Apoptosis induced by selenomethionine and methioninase is superoxide mediated and p53 dependent in human prostate cancer cells

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Abstract
Selenomethionine (SeMet) is the chemical form or major component of selenium used for cancer chemoprevention in several clinical trials. However, evidence from experimental studies indicates that SeMet has weaker anticancer effects than most other forms of selenium. Recent studies showed that the anticancer activity of SeMet can be enhanced by methioninase (METase), indicating that SeMet metabolites are responsible for its anticancer activity. In the present study, we showed that wild-type p53-expressing LNCaP human prostate cancer cells were more sensitive to cotreatment with SeMet and METase than p53-null PC3 human prostate cancer cells. SeMet and METase cotreatment significantly increased levels of superoxide and apoptosis in LNCaP cells. Cotreatment with SeMet and METase resulted in increased levels of phosphorylated p53 (Ser15), total p53, Bax, and p21Waf1 proteins. LNCaP cells treated with SeMet and METase also showed p53 translocation to mitochondria, decreased mitochondrial membrane potential, cytochrome c release into the cytosol, and activation of caspase-9. The effects of SeMet and METase were suppressed by pretreatment with a synthetic superoxide dismutase mimic or by knockdown of p53 via RNA interference. Reexpression of wild-type p53 in PC3 cells resulted in increases in superoxide production, apoptosis, and caspase-9 activity and a decrease in mitochondrial membrane potential following cotreatment with SeMet and METase. Our study shows that apoptosis induced by SeMet plus METase is superoxide mediated and p53 dependent via mitochondrial pathway(s). These results suggest that superoxide and p53 may play a role in cancer chemoprevention by selenium. [Mol Cancer Ther 2006;5(12):3275–84]

Introduction
Experimental and clinical studies have shown that selenium supplementation reduces cancer incidence, particularly prostate cancer (1–3). However, the underlying anticancer mechanism(s) of selenium is still not fully understood. Recent data suggest that selenium may prevent carcinogenesis by inhibiting cancer cell proliferation, promoting apoptosis, and modulating p53 functions (4–12). Induction of apoptosis is postulated to be a key event of cancer chemoprevention by selenium (5). Studies have shown that the effects of selenium on cancer cell growth inhibition and apoptosis in cultures and carcinogenesis in animals depend on the form and dose of selenium (13–16). Evidence from experimental studies suggests that selenium metabolites are responsible for the anticancer action (14, 15). In addition, studies have shown that reactive oxygen species (ROS) are produced by several selenium compounds through redox catalysis (7, 8, 14, 17–19). Thus, ROS, particularly superoxide, have been postulated to be key metabolites for induction of cancer cell apoptosis by some selenium compounds (14).

Animal studies have shown that most inorganic and organic forms of selenium compounds have anticancer activity (1, 2). Selenite and selenomethionine (SeMet) have been used in most experimental and clinical studies. SeMet is the major component in selenized yeast supplements and is the form of selenium used in clinical trials (1, 2, 6, 14). Both selenite and SeMet have anticancer activity, but SeMet is less effective than selenite, particularly in vitro (2, 14). Our previous studies showed that selenite-induced apoptosis of human prostate cancer cells was superoxide mediated and p53 dependent via mitochondrial pathways (7, 8). Several studies have shown that selenite-induced apoptosis was mediated by ROS production (14, 17–19). In contrast, SeMet has weaker anticancer activity than most other selenium compounds (14). The low anticancer activity of SeMet is most likely associated with its metabolism within cells. Recent studies showed that noneffective concentrations of SeMet in the presence of methioninase (METase) or methionine β-lyase induced apoptosis in human cancer cells (20–24), suggesting that active metabolites are generated from the catalysis of SeMet by these enzymes. Studies showed that overexpression of METase increased apoptosis and superoxide production by...
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SeMet in cancer cells and cotreatment with METase adenoviral constructs and SeMet inhibited tumor growth in nude mice (20, 21). A recent study showed that a mixture of SeMet and METase generated superoxide in an in vitro system (25). These combined data suggest that superoxide may be one of active metabolites of SeMet responsible for growth inhibition and apoptosis of cancer cells. The aim of the present study was to investigate the role of superoxide and p53 in SeMet- and METase-induced apoptosis in human prostate cancer cells. We compared cellular effects and superoxide production in the wild-type (wt) p53-containing LNCaP and p53-null PC3 human prostate cancer cell lines following cotreatment with SeMet and METase. We also analyzed effects of down-regulation or reexpression of p53 on cellular response to SeMet plus METase treatment and the interaction between superoxide and p53 in promoting apoptosis by SeMet plus METase in these two human prostate cancer cell lines. Our study not only confirms the observation of production of superoxide and induction of apoptosis by SeMet plus METase in previous studies but also shows that induction of apoptosis by SeMet plus METase is p53 dependent via mitochondrial pathway(s) and that superoxide production by SeMet plus METase is p53 dependent. Our results suggest that superoxide acts as both an activator and a downstream effector of p53 to promote apoptosis by SeMet plus METase treatment.

