Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in prostate cancer cells through DR4 and DR5 death receptors, but not in normal prostate cells, which do not express these receptors. Therefore, TRAIL has excellent potential to be a selective prostate cancer therapeutic agent with minimal toxic side effects. However, prostate cancer cells, as many other cancer types, develop resistance to TRAIL, and the underlying molecular mechanisms require further investigation. We hypothesize that selenium may sensitize TRAIL-resistant cells to undergo caspase-mediated apoptosis and increase therapeutic efficacy. Here, we report that TRAIL signaling in LNCaP prostate cancer cells stalled at downstream of caspase-8 and BID cleavage, as indicated by the lack of Bax translocation into mitochondria, and no subsequent activation of the caspase-9 cascade. Selenite induced a rapid generation of superoxide and p53 Ser^{15} phosphorylation and increased Bax abundance and translocation into the mitochondria. Selenite and TRAIL combined treatment led to synergistic increases of Bax abundance and translocation into mitochondria, loss of mitochondrial membrane potential, cytochrome *c* release, and cleavage activation of caspase-9 and caspase-3. Inactivating p53 with a dominant-negative mutant abolished apoptosis without affecting superoxide generation, whereas a superoxide dismutase mimetic agent blocked p53 activation, Bax translocation to mitochondria, cytochrome *c* release, and apoptosis induced by selenite/TRAIL. In support of Bax as a crucial target for cross-talk between selenite and TRAIL pathways, introduction of Bax into p53 mutant DU145 cells enabled selenite to sensitize these cells for TRAIL-induced apoptosis. Taken together, the results indicate that selenite induces a rapid superoxide burst and p53 activation, leading to Bax up-regulation and translocation into mitochondria, which restores the cross-talk with stalled TRAIL signaling for a synergistic caspase-9/3 cascade-mediated apoptosis execution. [Mol Cancer Ther 2006;5(7):1873–82]

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), the newest member of the tumor necrosis factor-α family, selectively induces apoptosis in a variety of cancer and transformed cells through the DR4 and DR5 death receptors, which are not expressed in most normal cells (1). Therefore, TRAIL has attracted intense interest as a promising agent under development for cancer therapy. Unfortunately, cancer cells develop resistance to TRAIL-induced apoptosis. For example, the human prostate cancer LNCaP cells are ~10 times more resistant to TRAIL-induced apoptosis than DU145 prostate cancer cells (2, 3). Elevated AKT in LNCaP cells has been shown as a major cause of resistance to TRAIL apoptosis (2, 3). Agents that can sensitize resistant cancer cells to TRAIL might be particularly important for developing combination regimens that can increase the overall therapeutic efficacy of TRAIL.

It has been well established that caspase-mediated apoptosis in most cells is induced through the activation of either the mitochondrial (intrinsic) pathway or the death receptor (extrinsic) pathway (4, 5). The mitochondrial pathway generally involves an induction of mitochondrial permeability transition and subsequent release of cytochrome *c* and other proapoptotic factors. Cytochrome *c*, the CARD adapter protein APAF-1, and propcaspase-9 assemble in the cytosol into the apoptosome, leading to caspase-9 activation, which in turn cleaves and activates the effector caspases, such as caspase-3. The death receptor pathway involves the engagement of the death receptors and the recruitment of the adaptor protein FADD and procaspase-8 to form a complex known as the death-inducing signaling complex. The consequent proximity of procaspase-8 proteins in the death-inducing signaling complex allows their autocleavage and activation. Active caspase-8 can directly activate caspase-3, the activity of which is often further regulated by the abundance of inhibitors of apoptosis. Efficient apoptotic signaling often requires a cross-talk between the two pathways via caspase-8 cleavage of BID, a BH3-only proapoptotic member of Bcl-2 family. Truncated BID translocates into the mitochondria and, through the

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activation of the proapoptotic Bcl-2 family members Bax and Bak, permeabilizes these organelles, leading to the release of cytochrome *c* and SMAC/Diablo. SMAC binds inhibitors of apoptosis, relieving caspase-3 for a full activation. It has been shown that TRAIL can activate caspase-8, but the signal fails to connect with the mitochondria for amplification in LNCaP cells (2, 3). Therefore, disabling the cross-talk between these two pathways may be one of the key mechanisms underlying the resistance of LNCaP cells to TRAIL. Conversely, agents that restore the cross-talk and mitochondria response may be able to overcome the resistance to TRAIL.

Selenium is an essential micronutrient for humans and animals. In the last three decades, selenium has been extensively studied as a cancer chemopreventive agent. A couple of human cancer prevention trials have indicated that a supranutritional selenium supplement might be a safe and effective preventive agent for several major cancers, including those of the prostate, lung, colon (6, 7), and liver (8). Several new selenium trials are under way to validate the prostate and the lung cancer preventive efficacies (9, 10) The potential use of selenium compounds for cancer combination chemotherapy has until recently received little attention (11–14). Animal and cell culture studies have shown that the anticancer activity of selenium mainly depends on the dosage and the chemical form (15–17). Recently, we have shown that a prototype monomethylated selenium, methylseleninic acid, but not the inorganic sodium selenite, specifically enhances apoptosis induced by diverse classes of chemotherapeutic drugs, such as Taxol, SN-38, and etoposide, in DU145 and PC-3 prostate cancer cells (12). Methylseleninic acid, but not selenite, has been also reported to enhance apoptosis induced by TRAIL in DU145 cells (13). Neither DU145 nor PC-3 cells contain a functional p53 (18). We and others have shown that methylselenium compounds, such as methylseleninic acid or methylselenocysteine, induce caspase-mediated apoptosis (17, 19) and the process seems to be independent of p53 functionality (20). Our earlier work has shown that selenite induces apoptosis of DU145 cells in the absence of caspase activity (17), but in the p53 wild-type LNCaP cells, selenite induces apoptosis predominantly through p53-dependent caspase activation (21). These observations suggest that the activity of selenium compounds to sensitize apoptosis by TRAIL or chemodrugs may depend on their efficacy to activate caspases. Because we and others have shown that selenite can induce DNA single-strand breaks (22–24) and that DNA damage invariably trigger p53 activation (25), we hypothesize that the status of p53 and/or its downstream transcriptional target Bax, which is crucial for regulating mitochondrial integrity and caspase-9 pathway, may be key determinants for selenite to sensitize cancer cells to TRAIL or drug-induced apoptosis.

