Expression of p53 Enhances Selenite-Induced Superoxide Production and Apoptosis in Human Prostate Cancer Cells

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Abstract

Although the anticancer effects of selenium have been shown in clinical, preclinical, and laboratory studies, the underlying mechanism(s) remains unclear. Our previous study showed that sodium selenite induced LNCaP human prostate cancer cell apoptosis in association with production of reactive oxygen species, alteration of cell redox state, and mitochondrial damage. In the present study, we showed that selenite-induced apoptosis was superoxide mediated and p53 dependent via mitochondrial pathways. In addition, we also showed that superoxide production by selenite was p53 dependent. Our study showed that wild-type p53–expressing LNCaP cells were more sensitive to selenite-induced apoptosis than p53-null PC3 cells. Selenite treatment resulted in high levels of superoxide production in LNCaP cells but only low levels in PC3 cells. LNCaP cells also showed sequential increases in levels of phosphorylated p53 (serine 15), total p53, Bax, and p21Waf1 proteins following selenite treatment. The effects of selenite were suppressed by pretreatment with sodium superoxide dismutase mimic or by knockdown of p53 via RNA interference. LNCaP cells treated with selenite also showed p53 translocation to mitochondria, cytochrome c release into the cytosol, and activation of caspase-9. On the other hand, restoration of wild-type p53 expression in PC3 cells increased cellular sensitivity to selenite and resulted in increased superoxide production, caspase-9 activation, and apoptosis following selenite treatment. These results suggest that selenite induces apoptosis by producing superoxide to activate p53 and to induce p53 mitochondrial translocation. Activation of p53 in turn synergistically enhances superoxide production and apoptosis induced by selenite. (Cancer Res 2006; 66(4): 2296-304)

Introduction

Selenium, an essential nutritional trace element, is a key element in maintenance of the activity of some antioxidant enzymes and redox-regulatory proteins (1, 2). A number of epidemiologic studies have shown an inverse association between selenium levels in toenails or serum and cancer risks in humans (3–6). Studies found that individuals with higher selenium levels in toenails or serum had significant reduction in prostate cancer incidence and the risk of advanced prostate cancer (7, 8). The most compelling findings were from a double-blind, placebo-controlled, randomized cancer prevention trial conducted by Clark et al. (9). In this study, human subjects given a daily dose of 200 μg of selenium supplementation showed a 63% decrease in prostate cancer incidence compared with the placebo group. The results of this study led to a current larger phase III, double-blind, placebo-controlled clinical trial, Selenium and Vitamin E Chemoprevention Trial (10).

At present, the anticancer mechanism(s) of selenium is still unclear. Several mechanisms have been postulated: (a) maintenance of glutathione peroxidase activity to protect against oxidative damage to DNA, proteins, and membrane lipids; (b) prevention and/or detoxification of carcinogenic intermediate metabolites of chemical carcinogens; (c) stimulation of the immune system; (d) effects of selenium intermediate metabolites on the cell cycle and apoptosis; (e) modulation of thioredoxin reductase activity and cell reduction/oxidation (redox) state, which in turn regulate cell signal transduction, transcription factor activation, and DNA repair; and (f) inhibition of angiogenesis (1, 2, 11, 12). Among these potential mechanisms of selenium action, cell cycle regulation and apoptosis have received the most attention and have been postulated to be critical for cancer chemoprevention by selenium compounds (13). Although apoptotic effects of selenium are dramatic and reproducible in experimental studies, the exact pathways have yet to be elucidated. There is an increasing interest in cell redox modulation by selenium because studies have shown that selenium treatment altered the intracellular redox state, resulting in alteration in the activity of transcription factors, cell cycle regulatory proteins, signal transduction molecules, and cell apoptosis (14–20).

Our previous study showed that selenite induced apoptosis and growth inhibition of human prostate cancer cells in association with production of reactive oxygen species (ROS), alterations in levels of intracellular reduced glutathione (GSH) and glutathione disulfide, and mitochondrial damage (15). Selenite treatment also up-regulated the antioxidant enzymes manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZn-SOD), glutathione peroxidase, and the cell cycle arrest gene p21Waf1 (16). These effects of selenite were suppressed by a synthetic superoxide dismutase (SOD) mimic. A number of studies from other laboratories have confirmed that selenite-induced apoptosis was mediated by ROS production (21–25). We also showed that overexpression of MnSOD prevented cell death from selenite treatment (26). In addition, studies from other laboratories have shown that selenium compounds modulated p53 (14, 27–29). Taken together, these results suggest that superoxide and p53 may be involved in apoptosis induced by selenite.

