Ursolic acid sensitizes prostate cancer cells to TRAIL-mediated apoptosis

Seoung Woo Shin, Jeen-Woo Park *

School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

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ABSTRACT

Prostate cancer is one of the most commonly occurring malignancies in men, and because existing treatments are not able to manage this neoplasm adequately, novel approaches are needed. Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has strong antitumor activity via the induction of apoptotic cell death in a wide range of tumor cell types and has negligible toxicity to most normal cells, some prostate carcinoma cells are resistant to the apoptotic effects of TRAIL. Therefore, combinatorial approaches with TRAIL and different chemotherapeutic agents have been developed to overcome the resistance of cancer cells to TRAIL. Here, we investigated the sensitizing effects of ursolic acid (UA), a pentacyclic triterpenoid found in many plants, on TRAIL-induced prostate cancer cell apoptosis. We found TRAIL-induced prostate cancer cells apoptosis was significantly enhanced by UA, and that UA induced CHOP-dependent DR5 up-regulation. This study shows the use of UA as a sensitizer for TRAIL-induced apoptotic cell death offers a promising means of enhancing the efficacy of TRAIL-based prostate cancer treatments.

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1. Introduction

Prostate cancer is the most frequently diagnosed, non-cutaneous neoplasm and the second leading cause of cancer related mortality in men [1]. Prostate cancer is unique among human cancers because of its striking age-dependent incidence and variable penetrance [2]. The development of prostate cancer in humans is viewed as a multistage process, involving at onset, a small latent carcinoma of low histological grade, which later progresses to a large metastatic lesion [3]. Treatment options available for this disease are limited because chemotherapy and radiation therapy are largely ineffective, and because metastatic disease frequently develops even after potentially curative surgery [4–6]. Thus, novel ways of treating prostate cancer must be developed.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF-superfamily, has strong antitumor activity via apoptotic cell death induction in a wide range of tumor cell types and negligible toxicity on most normal cells [7,8]. The apoptotic signal induced by TRAIL is triggered by its binding to death receptors 4 and 5 (DR4 and DR5), which results in receptor aggregation and the recruitment of Fas-associated protein with death domain (FADD) and procaspase-8 to form death-inducing signaling complex (DISC) [9]. Studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL [10–12], but combinatorial approaches based on TRAIL and different chemotherapeutic agents, such as small-molecule inhibitors, natural compounds, and drugs, have been developed to overcome the resistance of cancer cells to TRAIL [13–16].

Ursolic acid (UA), a pentacyclic triterpene carboxylic acid, is found in various plants in the forms of aglycones or glycosides [17]. UA has been found to have a number of important biological and biochemical functions, such as the inhibitions of tumorigenesis [18], tumor promotion [19], angiogenesis [20], tumor invasive activity [21], and the induction of tumor cell differentiation [22]. More recently, several studies have concluded that UA and its derivatives inhibit cancer cells growth by causing cell cycle arrest and the stimulating apoptosis [23,24].

Response to TRAIL is highly variable with resistance seen in many cancer cells. Although it was shown that UA potentiates TRAIL-induced apoptosis in various cancer cells including PC3 prostate cells [14], other human prostate cell lines like LNCaP and DU145 were completely resistant to the cytotoxic effect of TRAIL compared to PC3 cells. Modulation of apoptotic cell death in TRAIL-resistant LNCaP and DU145 cells by UA offers a promising therapeutic approach for the treatment of prostate cancer.

In this study, we investigated whether UA potentiates TRAIL-induced prostate cancer cell apoptosis. We demonstrate that combined treatment of TRAIL-resistant LNCaP and DU145 prostate cancer cells with subtoxic doses of UA and TRAIL were found to induce marked prostate cancer cell apoptosis. In addition, UA induced C/EBP homologous protein (CHOP)-dependent DR5 up-regulation, which is known to stimulate the partial priming of the proteolytic processing of caspase-3.

2. Materials and methods

2.1. Materials

UA, N-acetylcysteine (NAC), reduced glutathione (GSH), PEG-SOD, PEG-catalase, and propidium iodide (PI) were obtained from Sigma