Here, we report that selenite greatly sensitizes LNCaP cells, but not DU145 cells, to TRAIL-induced apoptosis. Our data show that TRAIL-induced death signaling in LNCaP cells stalls at downstream of BID cleavage. We also show that selenite-induced superoxide and p53 signaling are required for the sensitization effect in LNCaP cells, permitting TRAIL signaling (caspase-8 and BID) to converge on Bax to synergistically induce its up-regulation and mitochondria translocation, which in turn leads to the disruption of mitochondrial membrane potential, cytochrome *c* release, and caspase-9/3 activation. Ectopic expression of Bax in DU145 cells restores the enhancement effect of selenite on TRAIL-induced apoptosis in this Bax-deficient line. Our findings highlight selenite as a potential sensitizing agent for prostate cancer therapy with TRAIL in a p53/Bax-dependent manner.

Materials and Methods

Chemicals and Reagents

TRAIL (*Killer*TRAIL) and manganese(III) tetrakis(*N*-methyl-2-pyridyl)porphyrin (MnTMPyP), a superoxide dismutase mimetic chemical, were purchased from Alexis Biochemicals (San Diego, CA). Sodium selenite pentahydrate was purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Furosemide, etoposide, and an antibody for β-actin were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroethidine and 3,3′-dihexyloxacarbocyanine ($DIOC_6$) were purchased from Molecular Probes (Eugene, OR). An antibody against Bax was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against total p53 was purchased from PharMingen (San Jose, CA). The antibodies against DR4 and DR5 were purchased from Oncogene Research Products (San Diego, CA). The antibodies against caspase-8, caspase-9, caspase-3, BID, and phosphorylated p53 (Ser^{15}) were purchased from Cell Signaling Technology (Beverly, MA). A specific caspase-9 inhibitor z-LEHD-fmk was purchased from MP Biomedicals (Aurora, OH). An antibody against $p21^{WAF1}$ was purchased from Calbiochem (La Jolla, CA).

Cell Culture and Treatments

LNCaP and DU145 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Dominant-negative (DN) mutant p53 LNCaP-DN-P151S and the vector-transfected cells (26) were generously provided by Dr. Ralph W. deVere White (Department of Urology, University of California, Davis, CA). LNCaP, DN-P151S, and the vector cells were grown in RPMI 1640 with 10% fetal bovine serum. Regular DU145 and Bax-expressing DU145 cells were grown in DMEM with 10% fetal bovine serum. When cells were 50% to 60% confluent, the medium was changed before the treatment was initiated with selenite or the other agents.

Apoptosis Evaluation

Apoptosis was assessed by multiple methods as we recently reported (11, 20, 21). The first was a cell death ELISA kit purchased from Roche Diagnostics Corp. (Indianapolis, IN). This assay detects oligonucleosomes released after gentle lysis of the cells. Briefly, cells were cultured in T25 flasks for the desired duration. The spent medium containing floating cells was saved and kept on ice. The adherent cells were collected by gentle

trypsinization and were combined with the floaters for pelleting by centrifugation. After gentle lysis of the cells with the buffer provided with detection kit, the cell lysate was used for the ELISA test. The results were normalized by the protein content, which was determined by the Lowry method using a reagent kit from Sigma. The second method was Annexin V staining of externalized phosphatidylserine in apoptotic cells by flow cytometry using Annexin V/FITC staining kit (MBL International, Inc., Watertown, MA). The third method was immunoblot analysis of poly(ADP-ribose) polymerase cleavage of as described previously (17).

Analysis of Reactive Oxygen Species

Intercellular reactive oxygen species (ROS) generation was measured by flow cytometry following staining with hydroethidine, which has been shown to specifically detect superoxide (27). The hydroethidine is a reduced form of ethidium. Upon oxidation by superoxide, red fluorescent ethidium accumulates in the nucleus. The cells were treated with TRAIL and/or selenite with or without MnTMPyP for desired duration. At 30 minutes before harvest, hydroethidine was added to the medium to a concentration of 2 μmol/L. The cells were collected as described above, and the ethidium fluorescence intensities were measured using a Becton Dickinson (San Jose, CA) flow cytometer.

Immunoblot Analyses

Both floating and attached cells were harvested as described above. The cell pellet was washed twice in PBS and the lysate was prepared in radioimmunoprecipitation assay buffer as described previously (11, 21). Immunoblot analyses were essentially as described (11, 17), except that the signals were detected by enhanced chemifluorescence with a Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA).

Cell Fractionation

Mitochondrial isolation was done using ApoAlert cell fractionation kit purchased from Clontech (Palo Alto, CA). Briefly, cells were harvested by centrifuged at 600 × *g* for 5 minutes at 4°C. The pellets were washed once with ice-cold wash buffer provided with the kit and resuspended in 0.8 mL ice-cold fractionation buffer containing protease inhibitor mixture and DTT. After incubation on ice for 10 minutes, cells were homogenized using 2 mL Kontes Dounce tissue grinder. Homogenates were centrifuged at 700 × *g* for 10 minutes at 4°C. The supernatants were cytosolic fractions, and the pellets were lysed with the fractionation buffer as the mitochondrial fractions.

Measurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential was measured by flow cytometry following staining with $DIOC_6$, a cationic dye that has been shown to accumulate primarily in mitochondria with active membrane potential. The cells were harvested as described above. The cell pellet was resuspended in PBS with 40 nmol/L $DIOC_6$ and incubated at 37°C for 15 minutes. Fluorescence intensity was measured using a Becton Dickinson flow cytometer with excitation at 488 nm and emission at 520 nm.