The aims of the present study were to investigate the role of p53 and superoxide and apoptotic pathways in selenite-induced apoptosis in human prostate cancer cells. We compared cellular sensitivity to selenite between wild-type (wt) p53–containing LNCaP and p53-null PC3 human prostate cancer cell lines and...
determined that down-regulation or reexpression of wt p53 altered the cellular effects of selenite. We used an SOD mimick, manganese(III)tetraakis(N-methyl-2-pyridyl)porphyrin (MnTMPyP), to determine that activation of p53 by selenite treatment was mediated by superoxide production via posttranscriptional modification. Finally, we determined that superoxide production by selenite was also p53 dependent and apoptosis induced by selenite was via mitochondrial pathways.

Materials and Methods

Chemicals and antibodies. Sodium selenite and anti-p53-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Lucigenin (bis-N-methylacridinium nitrate) was purchased from Molecular Probes (Eugene, OR). MnTMPyP was purchased from Alexis Biochemicals (San Diego, CA). p53 and Bax small interfering RNAs (siRNA) and anti-p53 Ser15 antibody were purchased from Cell Signaling Technology (Beverly, MA). siRNA Duplex control (nonsilence) and RNAiFect Transfection Reagent were purchased from Qiagen (Valencia, CA). Apoptotic DNA-Ladder kit was purchased from Roche Diagnostic Co. (Indianapolis, IN). Caspase-Glo 9 Assay kit was purchased from Promega Co. (Madison, WI). SuperSignal West Pico Stable Peroxide and Luminol/Enhancer Solutions, M-per Mammalian Protein Extraction Reagent, and Mitochondria Isolation Kit were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Anti-p21(CDKN1A) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Bax and anti–phospho-histone H2AX (Ser139) antibodies were purchased from Upstate USA, Inc. (Charlottesville, VA).

Cell culture. LNCaP (ATCC CRL-1740) and PC3 (ATCC CRL-1435) cells were obtained from the American Type Culture Collection (Manassas, VA) and routinely maintained in 100-mm tissue culture dishes (Corning) in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies, Inc., Rockville, MD) at 37°C in a humidified atmosphere of 95% air and 5% CO2. For biochemical analyses, cells were collected by rinsing in PBS thrice, scraping with a rubber policeman in 10-mL PBS, and then centrifuging at 2,000 rpm for 5 minutes. After removing the PBS, the cell pellets were stored at −80°C until use.

Superoxide measurement. Lucigenin-dependent chemiluminescence in cells was measured by a modified method as previously described (30). The stock solution of lucigenin (10 mmol/L) was prepared in PBS and stored at −20°C in the dark. Lucigenin (100 μmol/L) was added to 1 × 10^6 cells in 100 μL PBS and incubated with or without 5 μmol/L MnTMPyP for 30 minutes before selenite treatment. The reaction was initiated by the addition of lucigenin and selenite to the cells and the chemiluminescence level was monitored as relative light units using a Lumat LB9501 luminometer (Berthold Tech USA, Oak Ridge, TN) for a total period of 15 minutes at 30-second intervals.

Flow cytometric analysis. Cell samples were prepared and analyzed as previously described (15). After selenite treatment and trypsinization, 1 × 10^6 cells were washed with PBS/EDTA/bovine serum albumin (BSA) buffer (PBS, 1 mmol/L EDTA, and 0.1% BSA) and fixed in 100 μL of PBS/EDTA/BSA buffer plus 900 μL of 70% ethanol for 30 minutes at −20°C. After washing with phosphate-citric acid buffer (0.192 mol/L Na2HPO4 and 4 mmol/L citric acid, pH 7.8), the cells were stained in 500 μL of propidium iodide staining solution (33 μg/mL propidium iodide, 200 μg/mL DNase-free RNase A, and 0.2% Triton X-100) overnight at 4°C. Both cell cycle distribution and apoptotic cells were simultaneously measured in a Becton Dickinson FACScan flow cytometer (San Jose, CA) using 488-nm laser excitation.

Apoptotic DNA ladder analysis. DNA isolation and gel electrophoresis were done according to the instructions of the manufacturer. Briefly, after selenite exposure, cells were scraped in PBS and harvested by centrifugation at 500 × g for 5 minutes at room temperature and then lysed in 400 μL lysis buffer for 10 minutes at room temperature. Following an addition of 100 μL isopropanol, the lysate was centrifuged through a filter and washed with the washing buffer. Genomic DNA was eluted with 100 μL elution buffer.

DNA samples were loaded onto a 1.5% agarose gel containing 0.1 mg/mL ethidium bromide and electrophoresed. The gel was photographed with Kodak Image Station 2000R using UV illumination and digitized with Kodak I1D 3.6 software (Eastman Kodak Company, Rochester, NY).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were seeded at 1 × 10^5 per well in 24-well plates overnight before treatment with different agents and then allowed to grow for an additional 5 days. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μL; 5 mg/mL in PBS) was added to each well of the plate and incubated for 3 hours at 37°C. MTT lysis buffer (100 μL of 10% SDS, 45% dimethyl formamide, adjusted to pH 4.5 by glacial acetic acid) was then added to dissolve the formazan. The absorbance was measured at 570 nm using a Beckman Coulter DU-640 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). The percentage of viable cells was calculated as the relative ratio of absorbance to the control.