* Corresponding author. Tel.: +82 53 950 6352; fax: +82 53 943 2762.
E-mail address: parkjw@knu.ac.kr (J.-W. Park).
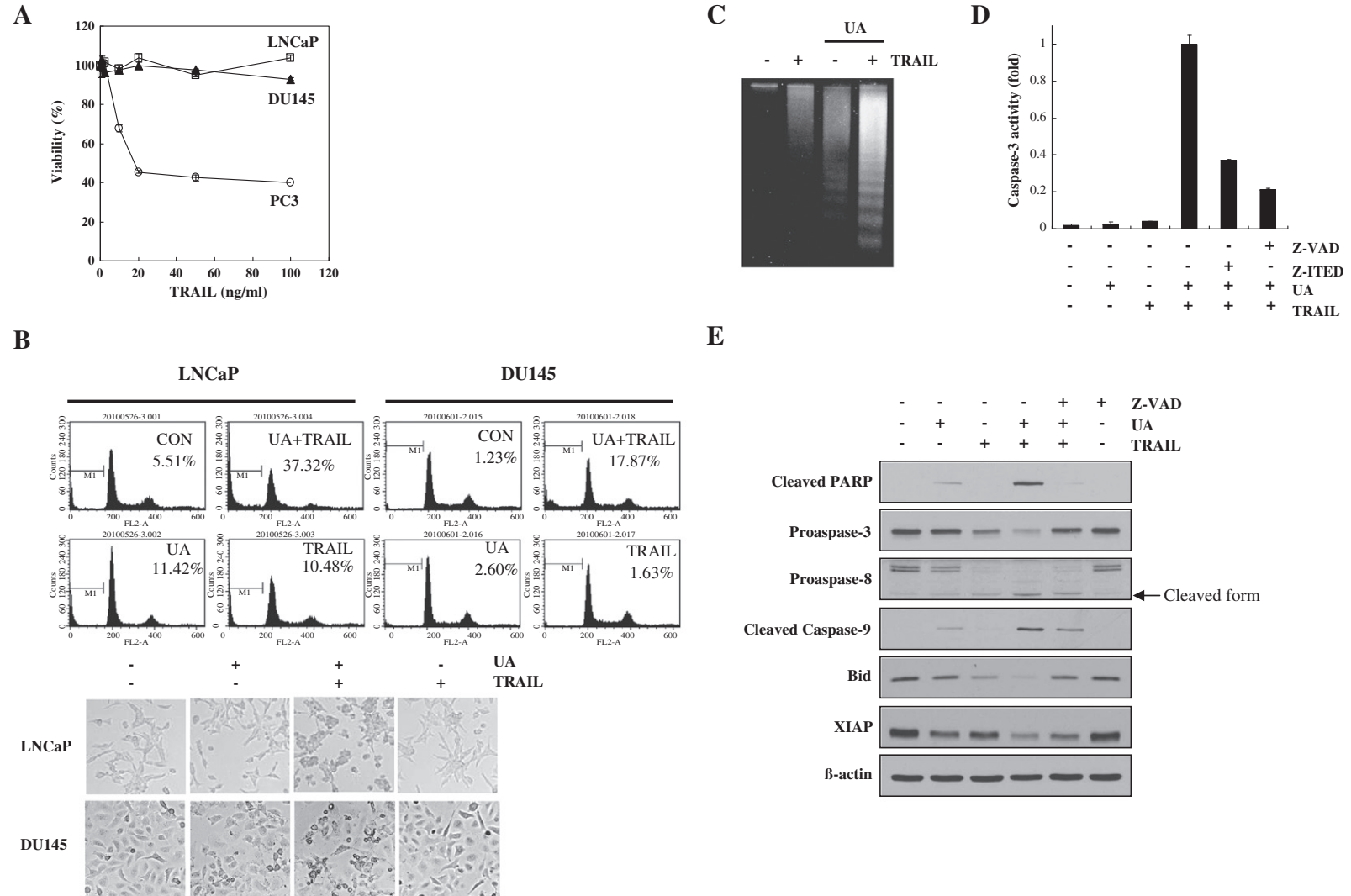
Fig. 1. UA sensitized human prostate cancer cells to TRAIL-induced apoptosis. (A) Effect of TRAIL on the viability of human prostate cancer cells. Three different human prostate cancer cell lines were treated with TRAIL for 24 h at the indicated concentrations and viabilities were assessed using an MTT assay. Data are presented as means ± S.D. of three separate experiments. (B) Flow cytometric analysis of apoptotic cells. LNCaP and DU145 cells were treated with 50 ng/ml TRAIL in the absence or presence of UA (20 μM) for 24 h. Levels of apoptosis were quantified using sub-G_1 fractions determined by FACS. Cell morphologies were determined by interference microscopy. (C) Fragmentation of genomic DNAs in LNCaP cells treated for 24 h with UA (20 μM) and TRAIL (50 ng/ml). Fragmented DNA was extracted and analyzed on 1% agarose gels. (D) LNCaP cells were treated with UA (20 μM) plus TRAIL (50 ng/ml) for 24 h in the presence or absence of Z-VAD-fmk (20 μM) or Z-ITED-fmk (20 μM). Enzymatic activities of caspase 3 were determined by incubating 20 μg of total protein with 200 μM of chromogenic substrate (DEVD-pNA) in 100 μl of assay buffer for 2 h at 37 °C. The release of chromophore p-nitroanilid (pNA) was monitored at 405 nm. Data are presented as means ± S.D. of three separate experiments. (E) LNCaP cells were treated with UA (20 μM) plus TRAIL (50 ng/ml) for 24 h in the presence or absence of Z-VAD-fmk (20 μM). Equal amounts of cell lysates (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against PARP, cleaved PARP, procaspase 3, procaspase 8, cleaved caspase 9, lamin B, Bid, and XIAP. β-Actin was used as the internal control.
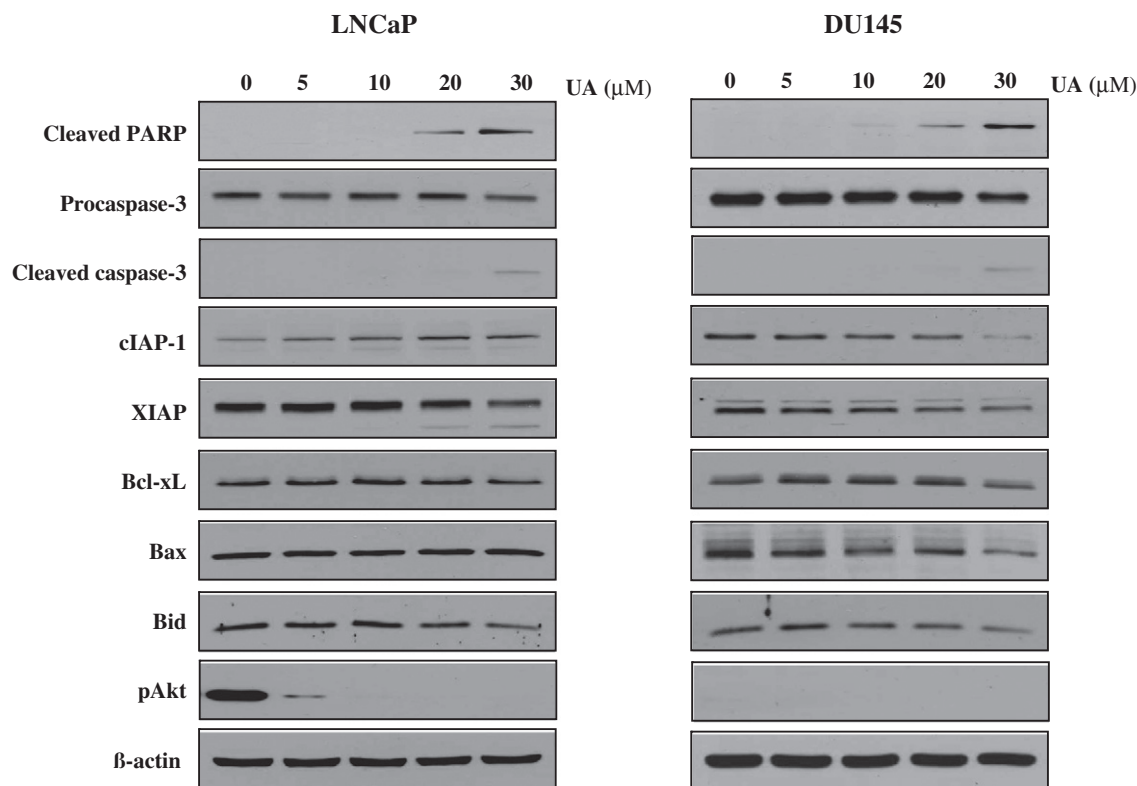
Fig. 2. Inhibitor of apoptosis proteins, c-IAP1, Bcl-xL, and XIAP were not involved in the sensitizing effect of UA on TRAIL-induced apoptosis. Cell extracts were prepared from LNCaP or DU145 cells treated with the indicated concentrations of UA for 24 h. Western blotting for markers and inhibitors of apoptosis was carried out. β-Actin was used as the internal control.