Results

Sodium Selenite Greatly Sensitizes LNCaP Cells, but not DU145 Cells, to TRAIL-Induced Apoptosis

LNCaP cells are more refractory to TRAIL-induced apoptosis than the DU145 cells (2, 3). We confirmed this differential sensitivity in dose-finding experiments (Fig. 1A). For example, apoptosis induced by 50 ng/mL TRAIL in LNCaP cells was equal to that induced by 12.5 ng/mL TRAIL in DU145 cells. To increase the likelihood of detecting a sensitization of TRAIL-induced death by selenium, we chose doses of selenite and TRAIL that by themselves would only induce minimal apoptosis: 50 ng/mL TRAIL and 3 μmol/L selenite for the combination treatment in LNCaP cells and 12.5 ng/mL TRAIL and 3 μmol/L selenite for the combination treatment in DU145 cells. As shown in Fig. 1B, TRAIL (50 ng/mL) or selenite (3 μmol/L) alone caused modest increases in apoptosis after 24 hours of treatment of LNCaP cells. Combining TRAIL with selenite increased apoptosis detected by Annexin V staining of externalized phosphatidylserine in

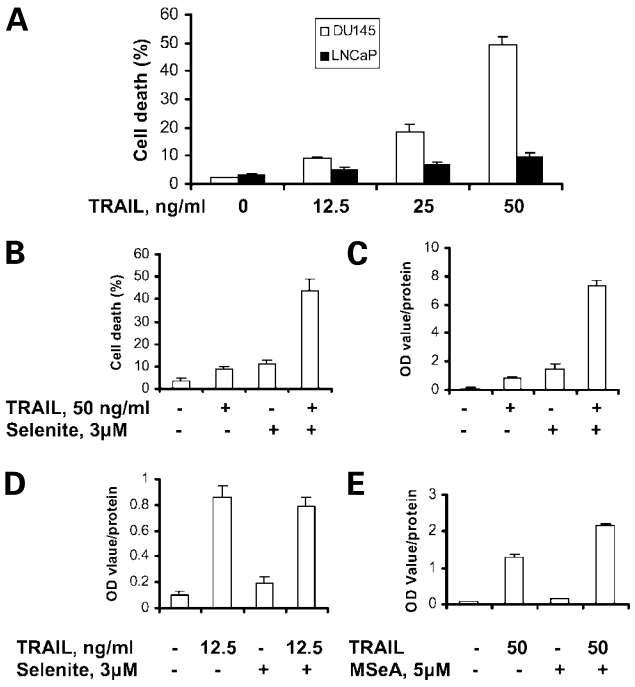

Figure 1. Selenite sensitizes LNCaP but not DU145 cells to TRAIL-induced apoptosis. Floating and attached cells were collected for apoptosis assays. **A,** dose response of TRAIL-induced apoptosis in LNCaP and DU145 cells. The cells were treated with TRAIL for 24 h and stained with Annexin V for externalized phosphatidylserine in apoptotic cells. **B** and **C,** selenite sensitizes LNCaP cells to TRAIL. The cells were treated with TRAIL and/or selenite for 24 h. Apoptosis was detected by Annexin V staining (**B**) and by ELISA kit for DNA fragmentation (**C**). **D,** selenite fails to sensitize DU145 cells to TRAIL. The cells were treated with TRAIL and/or selenite for 24 h and apoptosis was detected by ELISA kit for DNA fragmentation. **E,** methylseleninic acid (*MSeA*) modestly sensitizes LNCaP cells to TRAIL-induced apoptosis. The cells were treated with TRAIL and/or methylseleninic acid for 24 h and apoptosis was detected by ELISA kit for DNA fragmentation.

apoptotic cells by 3.2-fold over the sum achieved by the single-agent treatment alone (Fig. 1B). The apoptosis sensitization phenomenon was further confirmed using the Roche Death ELISA kit for DNA fragmentation (Fig. 1C). In contrast, combining TRAIL with selenite did not exert any enhancement effect in comparison with single-agent treatment in DU145 cells (Fig. 1D).

It has been shown that methylseleninic acid can sensitize DU145 and LNCaP cells to TRAIL-induced apoptosis, whereas selenite does not have this activity in DU145 cells (13). To compare the magnitude of the sensitization effects between the two forms of selenium, we used the same experimental design and apoptosis evaluation methods to measure the enhancement effects of methylseleninic acid on TRAIL-induced apoptosis in LNCaP cells. As shown in Fig. 1E, TRAIL and methylseleninic acid combination increased apoptosis by ~1.6-fold over the sum achieved by the single-agent treatment alone. These results indicated that selenite sensitized LNCaP cells, but not DU145 cells, to TRAIL-induced apoptosis and that the sensitization effect by selenite was much stronger than methylseleninic acid in LNCaP cells.

Selenite-Induced ROS Are Required for the Sensitization Effect in LNCaP Cells

ROS, mainly superoxide, has been implicated as a mediator of selenite-induced apoptosis in LNCaP cells (28) and DU145 cells (29). To examine whether ROS signaling was involved in the sensitization effects, we measured superoxide generation and determined the effect of a superoxide dismutase mimetic MnTMPyP on apoptosis induced by the combined treatment. Intracellular superoxide was measured by flow cytometry following staining with hydroethidine (Fig. 2A). As shown in Fig. 2B, TRAIL alone did not show any effect on ethidium fluorescence intensity (*column 2* versus *column 1*), whereas 3 μmol/L selenite increased ethidium fluorescence in a time-dependent manner, which was evident at 2 hours of treatment (*column 3* versus *column 1*). Combining TRAIL and selenite did not further increase the fluorescence intensity compared with selenite alone (*column 4* versus *column 3*). However, scavenging superoxide signaling by MnTMPyP (Fig. 2B, *column 6* versus *column 4*) completely blocked the sensitization effects on apoptosis (Fig. 2C). These results indicated that superoxide generation by selenite was necessary for its sensitization of LNCaP cells to TRAIL-induced apoptosis, but the synergy did not occur by generating more superoxide.