Western blot analysis. Cell pellets were lysed with M-PER mammalian protein extraction reagent and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA). Cell lysates (20-50 μg) were electrophoresed in 12.5% SDS polyacrylamide gels and then transferred onto nitrocellulose membranes. After blotting in 5% nonfat dry milk in TBS-Tween 20, the membranes were incubated with primary antibodies at 1:1,000 to 2,000 dilutions in TBS-Tween 20 overnight at 4°C, and then secondary antibodies conjugated with horseradish peroxidase at 1:10,000 dilution in TBS-Tween 20 for 1 hour at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Pierce Biotechnology).

siRNA transfection. Cells were seeded at 1 × 10^5 per well in six-well plates and allowed to grow to 60% confluence. Cells were transfected with 50 nmol/L of p53 or Bax siRNA with 2 μL RNAiFect Transfection reagent in 1 mL of serum-free medium for 12 hours, and then 1 mL of fresh medium with 10% fetal bovine serum was added to each well for 24 hours before selenite treatment. Cells were also transfected with nontargeting, negative control siRNA which has no known homology to mammalian genes and allows assessing the possibility of nonspecific gene silencing effects.

Adenoviral p53 transduction. PC3 cells were seeded at 4 × 10^5 in 60-mm tissue culture dishes for Western blot analysis and at 1 × 10^5 per well in 24-well plates for viability assay. Approximately 20 hours later, cells were infected with the indicated multiplicity of infection (MOI) of recombinant Ad5 cytomegalovirus wt p53-GFP adenoviral constructs (Ad-p53) or with media alone (mock) in serum-free medium. After 12 hours, an equal volume of fresh medium with 10% FBS was added to each dish or well for 24 hours before selenite treatment. Cells were also transduced with adenoviral empty constructs as a control.

Activity assay of caspase-9. Cells were seeded at 3 × 10^5 per well in a 96-well plate with 100 μL medium. Approximately 16 hours later, cells were treated with 2.5 μmol/L selenite for 18 hours to induce apoptosis. Caspase-Glo 9 Reagent (100 μL) was directly added into each well to a final volume of 200 μL/well. Chemiluminescence was measured using a Tropix TR717 Microplate Luminometer (Applied Biosystems, Bedford, MA).

Mitochondria fractionation. Cells were seeded at 6 × 10^6 in 100-mm tissue culture dishes and allowed to grow to 60% confluence. Cells were treated with or without 2.5 μmol/L selenite for 18 hours to induce apoptosis, and then mitochondria and cytosol fractions were separated from cells according to the instructions of the manufacturer (Pierce Biotechnology).

Laser scanning confocal microscopy of p53 mitochondrial translocation. Two thousand cells were seeded in eight-well Lab-Tek Chamber Slides (Nalge Nunc International, Rochester, NY) in 450-μL culture medium. Twenty-four hours after plating, cells were treated with 2.5 μmol/L selenite for 18 hours and then incubated with 200 μmol/L Mitotracker Red CM-H2XROS (Molecular Probes) in culture medium for 30 minutes. After washing thrice with PBS, the cells were fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature, and then incubated with a monoclonal anti-p53 antibody at a 1:100 dilution (Ab-6, EMD Biosciences, San Diego, CA) followed by incubation with antimouse immunoglobulin G (IgG)-FITC (BD Biosciences, San Diego, CA) at a 1:200 dilution for 1 hour at 37°C. After
rinsing with PBS, the slides were mounted with a 90% glycerol medium. Images were immediately observed and captured using a Bio-Rad MRC 1024 laser scanning confocal microscope at ×60 magnification (Bio-Rad Laboratories, Hercules, CA).

**Statistical analysis.** All data were presented as mean ± SD from at least three sets of independent experiments. ANOVA analysis with Tukey’s multiple comparisons was used to determine the significance of statistical differences between data at the level of P < 0.05 using SPSS computer statistics software (SPSS, Inc., Chicago, IL).

**Results**

Superoxide-mediated apoptosis in selenite-treated LNCaP cells. LNCaP cells were treated with different doses of selenite for different time periods and cell viability was assessed by MTT assay. Selenite treatment decreased cell viability in a dose-dependent manner (Fig. 1A). Significant cell viability decreases occurred in cells treated with 1.5 µmol/L and higher doses of selenite. Time-dependent decreases in viability which started at 24 hours were shown in cells treated with 2.5 µmol/L selenite over 120 hours (data not shown). Gel electrophoresis showed DNA laddering (fragmentation) in cells treated with 2.5 µmol/L selenite but not in cells treated with 0.5 µmol/L selenite (Fig. 1B). Flow cytometric analysis showed time-dependent increases in sub-G₁ cell populations in cells treated with 2.5 µmol/L selenite (Fig. 1C). These data showed that cells underwent apoptosis following selenite treatment in a dose- and time-dependent manner. To assess possible involvement of superoxide in selenite-induced apoptosis, cells were pretreated with a synthetic SOD mimic, MnTMPyP. As shown in Fig. 1D, pretreatment with MnTMPyP significantly reduced selenite-induced apoptosis in LNCaP cells. Using a lucigenin-dependent chemiluminescence assay for assessment of superoxide levels, chemiluminescence increased in cells treated with 2.5 µmol/L selenite in 3 minutes and reached a peak value in 6 minutes. Chemiluminescence produced by selenite treatment was suppressed by MnTMPyP pretreatment (Fig. 1E). There was only minimal chemiluminescence detected in a mixture of only the culture medium and lucigenin (data not shown). These results showed that selenite treatment increased superoxide production to trigger apoptosis in LNCaP cells.