Material and Methods

Chemicals and Antibodies

SeMet was purchased from Sigma Chemical Co. (St. Louis, MO). METase, a recombinant enzyme from the *Trichomonas vaginalis* gene produced from *Escherichia coli*, was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Manganese(III)tetrakis(N-methyl-2-pyridyl)-porphyrin (MnTMPyP) was purchased from Alexis Biochemicals (San Diego, CA). p53 small interfering RNAs (siRNA) were purchased from Cell Signaling Technology (Beverly, MA). siRNA Duplex control (nonsilencing) and RNAiFect Transfection Reagent were purchased from Qiagen (Valencia, CA). Apoptotic DNA Ladder kit was purchased from Roche Diagnostics (Indianapolis, IN).

Anti-β-actin monoclonal antibody was purchased from Sigma Chemical. Anti-phosphorylated p53 (Ser15) antibody was purchased from Cell Signaling Technology. Anti-p21<sup>WAF1</sup> (C-19) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Bax and anti-phosphorylated histone H2AX antibodies were purchased from Upstate USA, Inc. (Charlottesville, VA).

Cell Culture

LNCaP and PC3 cells were obtained from the American Type Culture Collection (Manassas, VA) and routinely maintained in 100-mm tissue culture dishes (Corning, Acton, MA) in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Inc., Rockville, MD) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

Superoxide Measurement

Lucigenin-dependent chemiluminescence in cells was measured by a modified method as described previously (26). 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<sup>6</sup> cells in 100 μL PBS and preincubated with or without 5 μmol/L MnTMPyP for 30 min. The reaction was initiated by the addition of lucigenin, SeMet, and METase to the cell suspension, and the chemiluminescence level was measured and recorded as relative light units by a luminometer (Lumat LB 9501, Berthold, Oak Ridge, TN) for a total period of 8 min at 30-s intervals.

Flow Cytometric Analysis

Cell samples were prepared and analyzed as described previously (8). After trypsinization, 1 × 10<sup>6</sup> cells were washed with PBS/EDTA/bovine serum albumin buffer (PBS, 1 mmol/L EDTA, 0.1% bovine serum albumin) and fixed in 100 μL of PBS/EDTA/bovine serum albumin buffer plus 900 μL of 70% ethanol for 30 min at −20°C. After washing with phosphate-citric acid buffer [0.192 mol/L Na<sub>2</sub>HPO<sub>4</sub>, 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 sub-G1 cells were simultaneously measured in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using 488-nm laser excitation.

To measure mitochondrial membrane potential (MMP), cells were resuspended in 1 mL of serum-free medium containing 2.5 mmol/L JC-1 dye and incubated at 37°C for 20 min. After washing twice with PBS, fluorescence in cells was immediately measured in a flow cytometer. Mitochondrial depolarization is indicated by the decrease in the ratio of the red signal at 590 nm emission to the green signal at 530 nm emission.

Apoptotic DNA Ladder Analysis

DNA isolation and gel electrophoresis were done according to the manufacturer’s instructions. Briefly, cells were scraped in PBS buffer and harvested by centrifugation at 500 × g for 5 min at room temperature and lysed in 400 μL lysis buffer for 10 min at room temperature. Following the 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. Equal amounts of DNA 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 (Eastman Kodak Co., Rochester, NY) using UV illumination and digitized with Kodak 1D 3.6 software.

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 and then treated with different agents 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 h at 37°C. MTT lysis buffer (100 μL of 10% SDS, 45% dimethyl formamide, adjusted to pH 4.5 by glacial 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 were 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 by the Bradford assay (Bio-Rad, Hercules, CA). 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 overnight at 4°C and then with secondary antibodies conjugated with horseradish peroxidase at 1:10,000 dilution in TBS-Tween 20 for 1 h 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 pS3 siRNA and 2 μL of RNAiFect transfection reagent in 1 mL serum-free medium for 12 h, and then 1 mL of fresh medium with 10% fetal bovine serum was added to each well for 24 h before SeMet and MET treatment. Cells were also transfected with the nonsilencing, negative control siRNA, which has no known homology to mammalian genes and allows assessment of nonspecific gene silencing effects.

Adenoviral 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 h later, cells were infected with the indicated multiplicity of infection of recombinant Ad5 cytomegalovirus wt p53-green fluorescent protein adenoviral constructs (p53-Ad) or empty control adenoviral constructs (control-Ad) in serum-free medium. After 12 h, an equal volume of fresh medium with 10% fetal bovine serum was added to each dish or well for 24 h before SeMet and METase treatment.