Selenite Sensitizes LNCaP Cells to TRAIL-Induced Apoptosis in a p53-Dependent Manner

Our previous studies showed that selenite induced p53 Ser^{15} phosphorylation and caspase-mediated apoptosis in LNCaP cells (21). To investigate whether selenite-induced p53 signaling was involved in the sensitization effect, we tested the effect of DN-P151S on apoptosis induced by the combination treatment. Inhibition of p53 activity was validated by the attenuation of p53-dependent $p21^{WAF1}$ expression induced by etoposide, a topoisomerase II inhibitor that causes DNA double-strand breaks. As shown

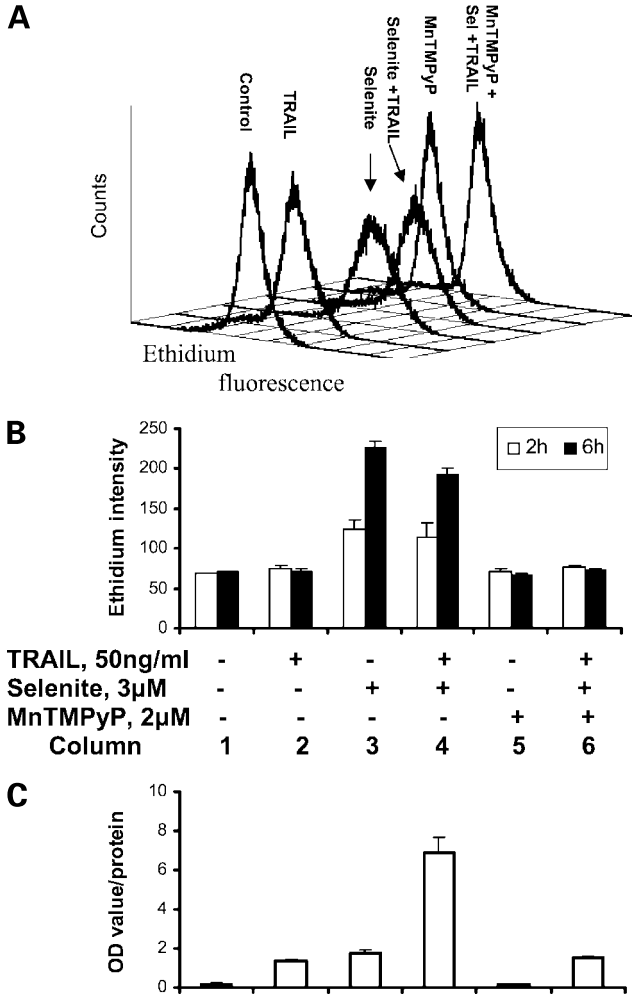

Figure 2. Role of superoxide signaling in the sensitization effect. **A,** superoxide detection by flow cytometry following staining with dihydroethidine. LNCaP cells were treated with TRAIL and/or selenite with or without MnTMPyP (pretreatment 1 h) for 6 h. **B,** mean fluorescence intensity of each group at 2 and 6 h. **C,** apoptosis outcome of the treated LNCaP cells at 24 h. Floating and attached cells were collected for apoptosis detection by ELISA kit for DNA fragmentation.

in Fig. 3A, etoposide treatment led to a significant elevation of p53 protein and $p21^{WAF1}$ in the vector transfectant. The same treatment caused only a slight increase of $p21^{WAF1}$ in DN-P151S cells. The vector and DN-P151S cells showed similar apoptosis responses to TRAIL alone, but DN-P151S cells were much less responsive to apoptosis induced by selenite than the vector cells (Fig. 3B). Moreover, the enhancement effect of selenite on TRAIL-induced apoptosis was completely abolished in P151S cells compared with the vector cells (Fig. 3B). These results indicate that the sensitization effect was heavily dependent on p53-mediated signaling.

To further clarify the role of p53 in the sensitization effect, we used Western blotting to analyze p53 phosphorylation and total p53 protein after combination treatment. As

shown in Fig. 3C, TRAIL alone did not affect the level of either p53 phosphorylation or total p53 (*lane 2* versus *lane 1*), whereas selenite increased both (*lane 3* versus *lane 1*). However, combining TRAIL with selenite did not further increase these variables over selenite alone (*lane 4* versus *lane 3*). The results indicated that the synergistic death signaling did not occur at p53 protein abundance or phosphorylation level, but selenite-induced p53 signaling was necessary and essential for the sensitization effects in LNCaP cells.

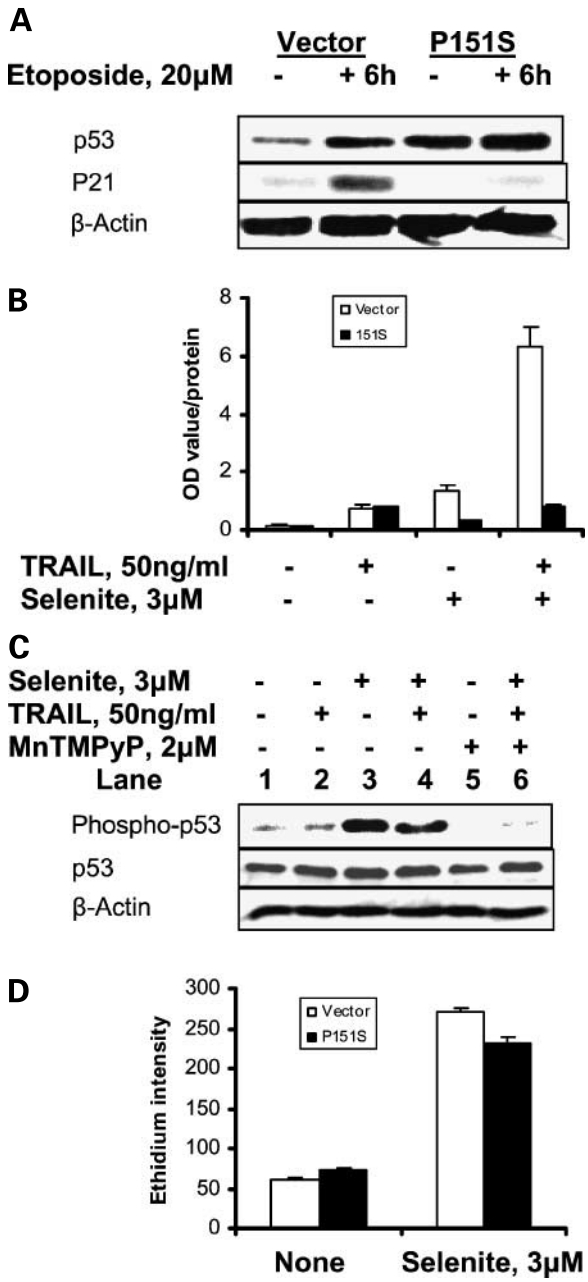

Figure 3. Role of p53 signaling in the sensitization effect. **A,** Western blotting analysis of induction of p53 and $p21^{cip1}$ after 6 h of treatment by etoposide in DN-P151S and the vector-transfected LNCaP cells. **B,** apoptosis induction by TRAIL and/or selenite in DN-P151S and vector-transfected cells after 24 h of treatment. Apoptosis was detected by ELISA kit for DNA fragmentation. **C,** Western blotting analysis of p53 induction by TRAIL and/or selenite in LNCaP cells after 8 h of treatment. **D,** comparison of superoxide generation between P151S and the mock cells induced by selenite after 6 h of treatment.