**Activation of p53 in LNCaP cells by selenite.** Our previous study showed that p21\(^{WAF1}\) was up-regulated in selenite-treated LNCaP cells (15), suggesting possible involvement of p53 in selenite-induced apoptosis. To determine whether p53 is activated by selenite treatment, Western blot analysis was used to detect

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**Figure 1.** Selenite causes apoptosis with superoxide accumulation in wt p53–expressing LNCaP cells. A, dose-dependent effect of selenite on cell viability as shown by the MTT assay. Cells were treated with 0 to 3.5 µmol/L selenite for 5 days. B, agarose gel electrophoretic detection of DNA fragmentation as a marker of cell apoptosis induced by selenite. Cells were treated with 2.5 µmol/L selenite for 24 hours. C, flow cytometric analysis of cell apoptosis by measuring the sub-G₁ cell population. Cells were treated with 2.5 µmol/L selenite for 24 and 48 hours. D, the SOD mimic MnTMPyP protected against cytotoxicity of selenite. Cells were treated with 0 to 3.5 µmol/L selenite with or without 5 µmol/L MnTMPyP for 5 days. E, chemiluminescence assay showing production of superoxide radicals in cells treated with selenite. Cells were treated with 2.5 µmol/L selenite, 5 µmol/L MnTMPyP, or selenite plus MnTMPyP. The treatment agents were added into the cell suspensions in test tubes and chemiluminescence was immediately measured. Points, mean of three independent experiments; bars, SD. *P < 0.05, compared with 0 µmol/L; #P < 0.05, compared with the corresponding concentration of selenite without MnTMPyP; **P < 0.05, compared with control (cells without treatment), MnTMPyP, Se + MnTMPyP, and Se at 1 minute.
levels of total p53 and phosphorylated p53 and its target genes p21Waf1 and Bax. As shown in Fig. 2A, selenite treatment resulted in elevations of total and phosphorylated p53 at serine 15 (p-p53 Ser15) in LNCaP cells in a dose-dependent manner. Significant elevations of p53 occurred in cells treated with selenite at doses of 2.0 \mu M/L and higher. The effect of selenite treatment on p53 was also time dependent (Fig. 2B). An elevation of total p53 was detected at 1 hour following 2.5 \mu M/L selenite treatment, reached a peak value at 6 hours, and was persistent up to 36 hours, although there was a slight decrease at 24 and 36 hours. An increase in p-p53 Ser15 was detected at 3 hours following selenite treatment, reached a peak value at 6 hours, and was maintained at a steady state up to 36 hours. Protein levels of both p21Waf1 and Bax were elevated in a dose- and time-dependent manner, corresponding to the elevation of p53 observed following selenite treatment (Fig. 2A and B). The effects of selenite on these proteins were suppressed by MnTMPyP pretreatment (Fig. 2C). It has been previously reported that selenite treatment caused DNA damage (31–33). A DNA damage marker, phosphorylated histone H2AX on serine 139 (H2AX), was analyzed by Western blotting (34). As shown in Fig. 2A and B, there were no significant increases in this phosphorylated protein in cells treated with different doses of selenite or 2.5 \mu M/L selenite for different times. This result suggests that DNA damage is not likely to be the mechanism of cell killing at the concentrations of selenite used in this study.

Role of p53 and Bax in selenite-induced apoptosis in LNCaP cells. We next determined the role of p53 and Bax in selenite-induced apoptosis using RNA interference to reduce mRNA of these two genes. As shown in Fig. 3A, cells transfected with p53 siRNA had decreased sensitivity to selenite whereas p53 sensitivity did not change in cells transfected with the control siRNA. The Western blot analysis showed that levels of total p53, p-p53 Ser15, p21Waf1, and Bax increased in cells treated with selenite and these effects of selenite were suppressed by p53 siRNA transfection, but not by the negative control siRNA, indicating that the effects of p53 siRNA were specific and selective (Fig. 3B). However, p53 siRNA treatment only partially suppressed Bax expression, suggesting up-regulation of Bax by selenite treatment may also be p53 independent. This conclusion is supported by the results of p53-null PC3 cells reported in the next figure, in which selenite treatment up-regulated Bax in the absence of p53 expression. Although Bax siRNA treatment also reduced cellular sensitivity to selenite, the magnitude was much lower than that of p53 siRNA (Fig. 3C). The Western blot analysis showed that Bax siRNA transfection decreased levels of Bax protein but did not affect elevation of p53 and p21Waf1 induced by selenite treatment, indicating that Bax is a downstream gene. The results showed that siRNA selectively down-regulated p53 and Bax, respectively. Down-regulation of p53 decreased the cellular sensitivity to selenite. Regulation of Bax was not completely p53 dependent and selenite-induced apoptosis was not completely mediated by Bax.