Chemical (St. Louis, MO). 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) were purchased from Molecular Probes (Eugene, OR). Antibodies to procaspase-3, Bcl-xL, Bax, Bid, DR4, DR5, HSP90β and β-actin were obtained from Santa Cruz (Santa Cruz, CA). Cleaved PARP, procaspase-8, caspase-3, caspase-9, pAkt, Grp78, CHOP, cIAP-1 and XIAP antibodies were purchased from Cell Signaling (Beverly, MA). Pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (Z-ITED-fmk), soluble recombinant TRAIL and DR5-specific blocking chimera antibody were purchased from R&D Systems (Minneapolis, MN).

2.2. Cell culture

LNCaP, PC3, DU145, A549, MCF7, HCT116, and HeLa cells were obtained from American Type Culture Collection (Manassas, VA), and HCT116 p53($-/-$) cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Cells were cultured as monolayers at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 using Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin.

2.3. Cytotoxicity

Cells (2×10^4) were grown until 80% confluent in 96-well plates, and cell viabilities after treatment with various concentrations of UA for 1–2 days were assessed using an MTT assay. Viabilities were expressed as percentages of untreated controls.

2.4. Cellular redox status

The productions of intracellular reactive oxygen species (ROS) and of the superoxide anion were monitored using the oxidant-sensitive fluorescent probes DCFH-DA and DHE, respectively, by fluorescence-activated cell sorting (FACS) after incubating cells in PBS for 30 min at 37 °C with 5 μM DCFH-DA or 5 μM DHE.

2.5. Flow cytometry

Cells were collected at 2000 ×g for 5 min, washed once with cold PBS, and fixed in 70% ethanol for at least 2 h at −20 °C. After removing the ethanol by centrifugation, cells were stained with 1 ml of PI staining solution (50 μg PI, 100 units RNase A, 1.5% Triton X-100) for at least 1 h in the dark at 4 °C, and analyzed with FACS, Coulter Elite ESP Cell Sorter (Beckman). Cells with sub-G_1 nuclear contents were considered apoptotic. Values are expressed as percentages of total cell counts.

2.6. DNA fragmentation assay

To determine whether chromosomal DNA has been degraded into nucleosome-sized fragments, a 500 μl aliquot of lysis buffer (100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to a cell pellet (2×10^5 cells) and incubated at 37 °C overnight. DNA was obtained by ethanol precipitation, separated in 0.8% agarose gel, and visualized under UV light.