To test whether superoxide generation was upstream of p53 activation, we examined the effects of MnTMPyP on p53 phosphorylation status. As shown in Fig. 3C, inhibition of superoxide generation completely blocked p53 phosphorylation induced by the combined treatment (*lane 6* versus *lane 4*). Yet, as shown in Fig. 3D, selenite induced a comparable level of superoxide in both DN-P151S and the vector cells. The results showed that superoxide generation was upstream of p53 activation and did not require p53 activation.

Combining TRAIL with Selenite Synergistically Induces Caspase-9/3 Activation

It has been shown that TRAIL resistance in LNCaP cells was due to inefficient signaling from caspase-8 to downstream executioner caspase-3, which can be overcome by an inhibition of the phosphatidylinositol 3-kinase/AKT survival pathway (2, 3). We have shown that selenite-induced apoptosis in LNCaP cells involves both caspase-8 and caspase-9 pathways (21). We hypothesize that selenite-induced caspase-9 activation (mitochondrial response) amplifies TRAIL-induced apoptosis by restoring cross-talk between caspase-8 and caspase-9 pathways. To test this hypothesis, we analyzed by Western blotting the expression or cleavage status of caspase-8, caspase-9, caspase-3, and a caspase-8 substrate, the BH3-only proapoptotic protein BID. As shown in Fig. 4A, TRAIL alone induced a significant cleavage of caspase-8 and its substrate BID without a detectable cleavage of caspase-9 (*lane 2* versus *lane 1*), whereas 3 μmol/L selenite alone induced a modest cleavage of caspase-8 and caspase-9 without the cleavage of BID (*lane 3* versus *lane 1*). TRAIL and selenite combined caused a slight further increase of cleavage of caspase-8 and BID but dramatically increased caspase-9 and caspase-3 cleavage (*lane 4* versus *lane 3*). To verify the role of caspase-9 activation in apoptosis induction by the combination treatment, we tested the effect of a specific caspase-9 inhibitor on apoptosis induced by combination treatment. As shown in Fig. 4B, 20 μmol/L z-LEHD-fmk completely blocked the enhanced apoptosis (*column 5* versus *columns 2* and *4*). The results indicated that TRAIL-induced death signaling was blocked downstream of BID and upstream of caspase-9. Combining selenite with TRAIL enabled TRAIL-induced death signaling to go through mitochondria to synergistically induce caspase-9 activation.

To determine whether the activation of mitochondria/caspase-9 pathway by combining TRAIL with selenite involved a decrease of AKT activity, we carried out Western blotting to analyze the phosphorylation level of AKT. As shown in Fig. 4C, TRAIL alone did not affect AKT phosphorylation level (*lane 2* versus *lane 1*), whereas selenite alone or combining with TRAIL even increased

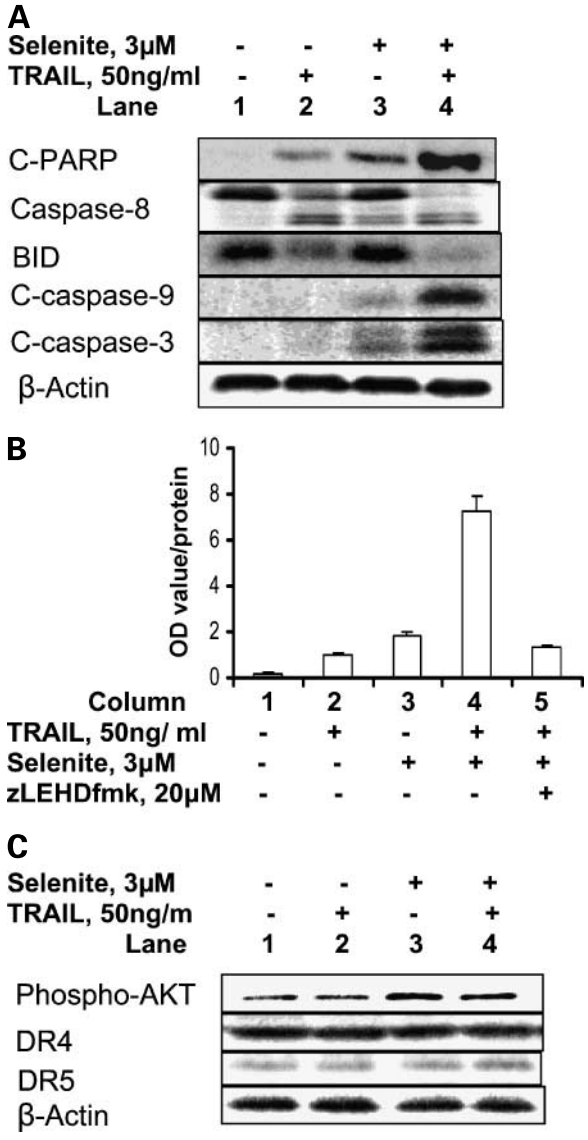

Figure 4. Role of caspase-9 activation in the sensitization effect. **A,** Western blotting analysis of caspase-8, caspase-9, caspase-3, BID, and poly(ADP-ribose) polymerase cleavage in LNCaP cells after 24 h of treatment with TRAIL and/or selenite. **B,** effect of caspase-9 inhibitor z-LEHD-fmk on apoptosis induction by combined TRAIL/selenite treatment 24 h. Apoptosis was detected by ELISA kit for DNA fragmentation. **C,** Western blotting analysis of the levels of AKT phosphorylation and death receptors DR4 and DR5 after 8 h of treatment with TRAIL and/or selenite.