Effect of wt p53 on cellular response to selenite in p53-null PC3 cells. To verify that cellular sensitivity to selenite is dependent on p53, we next tested the sensitivity of p53-null PC3 cells to selenite and whether restoration of wt p53 expression altered sensitivity of PC3 cells to selenite. Figure 4A shows the dose- and time-dependent effects of selenite in PC3 cells. PC3 cells were 2-fold more resistant to selenite than LNCaP cells (Fig. 1A). To reduce cell viability by 50% required 1.5 \mu M/L selenite for LNCaP cells (Fig. 1A) and 3 \mu M/L selenite for PC3 cells (Fig. 4A). Time course studies showed that 50% of LNCaP cells were killed by 2.5 \mu M/L selenite at 48 hours (data not shown) whereas only 20% of PC3 cells were killed at 48 hours and <40% of cells were killed at 120 hours (Fig. 4B). Western blot analyses showed that PC3 cells had no detectable p53 and p21Waf1 and had very low levels of Bax (Fig. 4B). Following transduction of Ad-p53, PC3 reexpressed p53 and p21Waf1 in a dose-dependent manner. At 0.5 MOI of Ad-p53 transduction, selenite treatment induced phosphorylation of p53 Ser15 whereas p53 Ser15 was also phosphorylated in cells transduced with \geq 1 MOI Ad-p53 without selenite treatment. Selenite treatment alone or transduction of control adenoviral constructs alone significantly increased levels of Bax. Levels of Bax were further increased in cells transduced with 2 or 4 MOI of Ad-p53. These data showed that expression of p21Waf1 was p53 dependent whereas Bax was not completely p53 dependent. These results were consistent with the results of LNCaP cells (Fig. 3B and D). Figure 4D shows that reexpression of wt p53 enhanced sensitivity of PC3 cells to selenite in a dose-dependent manner. Transduction of control adenoviral constructs alone did not alter cellular response to selenite.

Dependence of selenite-induced superoxide production on p53 status in LNCaP and PC3 cells. Because selenite-induced cell apoptosis was more dependent on p53 than Bax, we next analyzed
other mechanisms by which selenite induced p53-dependent apoptosis. We first reduced levels of p53 in LNCaP cells by siRNA transfection. As shown in Fig. 5A, transfection of p53 siRNA suppressed selenite-induced elevation of superoxide although p53 siRNA alone increased levels of superoxide. The latter was mostly likely due to the combined effects of RNaiFect and siRNA, suggesting that the transfection reagent may cause oxidative stress in cells. Similar effects on protein levels of p53 and p21WAF1 were also seen in RNA interference studies (Fig. 3B and D). In contrast, reexpression of wt p53 in PC3 cells significantly increased superoxide production following selenite treatment (Fig. 5B). There were no significant changes in superoxide production in cells treated with 0.5 μmol/L selenite (data not shown). These results showed that superoxide production and apoptosis by selenite treatment were enhanced by p53. Superoxide may act as a downstream mediator of apoptosis as well as a mediator of p53 phosphorylation and activation.

p53-dependent, superoxide-mediated mitochondrial pathways of apoptosis induced by selenite treatment. To explore involvement of mitochondrial pathways in cell apoptosis, mitochondria were isolated from the cytosol of LNCaP cells. Mitochondrial translocation of p53 and release of cytochrome c from the mitochondria into the cytosol were determined by the Western blot analysis. As shown in Fig. 6A, protein levels of p53, p-p53 Ser15, p21WAF1, and Bax in cells with and without selenite treatment. C, Western blot analysis showing suppressive effects of p53 siRNA transfection on expression of p53, p21WAF1, and Bax in cells with and without selenite treatment. Cells were transfected with 50 nmol/L of p53 or Bax siRNA for 36 hours and then treated with selenite for 5 days for cell viability analysis or with 2.5 μmol/L selenite for 18 hours for Western blot analysis. Protein loading: 40 μg for p53, p-p53 Ser15, p21WAF1, Bax, and H2AX and 20 μg for β-actin. Points, mean of three independent experiments; bars, SD. *, P < 0.05, compared with control siRNA.