2.7. Western blot analysis

Cell lysates were resolved on a 10–12.5% SDS-PAGE and transferred to nitrocellulose membranes, which were incubated with primary antibodies and then with horseradish peroxidase-labeled anti-rabbit IgG. Immune complexes were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

2.8. Small interfering RNA (siRNA)

The siRNA duplexes used in this study were purchased from Invitrogen and had the following sequences: DR5, UUU AGC CAC CUU UAU CUC AUU GUC C; CHOP, AAG ACC CGC GGC GAG GUG AAG; Control, AAG ACC CGC GCC GAG GUG AAG. Cells were transfected with 20 nM oligonucleotide using Lipofectamine RNAi MAX (Invitrogen) in serum-free conditions according to the manufacturer's protocol. After incubation for 24 h, the cells were washed and supplemented with fresh medium containing 10% FBS.

2.9. Caspase activity assay

Cell were washed three times with chilled PBS and incubated with 75 μl of lysis buffer (50 mM Tris–Cl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM PMSF) for 30 min at 37 °C. The contents of three wells were then pooled and centrifuged at 20,000 ×g for 20 min at 4 °C. The supernatant was mixed (1:1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM DTT, 0.5 mM PMSF, 10% glycerol), and the reaction was initiated by adding 5 μl (5 mg/ml) of the colorimetric agent Ac-DEVD-pNA (caspase-3 substrate). The mixture was then incubated for 1 h at 37 °C, and caspase activity was measured by measuring absorbance at 405 nm. Caspase activities were calculated as (absorbance/mg of protein in a treated sample/ (absorbance/mg of protein in the untreated control sample).

2.10. Statistical analysis

Differences between two mean values were evaluated using the Student's t-test, and were considered significant when p values were <0.05.

3. Results and discussion

3.1. UA sensitized TRAIL-mediated apoptosis

TRAIL is considered a promising tumoricidal drug because it is known that it induces apoptosis specifically in malignant or transformed cells, but is not cytotoxic to normal cultured cells [25]. However, a considerable number of cancer cells are resistant to TRAIL, perhaps due to the expressional deregulation of antiapoptotic molecules [26,27]. Furthermore, several studies have shown that chemotherapeutic agents including dietary polyphenols and ionizing radiation can sensitize TRAIL-induced cytotoxicity [16,28,29], suggesting that TRAIL resistance in cancer cells can be overcome by effective sensitizers.

In our search of novel strategies to overcome cancer cell resistance, we investigated the anti-cancer effect of UA on human prostate cancer cell lines. In contrast to PC3 cells, LNCaP and DU145 cells were resistant to the cytotoxic effect of TRAIL (Fig. 1A). However, co-treatment with UA and TRAIL affected morphologic changes of LNCaP cells and DU145 cells. More specifically, apoptotic characteristics, such as, cell shrinkage, apoptotic bodies, and detachment from plates, were
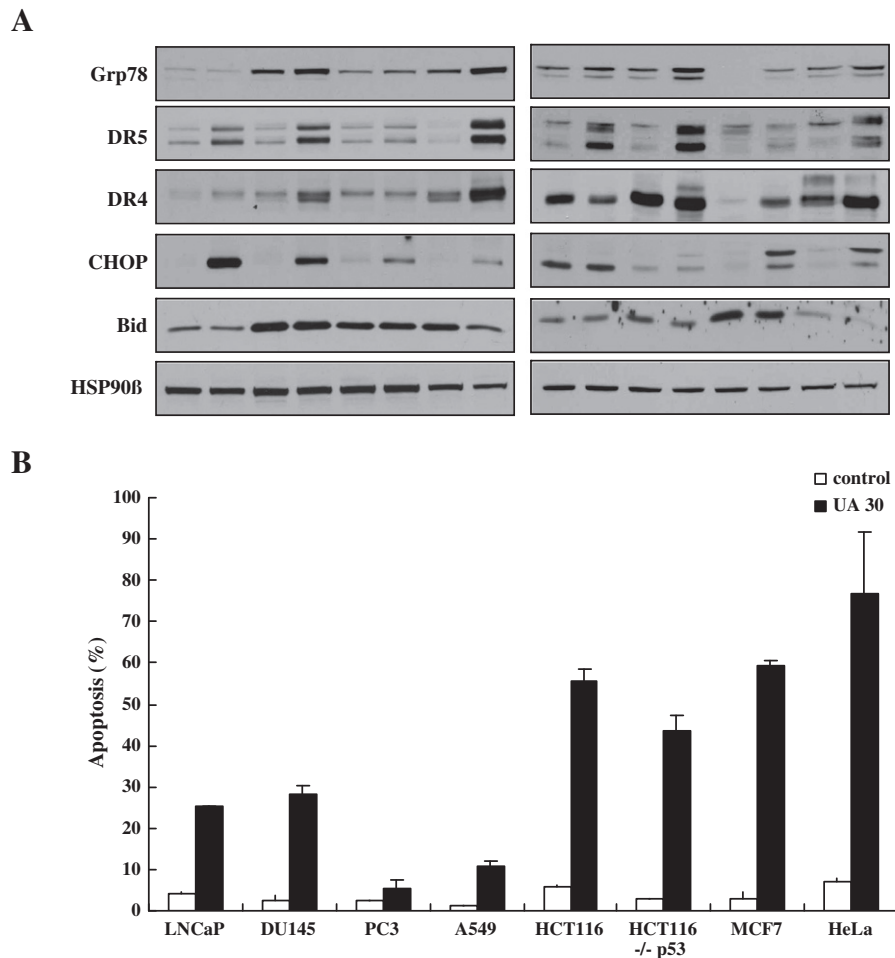