AKT phosphorylation level (*lanes 3* and *4* versus *lane 1*). Previous studies have shown that DR5 was up-regulated by selenite in DU145 cells (30). Thus, we examined whether selenite might up-regulate death receptors to contribute to the sensitization of LNCaP cells to apoptosis induced by TRAIL. Selenite or TRAIL treatment alone or their combination did not significantly change the levels of DR4 and DR5 (Fig. 4C, *lane 3* or *4* versus *lane 1*), ruling out DR4 and DR5 up-regulation as targets for the synergy. These results together indicate that combining TRAIL with selenite increased cross-talk with mitochondrial caspase-9 pathway independently of the AKT pathway and without up-regulating DR4 and DR5 protein abundance.

Combining TRAIL with Selenite Synergistically Induces Bax Up-Regulation and Activation in LNCaP Cells

To further identify potential molecular target(s) through which TRAIL and selenite act synergistically to induce caspase-9 activation, we next focused on Bax because it is a known transcriptional target of p53 and a binding partner for BID (31, 32). As shown in Fig. 5A, TRAIL alone did not affect Bax protein level (*lane 2* versus *lane 1*), whereas selenite moderately increased Bax level (*lane 3* versus *lane 1*). Combining TRAIL with selenite caused a further increase in Bax level (*lane 4* versus *lane 3*). Furthermore, Bax mostly remained in the cytosol of TRAIL-treated cells (Fig. 5B, *lane 2* versus *lane 1*), whereas selenite treatment increased Bax translocation from the cytosol into the mitochondria (Fig. 5B, *lane 3* versus *lane 1*). Combining TRAIL with selenite greatly increased Bax translocation to mitochondria, which in turn led to significant decrease of the cytosolic Bax (*lane 4* versus *lanes 1* and *3*). This event was blocked by MnTMPyP (Fig. 5B, *lane 6* versus *lane 4*).

To test the function of Bax translocation, we examine the effect of furosemide, a chloride channel inhibitor that can prevent Bax mitochondria translocation (33), on apoptosis induction by the combined treatment. As shown in Fig. 5C, furosemide significantly blocked Bax translocation to the mitochondria induced by TRAIL with selenite and decreased apoptosis in proportional to the inhibition of Bax translocation (*lane 4* versus *lane 2*). The results suggest that a ROS-p53-dependent synergistic activation of Bax was crucial for TRAIL and selenite-induced apoptosis.

TRAIL and Selenite Synergistically Induce ROS-Dependent Disruption of Mitochondrial Membrane Potential and Cytochrome *c* Release

Based on the literature, we expected that increased Bax translocation to mitochondria induced by the combined treatment would disrupt mitochondrial integrity. To test this, we measured mitochondrial membrane potential by flow cytometry after staining the cells with the strong cationic dye $DIOC_6$ (Fig. 5D). As shown in Fig. 5E, TRAIL alone did not affect mitochondrial membrane potential (*column 2* versus *column 1*), whereas selenite induced a modest increase of mitochondrial permeability transition compared with control (*column 3* versus *column 1*). In contrast, TRAIL and selenite combined treatment dramatically increased mitochondrial permeability transition (*column 4* versus *columns 1* and *3*), which was completely blocked by MnTMPyP (*column 6* versus *column 4*). Consistent with mitochondrial permeability transition response patterns, TRAIL and selenite synergistically increased cytochrome *c* release from mitochondria to cytosol (Fig. 5E, *lane 4* versus *lanes 1* and *3*), which also was nearly blocked by an inhibition of ROS (*lane 6* versus *lanes 3* and *4*). The results provided strong evidence that the mitochondria were central targets in TRAIL and selenite combination-induced apoptosis in LNCaP cells.

Selenite Sensitizes Bax-Expressing DU145 Cells to TRAIL-Induced Apoptosis

Selenite did not sensitize DU145 cells, which do not express Bax, to TRAIL-induced apoptosis (Fig. 1D). Because Bax plays a key role in TRAIL and selenite combination-induced apoptosis in LNCaP cells, we hypothesize that introducing Bax into the DU145 cells can restore the sensitization effect of selenite on TRAIL in this cell line. The retroviral vector HKTK-Bax was employed to infect DU145 cells to reconstitute Bax protein expression as described previously (34). Western blotting showed that Bax was undetectable in mock cells but highly expressed in Bax/DU145 cells (Fig. 6A). Whereas selenite did not sensitize the mock cells to TRAIL induction of apoptosis, Bax/DU145 cells restored the ability for selenite to sensitize TRAIL-induced apoptosis (Fig. 6B).

Discussion

Prostate cancer is the most common malignancy and second leading cause of cancer-related death in American men. Because conventional antiproliferative chemotherapeutic drugs are not effective for prostate cancer treatment due to its slow growth, apoptosis induction is an attractive approach for clinical therapy of prostate cancer. Because the normal cells do not express the TRAIL receptors DR4 and DR5 (1), TRAIL has been shown to induce apoptosis in prostate cancer cells but not in normal prostate cells (2, 3). The differential apoptosis response between normal prostate and prostate cancer cells to TRAIL increases enormously the usefulness of TRAIL as a selective biological therapeutic agent. However, an obstacle to effective therapy is that prostate cancer, as many other cancers, develops resistance to TRAIL. Novel agents are needed to overcome the resistance to improve TRAIL efficacy.

To this end, we found, for the first time, that a minimal apoptotic dose of selenite greatly sensitized p53 wild-type LNCaP cells to TRAIL-induced apoptosis (Fig. 1B and C). A strong sensitization effect was also observed in p53 mutant DU145 cells that were infected with a retroviral vector expressing Bax (Fig. 6), and androgren-independent Bax-positive LNCaP-C4-2 cells (data not shown). Because Bax is a downstream transcriptional target of p53 (31), these results indicate that the enhancement of the therapeutic efficacy of TRAIL by selenite may be more effective in patients with p53 wild-type prostate cancer. Our findings here in concert with the reported enhancement of TRAIL-induced apoptosis by methylseleninic acid in p53 nonfunctional prostate cancer cells (13) suggest that combining these two forms of selenium may be merited to increase the response spectrum of prostate cancers to TRAIL therapy. These predictions should be investigated in the future in appropriate animal models before translation into the clinic.