Figure 3. Down-regulation of p53, but not Bax, by RNA interference causes resistance to selenite-mediated cytotoxicity in LNCaP cells. A, MTT assay of viability of LNCaP cells with p53 siRNA transfection and selenite treatment. B, Western blot analysis showing suppressive effects of p53 siRNA transfection on expression of p53, p21WAF1, and Bax in cells with and without selenite treatment. C, MTT assay of cellular response to selenite following down-regulation of Bax by siRNA transfection. D, Western blot analysis of effects of Bax siRNA transfection on p53, p21WAF1, and Bax in cells with and without selenite treatment. A mixture of culture media and chemical reagents without cells produced only minimal background chemiluminescence (data not shown). These data show that apoptosis induced by selenite occurred in intact LNCaP cells following selenite treatment.

We next analyzed caspase-9 activity with a chemiluminescence assay. As shown in Fig. 6C, caspase-9 activity significantly increased in LNCaP cells treated with 2.5 μmol/L selenite for 18 hours. Activation of caspase-9 by selenite treatment was suppressed by transfection of p53 siRNA. In contrast to LNCaP cells, PC3 cells showed no significant increase in caspase-9 activity following selenite treatment (Fig. 6D). After restoration of wt p53 expression by adenoviral transduction, selenite treatment significantly increased caspase-9 activity in PC3 cells (Fig. 6D). A mixture of culture media and chemical reagents without cells produced only minimal background chemiluminescence (data not shown). These data show that apoptosis induced by selenite was p53 dependent via mitochondrial pathways.

Discussion

Studies have shown that ROS, particularly superoxide, are produced by several selenium compounds when they interact with thiols (21). The prooxidant effects of these selenium compounds have been proposed to be one of the mechanism(s) by which selenium exerts the anticancer effect (21). Selenite is one of these selenium compounds. Metabolism of selenite to elemental selenium is involved in GSH oxidation and superoxide production in biological systems (1, 21, 35). Proposed mechanisms are as follows:

by Mitotracker and an elevation of p53 in LNCaP cells at 12 hours following 2.5 μmol/L selenite treatment was shown by the immuno-fluorescent stain using a monoclonal anti-p53 antibody and FITC-conjugated antimouse IgG as shown in green. Figure 6B (overlay) shows increased orange fluorescence in LNCaP cells treated with selenite, indicating a colocalization of Mitotracker (red fluorescence) and p53 (green fluorescence) and also indicating mitochondrial translocation of p53. The results confirmed that the level of p53 increased and the translocation of p53 to mitochondria occurred in intact LNCaP cells following selenite treatment.
H$_2$SeO$_2$ + 4GSH $\rightarrow$ GSSeSG + GSSG \hspace{1cm} (A)

GSSeSG + GSH or NADPH $\rightarrow$ H$_2$Se + GSSG or
+ GSH + NADP$^+$ \hspace{1cm} (B)

H$_2$Se + O$_2$ $\rightarrow$ Se$^0$ + O$_2^*$ + H$_2$O \hspace{1cm} (C)

Balances in Eqs. (A) – Eqs. (C) are incomplete. As shown in Eq. (A), selenite nonenzymatically reacts with GSH to form selenodiglutathione (GSSeSG) and glutathione disulfide (GSSG). Selenodiglutathione reacts with NADPH or GSH to produce hydrogen selenide (H$_2$Se) in Eq. (B). Hydrogen selenide is oxidized by O$_2$ to produce elemental selenium (Se$^0$) and superoxide radical (O$_2^*$) in Eq. (C). It has been proposed that the intermediate metabolite selenotrisulfide from interaction of selenite with GSH may also produce superoxide and other ROS (21). One study reported that selenocystamine (RSeSeR) can interact with GSH to form the reduced diselenide (RSe$^-$) that interacts with O$_2$ to produce superoxide (35), suggesting that the selenopersulfide anion (GSSe$^-$) formed from selenite may react with O$_2$ to produce superoxide in a similar pathway (21). Therefore, selenite redox catalysis can result in GSH oxidation and superoxide production with resultant oxidative shift in the redox state of cells. Superoxide is most likely to play a major role in selenite-induced prooxidant effect in cells. Several studies have shown that some selenium compounds can produce superoxide and induce cell apoptosis (21–25). Selenite-induced cell death can be inhibited by treatment with a SOD mimic or by overexpression of MnSOD (15, 26). Studies also found that selenium compounds with superoxide production generally have better anticancer activity (21), suggesting that the subtoxic yet prooxidative effect of selenium compounds may be the anticancer effect of selenium by induction of cell cycle arrest and apoptosis (13, 14).

Our study showed that selenite treatment resulted in immediate elevation of superoxide, subsequent p53 activation, and apoptosis.