Fig. 3. The effects of UA on DR5 levels in various cancer cell lines. (A) Several cancer cells were treated with 30 μM UA for 24 h. Expression levels of DR5, DR4, Bid and CHOP were determined by Western blotting. HSP90β was used as the internal control. (B) LNCaP, DU145, PC3, A549, HCT116, HCT116(p53 −/−), MCF7, and HeLa cells were treated with 30 μM UA for 24 h, PI stained, and evaluated for DNA content. Fractions of apoptotic cells are shown. Data are presented as means ± S.D. of three separate experiments.

observed (Fig. 1B). To confirm the effect of UA on TRAIL-induced apoptosis, we measured apoptosis by FACS analysis of sub-G_1 fractions. The apoptosis rate increased markedly after co-treatment with UA and TRAIL as compared with either agent alone. Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA fragmentation in LNCaP cells co-treated with 20 μM UA and 50 ng/ml TRAIL (Fig. 1C). In addition, we examined whether co-treatment with UA and TRAIL activated DEVDases, key executioners of apoptosis. It was found that co-treatment of LNCaP cells with UA and TRAIL strongly stimulated DEVDase activity (Fig. 1D) and led to a reduction in the protein level of procaspase-3 with concomitant cleavage of PARP, a caspase substrate (Fig. 1E). The levels of cleaved forms of caspase-8 and -9 also increased after co-treatment. In addition, we examined the cleavages of apoptotic regulators, such as, XIAP and Bid and found that co-treatment degraded both. Furthermore, the pretreatment of LNCaP cells with z-VAD-fmk, a pancaspase inhibitor, or z-IETD, a caspase-8 inhibitor, significantly inhibited UA/TRAIL-activated DEVDase activity and the expression of apoptosis marker proteins (Fig. 1D and E).