The molecular mechanisms underlying the resistance of prostate cancer cells to TRAIL have been investigated in a limited number of studies. Expression of certain apoptotic regulating genes, such as AKT (35, 36), nuclear factor-κB

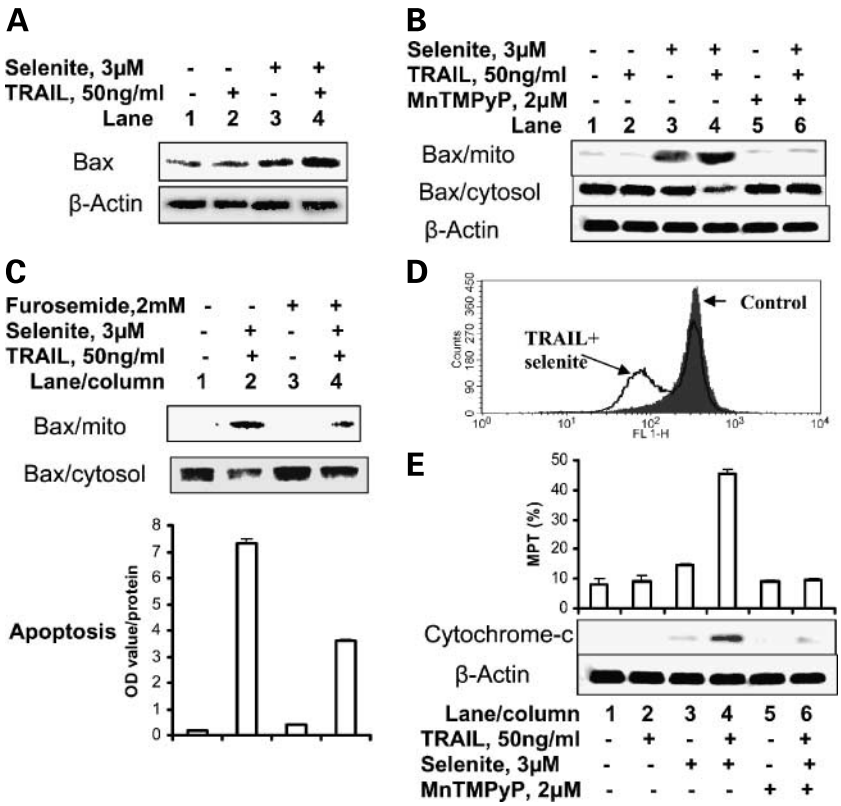

Figure 5. Role of Bax in TRAIL/selenite induction of synergistic apoptosis in LNCaP cells. **A,** Western blotting analysis of total Bax protein level after 24 h of treatment with TRAIL and/or selenite. **B,** Western blotting analysis of Bax distribution in cytosol and mitochondria after 8 h of treatment with TRAIL and/or selenite with or without MnTMPyP. **C,** effects of furosemide on Bax translocation and apoptosis induced by TRAIL/selenite for 12 h. **D,** detection of mitochondrial permeability transition in LNCaP cells treated with TRAIL/selenite for 8 h. Mitochondrial uptake of $DIOC_6$ was measured by flow cytometry following staining. **E,** percentage of cells with decreased $DIOC_6$ uptake (% *MPT*) after treatment with TRAIL and/or selenite with or without MnTMPyP for 8 h (*columns*) and Western blotting analysis of cytochrome *c* in cytosol.

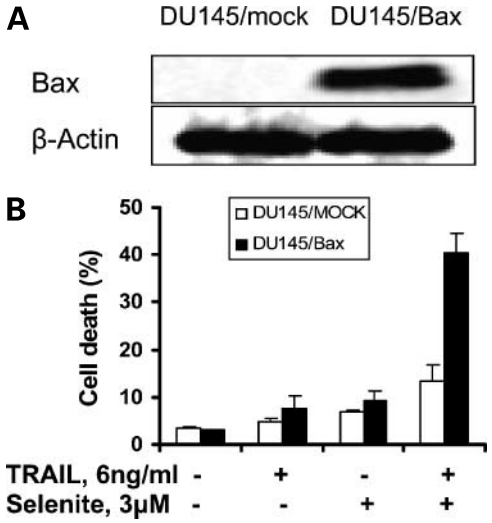

Figure 6. Effect of restoring Bax expression in DU145 cells on TRAIL/selenite-induced apoptosis. **A,** Western blotting analysis of Bax protein abundance in stable cell lines obtained by infection with retroviral vector (mock) or Bax-expressing construct. **B,** apoptosis induction by TRAIL and/or selenite in Bax-expressing and the mock cells after 48 h of treatment. Apoptosis was measured by Annexin V staining.

(37, 38), Bcl-2 (39), Bax (40), and c-FLIP (41, 42), has been suggested to modify the sensitivity of cancer cells to TRAIL-induced apoptosis. The lack of response of LNCaP cells to TRAIL seems to be due to elevated AKT activity, which presumably blocks TRAIL-induced signaling at BID level (2, 3). It is known that truncated BID can activate the conformation of Bax and related protein Bak (31). This activation of Bax and Bak by truncated BID is required to initiate mitochondrial dysfunction and apoptosis (32). We have found that TRAIL alone induced significant caspase-8 and BID cleavage but without a detectable induction of mitochondrial permeability transition, cytochrome *c* release, and caspase-9 activation (Figs. 4A and 5E). Such findings are consistent with recent studies showing that the mitochondrial response to TRAIL is low in these cells (43). Moreover, recent work shows that an activation of Bax (40) or a down-regulation of the prosurvival mitochondrial protein Bcl-2 (44) can sensitize DU145 and LNCaP cells to TRAIL, suggesting that mitochondrial response plays an important role for the sensitivity of prostate cancer cells to TRAIL. Our data therefore suggest that although TRAIL signaling transduces to BID in the LNCaP cells, the truncated BID alone cannot trigger mitochondrial activation with the regular abundance of Bax (Fig. 5B). The reasons that truncated BID cannot efficiently activate Bax translocation in LNCaP cells need to be further investigated. In contrast to TRAIL treatment alone, combining TRAIL with selenite caused a significant induction of mitochondrial permeability transition, cytochrome *c* release, and caspase-9 activation (Figs. 4A and 5E). These events correlated excellently with increased Bax abundance and Bax translocation to mitochondria (Fig. 5B). Functional blocking of Bax translocation confirmed its important role in the enhanced apoptosis by the combination treatment (Fig. 5C). Therefore, selenite treatment bridges Bax to truncated BID, allowing for efficient TRAIL signaling to mitochondria for synergistically enhanced caspase-9 activation and apoptosis execution. The central importance of this caspase cascade was confirmed by the complete reversal of synergistic apoptosis by the caspase-9 inhibitor (Fig. 4B).