Figure 4. Forced p53 expression sensitizes PC3 cells to selenite-mediated cytotoxicity. A, MTT assay of a dose-dependent effect of selenite on cell viability. Cells were treated with selenite for 5 days. B, MTT assay of a time-dependent effect of selenite on cell viability. Cells were treated with 2.5 $\mu$mol/L selenite for 36 hours and then treated with 2.5 $\mu$mol/L selenite in suspension. Superoxide production was immediately measured using a luminometer. Points, mean of three independent experiments; bars, SD. *, $P < 0.05$, control (cells without treatment) versus p53 siRNA and p53 siRNA + Se. **, $P < 0.05$, 2.5 $\mu$mol/L Se versus control, p53 siRNA, and p53 siRNA + Se. #, $P < 0.05$, control (cells without treatment) versus 2.5 $\mu$mol/L Se and Ad-p53. ##, $P < 0.05$, Ad-p53 + Se versus control, 2.5 $\mu$mol/L Se, and Ad-p53.

Figure 5. p53 modulates selenite-induced production of superoxide radical in LNCaP and PC3 cells. A, chemiluminescence assay of suppression of superoxide production by p53 siRNA transfection in LNCaP cells treated with selenite. B, enhancement of superoxide production by Ad-p53 transduction in PC3 cells treated with selenite. Cells were transfected with 50 mmol/L p53 siRNA or transduced with 4 MOI Ad-p53 for 36 hours and then treated with 2.5 $\mu$mol/L selenite in suspension. Superoxide production was immediately measured using a luminometer. Points, mean of three independent experiments; bars, SD. *, $P < 0.05$, control (cells without treatment) versus p53 siRNA and p53 siRNA + Se. **, $P < 0.05$, 2.5 $\mu$mol/L Se versus control, p53 siRNA, and p53 siRNA + Se. #, $P < 0.05$, control (cells without treatment) versus 2.5 $\mu$mol/L Se and Ad-p53. ##, $P < 0.05$, Ad-p53 + Se versus control, 2.5 $\mu$mol/L Se, and Ad-p53.
of LNCaP cells. These effects of selenite were inhibited by the superoxide scavenger MnTMPyP or by MnSOD overexpression as shown by this and previous studies (15, 26). These results suggest that superoxide acts as a mediator for p53 activation and apoptosis induced by selenite treatment. Activation of p53 was via posttranslational modification evidenced by p53 phosphorylation and up-regulation of p21Waf1 and Bax by the Western blot analysis. One study reported that apoptosis induced by selenite was mediated by DNA damage with involvement of the ataxia telangiectasia mutated protein and induction of H2AX phosphorylation (31). Our study shows that selenite treatment did not induce H2AX phosphorylation and pretreatment with caffeine, an ataxia telangiectasia mutated inhibitor, did not alter the effects of selenite (data not shown), suggesting that DNA damage was not the underlying mechanism of selenite-induced p53 activation and cell apoptosis in our study. These experimental differences are most likely due to the different doses of selenite used between studies (31). The doses of selenite used by others were 100 and 500 μmol/L, which were extremely toxic levels of selenite to all cells and therefore induced DNA damage. In our study, we used only 2.5 and 3 μmol/L selenite, which were 30- to 160-fold lower but higher than the requirement for maintenance of the levels of glutathione peroxidase in tissue culture (15). The doses of selenite used in our studies most likely induced a prooxidative effect resulting in an oxidative shift in the cell redox state and growth inhibition or apoptosis, as described, instead of DNA damage. Our results suggest that selenite treatment alters the cell redox state by production of superoxide and activates p53 triggering apoptosis.

The tumor suppressor p53 protein plays an important role in DNA repair, cell cycle arrest, and apoptosis (35–38). Induction of apoptosis is considered to be central to the tumor suppressive function of p53. Through transcription-dependent pathways, p53 functions as a transactivator to up-regulate downstream proapoptotic genes, such as Bax, and/or functions as a repressor to down-regulate antiapoptotic genes, such as Bcl-2, promoting apoptosis. Through transcription-independent pathways, p53 can translocate to mitochondria in response to DNA damage or other stressors, resulting in apoptosis via interaction with antiapoptotic Bcl-2 and Bcl-X proteins that alter the mitochondrial membrane potential and induce cytochrome c release into the cytosol with resultant caspase activation (39). p53-dependent apoptosis has also been shown to be mediated by ROS (40, 41). Apoptosis triggered by p53 has been reported to be dependent on an increase in ROS and the release of apoptotic factors from mitochondrial damage (42). These studies suggest that ROS are downstream mediators in p53-dependent apoptosis in transcription-dependent or transcription-independent pathways. ROS are known to play an important role in apoptosis (43, 44). When cells are exposed to oxidative stress, such as hypoxia or genomic damage, p53 is expressed at high levels by posttranslational modifications, including phosphorylation, acetylation, and glycosylation (45, 46). These modifications occur rapidly and lead to the activation of p53, resulting in either G1 or G2-M cell cycle arrest or apoptosis. Therefore, ROS can function as p53 activators or p53 downstream effectors.

Studies have shown that selenite-induced cell apoptosis was associated with superoxide production and was suppressed by antioxidants, especially by MnSOD or SOD mimics (15, 23, 25, 26). Recent studies have shown that selenite treatment up-regulated p53 and induced p53 phosphorylation on Ser15 to induce caspase-9 in LNCaP cells. These effects of selenite were also up-regulated by selenite treatment. These effects of selenite were suppressed by pretreatment with the SOD mimic MnTMPyP. These results showed that treatment with selenite produced superoxide and subsequently activated p53 via phosphorylation, suggesting that superoxide may be a mediator for p53 phosphorylation/activation in anticancer effects of selenite.