## 3.2. UA upregulated DR5 in different cancer cells

Recent studies using affinity assays and phage displays of DR-selective TRAIL variants have revealed that DR5 may play a more prominent role than DR4 in TRAIL-mediated apoptotic signaling [30,31]. To assess the mechanism underlying this synergistic induction of apoptosis by UA and TRAIL in LNCaP cells and DU145 cells, we examined the effect of UA (5–30 μM) on the expression of apoptosis regulatory proteins by Western blotting. Treatment with subtoxic concentrations of UA (<20 μM) alone did not induce any significant changes in the protein levels of Bcl-xL, Bax, XIAP, Bid, caspase-3, or cIAP-1 (Fig. 2). In the recent study, it was shown that As_2O_3 induces an inhibition of Akt and increases the cell surface expression of DR5 [32]. Therefore, we examined whether these signaling molecules are similarly regulated by UA in prostate cancer cells. LNCaP cells lack active lipid phosphatase PTEN, a negative regulator of the PI3K/Akt pathway, and demonstrate a high constitutive Akt activity, whereas DU145 cells display high PTEN levels, which correlated with no detectable levels of phosphorylated Akt [33]. We found that Akt activity was down-regulated in LNCaP cells (Fig. 2). In contrast, UA treatment produced a significant increase in the DR5 protein expression in LNCaP and DU145 prostate cancer cells. We also examined whether DR5 was up-regulated by UA in other cancer cells, and found that DR5 was up-regulated in number of cancer cell types, such as, colon cancer cells (HCT116 and HCT116 p53 −/−), prostate cancer cells (LNCaP, DU145 and PC3), breast cancer cells (MCF7), cervical carcinoma cells (HeLa), and lung carcinoma cells (A549). In fact, UA induced apoptosis and DR5 up-regulation in all the cancer cell lines tested in this study (Fig. 3A), which suggests that DR5 up-regulation is common response to UA cancer cells and that UA-induced DR5 expression is associated with UA-induced apoptosis (Fig. 3B). Several studies have provided evidence that DR5 up-regulation is a promising
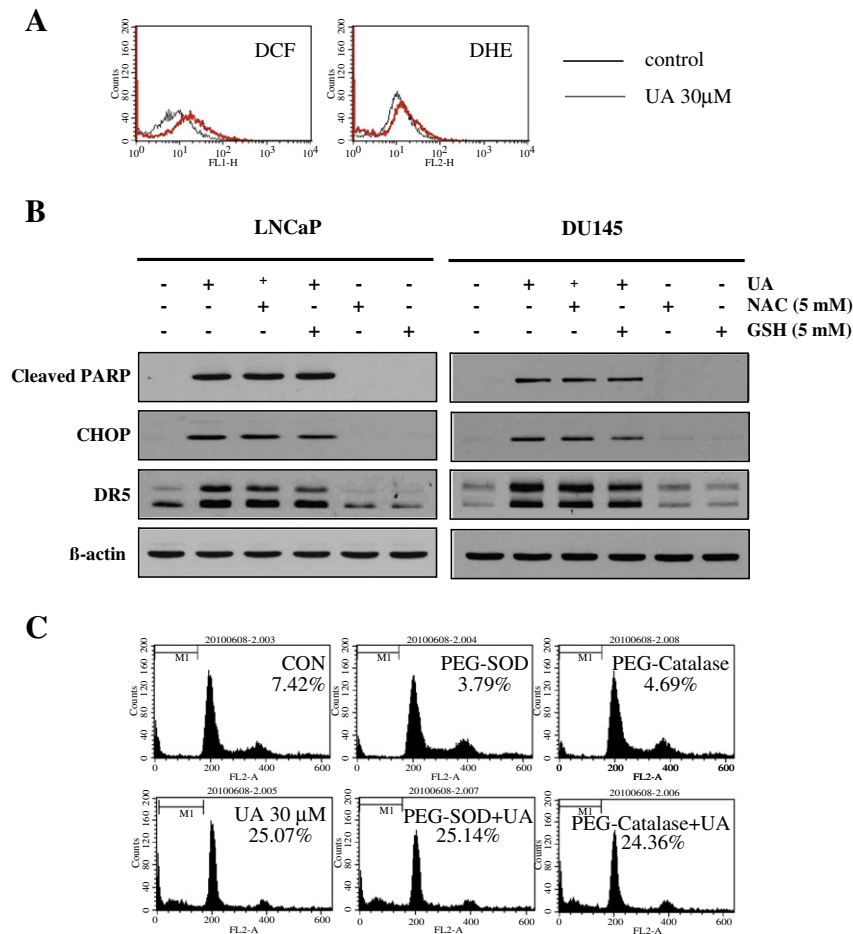
Fig. 4. ROS generation was not critical for the UA-induced up-regulation of DR5 or apoptosis. (A) Generation of ROS by UA. LNCaP cells were treated with 30 μM UA for 6 h and ROS levels were measured by FACS after staining with H_2DCF-DA or dihydroethidium, as described in Materials and methods. (B) Effects of NAC and GSH on UA-induced DR5 and CHOP protein levels. LNCaP cells were incubated with the indicated concentration of NAC or GSH for 1 h and then challenged with UA (30 μM) for 24 h. Equal amounts of cell lysates (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-CHOP, anti-DR5, and anti-cleaved-PARP antibodies. β-Actin was used as the internal control. (C) LNCaP cells were pretreated with PEG-SOD (200 U) or PEG-catalase (1000 U) for 1 h and then further treated with 30 μM UA for 24 h. Apoptosis was analyzed by FACS.
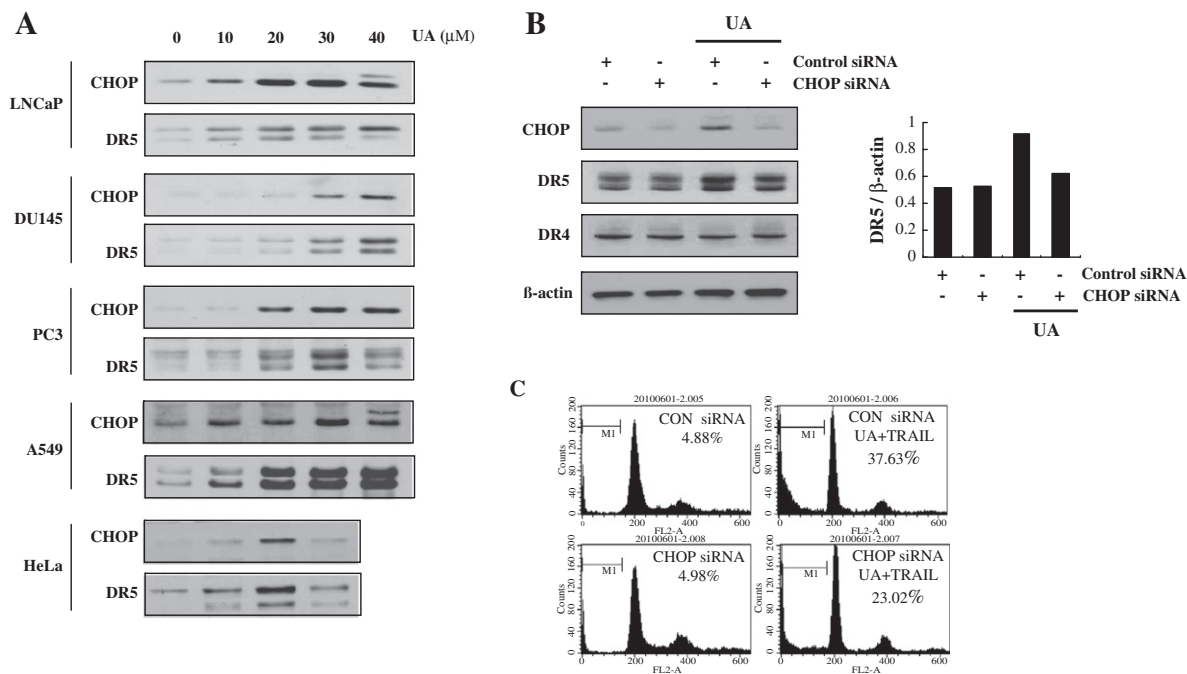
Fig. 5. CHOP induced DR5 up-regulation in various cancer cell lines exposed to UA. (A) Cell extracts from various cancer cell lines were treated with the indicated concentrations of UA for 24 h. Western blotting of CHOP and DR5 was performed. (B) Suppression of CHOP by siRNA reduced UA-induced DR5 up-regulation and UA-stimulated TRAIL-induced apoptosis. LNCaP cells were transfected with scrambled negative control RNA or siRNA duplexes against CHOP, incubated for 24 h, and then treated with 20 μM UA for 24 h. Western blotting of DR5 and DR4 was performed. DR5 bands were normalized to β-actin. (C) To examine the effect of CHOP down-regulation on UA-sensitized TRAIL-induced apoptosis, transfected cells with scrambled negative control RNA or CHOP siRNA were treated with 20 μM UA plus 50 ng/ml TRAIL for 24 h. Apoptosis was analyzed by FACS.

