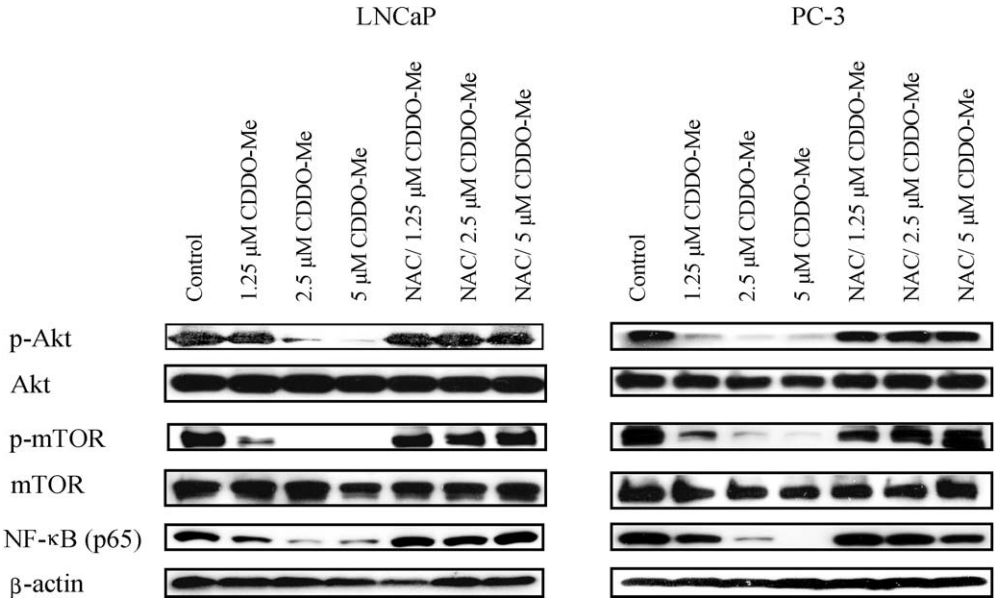

Fig. 6. NAC prevents CDDO-Me-mediated inhibition of Akt, mTOR and NF-κB. LNCaP and PC-3 cells were pretreated with NAC (200 mM) for 2 h before treating with CDDO-Me at concentrations of 1.25–5 μM for 20 h. After treatment, cell lysates and nuclear extracts were prepared and analyzed for p-Akt, p-mTOR, and NF-κB (p65) by Western blotting. The uniformity of sample loading was determined by anti-β-actin antibody. Similar results were obtained in three independent experiments.

prevent the activation (phosphorylation) of Akt and subsequently mTOR by directly targeting PI3K, an upstream kinase that regulates Akt/PBK activation. Clearly more work is needed to precisely define the role of ROS in the inhibition of prosurvival Akt, mTOR and NF-κB signaling proteins in cancer cells by CDDO-Me.

5. Conclusion

The present study provides an insight into the role of reactive oxygen species in apoptotic death and inhibition of survival and growth favoring Akt, mTOR and NF-κB signaling proteins by CDDO-Me in prostate cancer cells. A better understanding of the mechanism of action of CDDO-Me could potentially facilitate clinical development of CDDO-Me for prostate cancer.

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