**Figure 6.** Selenite causes p53 mitochondrial translocation, cytochrome c release from mitochondria, and activation of caspase-9 in LNCaP and PC3 cells. A, Western blot analysis of selenite-induced p53 accumulation in mitochondria and cytochrome c release into cytosol in LNCaP cells. WC, whole-cell lysate; Cyto, cytosolic extract; Mito, mitochondrial extract. Cells were treated with 2.5 μmol/L selenite for 18 hours. p21Waf1 was used as a control for the purity of mitochondrial extracts and also for p53 transcriptional activity. β-Actin was used as a control for equal sample loading. Protein loading: 40 μg for p53, p-p53 Ser15, p21Waf1, Bax, and cytochrome c and 20 μg for β-actin. B, laser scanning confocal microscopic photographs showing colocalization of p53 and MitoTracker in mitochondria of LNCaP cells. Red, MitoTracker as a mitochondrial marker; green, p53 labeled with a p53 antibody and a FITC-conjugated secondary antibody; overlay, merging of MitoTracker (red) and p53 (green). C, chemiluminescence assay of activation of caspase-9 by selenite treatment and the suppressive effect of p53 siRNA transfection in LNCaP cells. Cells were transfected with 5 nmol/L p53 siRNAs or transduced with 4 MOI p53 Ad for 36 hours and then treated with 2.5 μmol/L selenite for 18 hours. Chemiluminescence was measured using a luminometer. Columns, mean of three independent experiments; bars, SD. *, P < 0.05, compared with control (cells without treatment), p53 siRNA, and p53 siRNA + Se or control (LNCaP), Se, and Ad-p53 (PC3).
Transfection of p53 siRNA down-regulated p53, p21<sup>Waf1</sup>, and Bax and decreased superoxide production by LNCaP cells. Conversely, PC3 cells transduced with Ad-p53 reexpressed wt p53 and p21<sup>Waf1</sup>, elevated Bax protein, and produced high levels of superoxide following selenite treatment. Cell viability showed that LNCaP cells transfected with p53 siRNA decreased sensitivity to selenite whereas PC3 cells with restoration of p53 expression increased sensitivity to selenite. Our study showed that superoxide production and apoptosis by selenite were p53 dependent and p53 synergistically enhanced superoxide production by selenite. These results suggest that p53 may function to regulate superoxide production from selenite treatment and selenite may also act as a downstream effector for apoptosis. Therefore, p53 levels would affect selenite-induced apoptosis in cancer cells.

Results of p53 mitochondrial translocation, increased Bax protein, cytochrome c release, and caspase-9 activation suggest that Se-induced apoptosis is via p53-dependent mitochondrial pathways. Bax is a mitochondrial proapoptotic protein and is regulated by p53 (36–38, 47). Our results showed that Bax was up-regulated in LNCaP cells corresponding to p53 up-regulation and phosphorylation following selenite treatment or after restoration of p53 expression in PC3 cells. These findings are consistent with the results of previous studies showing that Bax is a p53 target gene. In addition, our study showed that Bax expression was also p53 independent, p53-null PC3 cells expressed low levels of Bax and Bax was up-regulated by selenite treatment in the absence of p53 in PC3 cells. Bax siRNA transfection only slightly decreased selenite-induced apoptosis in LNCaP cells although levels of Bax protein were down-regulated. In contrast, p53 siRNA transfection and sod mimetic treatment were more effective in reducing apoptosis by selenite treatment. These results suggest that p53 and superoxide play an important role in selenite-induced apoptosis whereas Bax plays a minor role.

In summary, our study shows that induction of apoptosis by selenite is superoxide mediated and p53 dependent via mitochondrial pathways. Activation of p53 by selenite treatment is mediated by superoxide production. On the other hand, p53 can synergistically enhance superoxide production and cell apoptosis by selenite. The mitochondrial translocation of p53 may alter mitochondrial functions with resultant increased levels of superoxide triggering apoptosis. Therefore, cells with wt p53 expression are more sensitive to selenite than p53-null cells. These results suggest that superoxide production and p53 may enhance selenite-induced apoptosis and are crucial for apoptotic chemoprevention of prostate cancer by selenite. Our results provide a link between superoxide and p53 in selenite-induced apoptosis in cancer cells. Our results also suggest that levels of intracellular antioxidants, particularly SOD, may influence the chemopreventive efficacy of selenite.

Acknowledgments

Grant support: The University of Wisconsin-Madison, the American Cancer Society, and National Cancer Institute grant CA111428-1 (W. Zhong). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the William S. Middleton Veterans Administration Hospital (Madison, WI) for the use of their resources and facilities.

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