strategy for sensitizing cancer cells to TRAIL-induced apoptosis [34–36]. DR5 is regulated by either a p53-dependent or a p53-independent mechanism [37–39]. In the present study, UA induced the expression of DR5 in all cancer cell lines, regardless of p53 status, that is in; LNCaP and HCT116 (wild-type p53), DU145 cells (mutant p53), and PC3 and HCT116 p53 (−/−) cells (null p53 cells), indicating that UA up-regulates DR5 expression in a p53-independent manner.

3.3. ROS generation was not involved in UA/TRAIL-mediated apoptosis

ROS generation has been proposed to be involved in DR5 up-regulation by cancer chemopreventive agents, including curcumin and sulforaphane [34,35]. Hence, we examined whether UA-induced DR5 up-regulation is dependent on ROS levels in our systems. We used flow cytometric analysis to quantify ROS levels in UA-treated cells using DCFHDA. Results revealed that DCF fluorescence was significantly higher in LNCaP cells treated with 30 μM UA for 6 h than in untreated cells (Fig. 4A). Furthermore, flow cytometric analysis using the fluorescent probe DHE, which detects intracellular O_2^- production, showed an increase in O_2^- levels after UA treatment (Fig. 4A). Interestingly, however, pretreatment with the antioxidant NAC or GSH failed to inhibit UA-induced DR5 upregulation (Fig. 4B). Furthermore, neither PEG-catalase nor PEG-SOD blocked UA-induced apoptosis (Fig. 4B). Taken together, these results suggest that UA-generated ROS is not required for UA-induced DR5 up-regulation and UA/TRAIL-induced apoptosis. Alternatively, down-regulation of Akt by UA could be another mechanism of potentiation of TRAIL-induced apoptosis.

3.4. The UA-induced DR5 up-regulation was CHOP-dependent in prostate cancer cells

It has been suggested that CHOP is a potential transcription factor for DR5 [36,40,41]. Thus, CHOP protein levels were examined to investigate further the underlying mechanisms by which UA induces DR5 up-regulation. CHOP has recently been reported to be involved in

DR5 up-regulation mediated by several TRAIL sensitizers, such as proteasome inhibitor and tunicamycin [41,42]. Our results show that CHOP protein levels were significantly elevated in prostate cancer cells treated with UA, and that this increase preceded UA-mediated DR5 induction (Fig. 5A). Furthermore, UA induced CHOP up-regulation dose-dependently in the LNCaP, DU145, and PC3 cell lines, suggesting that CHOP up-regulation is a common response of prostate cancer cells to UA. CHOP siRNA was then transfected to clarify the functional roles of CHOP in UA-induced DR5 up-regulation. It was found that whereas DR5 was up-regulated by UA in LNCaP cells transfected with scrambled siRNA, transfection with CHOP siRNA abrogated the up-regulation of DR5 (Fig. 5B). In addition, UA/TRAIL-mediated apoptosis was significantly reduced by CHOP siRNA transfection (Fig. 5C). These results suggest that CHOP-dependent DR5 up-regulation contributes to the sensitizing effect of UA on TRAIL-induced apoptosis in human prostate cancer cells.

3.5. The up-regulation of DR5 by UA contributed to the enhancement of TRAIL-induced apoptosis in LNCaP and in DU145 cells