In terms of upstream proximal signaling, our study supports a critical role of superoxide generation and p53 activation induced by selenite to bridge TRAIL signaling to the mitochondria. The role of ROS and p53 in apoptosis induction has been documented in a few cell types (21, 45, 46). Sodium selenite was capable of inducing rapid superoxide generation (Fig. 2B) and p53 phosphorylation (21), and both have been implicated in selenite-induced apoptosis (21, 28). Combining selenite with TRAIL failed to further increase either of them beyond that induced by selenite alone. However, inhibition of either ROS signaling by MnTMPyP (Fig. 2C) or p53 signaling by a DN mutant (Fig. 3B) completely blocked the enhancement effects.

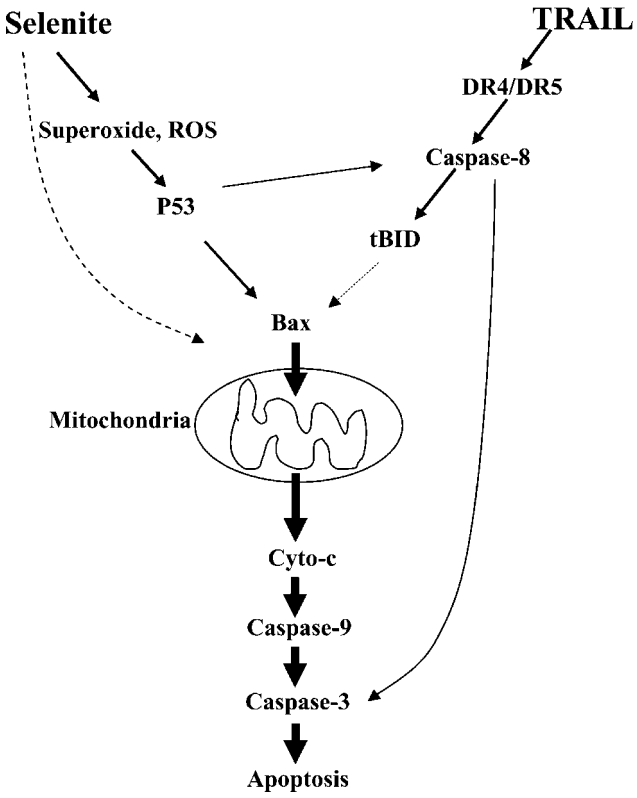

Figure 7. Signaling pathways underlying TRAIL and/or selenite-induced apoptosis in LNCaP prostate cancer cells. TRAIL induces caspase-8 activation and BID cleavage, whereas selenite induces superoxide generation and p53 activation. These two death signaling pathways synergistically induce Bax expression and mitochondrial translocation, which in turn trigger mitochondrial pathway to amplify the death execution.

These results together rule out ROS and p53 as the targets for the combination treatments to exert synergistic apoptosis but do affirm them as essential and necessary initial death signaling for selenite to amplify TRAIL-induced death at the mitochondria level through Bax.

Concerning the specificity of selenium compounds for the enhancement effect, our previous studies have shown that selenite and methylseleninic acid exert distinct effects on apoptosis, cell cycle, and protein kinases in LNCaP cells (20) and DU145 cells (29). Our present work showed that the sensitization effects of selenite on TRAIL were much stronger than methylseleninic acid in LNCaP cells (Fig. 1B and C versus Fig. 1E). The differential effects correlated with superoxide generation and p53 signaling, which can be induced by selenite but not methylseleninic acid (20, 21). It has been shown that inhibiting AKT sensitized LNCaP cells to TRAIL-induced apoptosis (2, 3). Therefore, a decrease of AKT phosphorylation by methylseleninic acid (20, 29) might be one of the mechanisms for its sensitization of LNCaP cells to TRAIL. However, an increase of AKT phosphorylation by selenite was observed here (Fig. 4C), suggesting that selenite sensitization of LNCaP cells to TRAIL was independent of the AKT activity. A recent study has shown that methylseleninic acid sensitizes DU145 cells to TRAIL-induced apoptosis through a decrease of c-FLIP and TRAIL-medicated phosphorylation of Bad (13). We found that selenite did not decrease the expression of c-FLIP and that TRAIL did not induce Bad phosphorylation in LNCaP cells (data not shown). Collectively, it is very likely that selenite and methylseleninic acid sensitize prostate cancer cells to TRAIL-induced death signaling through different mechanisms of caspase activations. It could be expected that combining these two types of selenium with TRAIL may widen the spectrum of prostate cancers responsive to the combination therapy.

Finally, selenite sensitizes Bax-expressing DU145 (Fig. 6), but not the mock-infected cells (Fig. 6) or Bax-deficient regular DU145 cells (Fig. 1D), supporting Bax as a critical target/mediator of the sensitization effects. It is important to point out that the sensitization could be detected within 5 to 6 hours in LNCaP cells but took 20 hours in DU145/Bax cells (data not shown). The different p53 status between LNCaP (wild-type) and DU145 (mutant) may account for the different kinetics of response. We speculate that combining TRAIL with selenite induces a rapid Bax activation through p53-dependent pathways in LNCaP cells and a delayed Bax activation through p53-independent pathway in DU145 cells. This hypothesis is currently being investigated.

In summary, we showed (see scheme in Fig. 7) that TRAIL signaling in LNCaP cells stalled downstream of BID cleavage in that truncated BID failed to promote Bax translocation into mitochondria and the subsequent activation of caspase-9 cascade. Selenite-induced superoxide and p53 signaling sensitized LNCaP cells to TRAIL by increasing Bax abundance and mitochondrial translocation, restoring cross-talk with truncated BID. Our findings suggest that combining TRAIL with selenium forms that target p53-Bax activation may be useful to improve the efficacy of TRAIL for the chemotherapy of prostate cancer with intact p53 or Bax pathway. Use of a mixture of selenite and methylseleninic acid with TRAIL would be expected to target a broader spectrum of prostate cancer.

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