To confirm the biological significance of DR5 induction by UA in LNCaP cells, we knocked down DR5 expression using a siRNA duplex targeting DR5 mRNA and examined the effect of this on TRAIL-induced apoptosis. As shown in Fig. 6A, transfection of DR5 siRNA, but not transfection of scrambled siRNA, result in the suppression of UA induced DR5 up-regulation. Furthermore, the apoptotic population and the PARP cleavage induced by UA plus TRAIL were significantly inhibited in cells transfected with DR5 siRNA as compared with cells transfected with scrambled siRNA (Fig. 6B and C). To examine the functional role played by DR5 in the enhancement of TRAIL-induced apoptosis by UA, we used a recombinant human DR5/Fc chimeric protein, which has a dominant-negative effect by competing with endogenous DR5. As shown in Fig. 6D, the UA mediated enhancement of TRAIL-induced apoptosis was significantly blocked by DR5/Fc chimeric protein. In addition, treatment with DR5/Fc chimeric protein significantly attenuated cleavages of PARP, caspase-3 and caspase-8. Taken together, these results suggest that UA-induced DR5 is critical for the
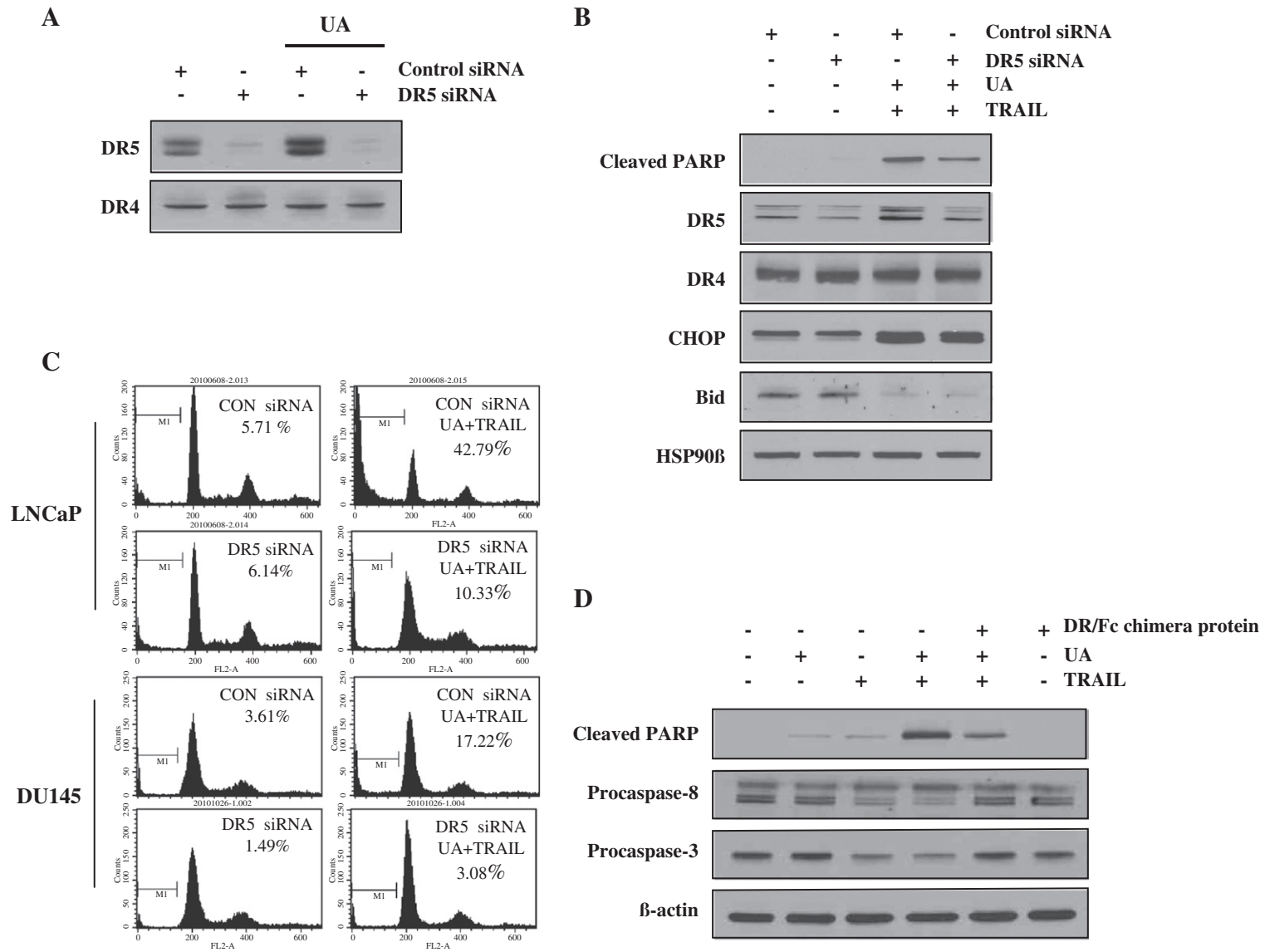
Fig. 6. Effects of DR5 down-regulation and of DR5/Fc chimeric protein on UA plus TRAIL-induced apoptosis. (A) Suppression of DR5 expression using DR5 siRNA reduces UA-induced DR5 up-regulation. LNCaP cells were transfected with scrambled negative control RNA or siRNA duplexes against DR5, incubated for 24 h, and treated with 20 μM UA for 24 h. Western blotting of DR5 was performed. (B) LNCaP cells were transfected with DR5 siRNA or scrambled control siRNA. At 24 h post-transfection, cells were treated with 20 μM UA plus 50 ng/ml TRAIL for 24 h. Equal amounts of cell lysates (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against cleaved PARP, DR5, DR4, CHOP, and Bid. HSP90β was used as the internal control. (C) Apoptosis was analyzed by FACS. (D) LNCaP cells were treated with UA plus TRAIL in the presence or absence of DR5/Fc chimeric protein (20 ng/ml). Equal amounts of cell lysates (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against cleaved PARP, procaspase-3, and procaspase-8. HSP90β was used as the internal control.

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enhancement of TRAIL sensitivity in LNCaP cells. Furthermore, DR5 knockdown by siRNA duplexes or by DR5-specific blocking chimeric antibody effectively attenuated the cell death induced by UA plus TRAIL.

4. Conclusions

The present study shows that co-treatment with UA and TRAIL induces apoptosis in human prostate cancer cells. Although animal studies are required to support its clinical application, our data provide mechanistic evidence that UA treatment results in CHOP-mediated DR5 upregulation and renders cancer cells more sensitive to the cytotoxic activities of TRAIL. Thus, our findings suggest that co-treatment with UA and TRAIL offers a novel strategy for treating a variety of human cancers resistant to chemotherapy.

Acknowledgments

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