Caspase-9 Activity Assay
Cells were seeded at 3 × 10^4 per well in a 96-well plate with 100 μL medium. Approximately 16 h later, cells were treated with SeMet and METase for 18 h 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 Lumimeter (Applied Biosystems, Bedford, MA).

Mitochondria Fractionation
LNCaP cells were treated with different doses of SeMet plus 0.1 unit/mL METase for different times, and cell viability was assessed by the MTT assay. As shown in Fig. 1A and B, SeMet and METase cotreatment decreased cell viability in a dose- and time-dependent manner. Significant cell viability decreases occurred in cells treated with 1.5 μmol/L and higher doses of SeMet with 0.1 unit/mL METase (Fig. 1A) or in cells treated with 3.0 μmol/L SeMet with 0.1 unit/mL METase for 36 h and longer times (Fig. 1B), with a IC_50 of 2.5 μmol/L after 72 h of treatment. METase alone did not cause significant cell death (Fig. 1B). Analyses of apoptosis by flow cytometry and gel electrophoresis showed that cells treated with 3.0 μmol/L SeMet plus 0.1 unit/mL METase showed a 45-fold increase in the sub-G1 cell population compared with the control, and DNA laddering (fragmentation) was observed in cells treated with 3.0 μmol/L SeMet plus 0.1 unit/mL METase (Fig. 1C and D). These data showed that cells underwent apoptosis following treatment with SeMet plus METase. To assess the involvement of superoxide in apoptosis, cells were pretreated with a chemical superoxide dismutase (SOD) mimic, MnTMPyP. Pretreatment with 3 μmol/L MnTMPyP significantly reduced SeMet plus METase-induced DNA fragmentation (Fig. 1D) and cell death (Fig. 1E). Treatment with SeMet, METase, or MnTMPyP alone did not cause significant cell death (Fig. 1E). Lucigenin-dependent chemiluminescence assay showed that treatment with 3.0 μmol/L SeMet and 0.1 unit/mL METase resulted in an increase in intracellular chemiluminescence in 4 min with a peak value at 6 min (Fig. 1F). SeMet or METase alone did not cause significant increases in chemiluminescence. Chemiluminescence produced by SeMet and METase treatment was suppressed by MnTMPyP pretreatment. There was only minimal chemiluminescence detected in the mixture of the culture media.
medium and lucigenin in the absence of cells (data not shown). These combined results indicate that SeMet and METase treatment triggers cell apoptosis by producing superoxide.

p53 Regulation and p53-Dependent Cell Death by SeMet plus METase in LNCaP Cells

To determine whether p53 is activated by SeMet and METase treatment, Western blot analysis was used to detect immunoreactive levels of total p53 and phosphorylated p53 at Ser15 (P-p53 Ser15) and p53 target genes p21Waf1 and Bax. As shown in Fig. 2A and B, cotreatment with SeMet and METase resulted in elevations of total p53 and P-p53 Ser15 in LNCaP cells in a dose- and time-dependent pattern. Protein levels of both p21Waf1 and Bax were also elevated corresponding to the elevation of p53 observed following SeMet plus METase treatment. Detectable elevations of total and phosphorylated p53 occurred at 1.5 μmol/L and higher concentrations of SeMet, whereas elevations of p21Waf1 and Bax were detected at the 0.5 μmol/L concentration. Elevations of total p53, P-p53 Ser15, and Bax were observed at 1 h and thereafter following treatment with SeMet and METase, whereas an elevation of p21Waf1 was first observed at 3 h. To exclude an involvement of DNA damage by SeMet and METase treatment, a DNA damage Figure 1. Effects of SeMet and METase on apoptosis and superoxide production in LNCaP cells. A, MTT assay showing a dose-dependent effect of SeMet and METase on cell viability. Cells were treated with SeMet and METase for 5 d. B, MTT assay showing a time-dependent effect of SeMet, METase, or combination on cell viability. C, flow cytometric analysis showing apoptosis (sub-G1 cell population) induced by SeMet and METase. Cells were treated with 3 μmol/L SeMet and 0.1 unit/mL METase for 24 h. D, agarose gel electrophoretic detection of DNA fragmentation as a marker of cell apoptosis induced by SeMet and METase. Cells were treated with 3 μmol/L SeMet, 0.1 unit/mL METase, and 3 μmol/L MnTMPyP alone or in combinations for 24 h. E, protection by MnTMPyP against cytotoxicity of SeMet and METase. Cells were treated with 3.0 μmol/L SeMet, 0.1 unit/mL METase, and 3.0 μmol/L MnTMPyP alone or in combinations for 5 d, and cell survival was measured by the MTT assay. F, superoxide production in cells cotreated with SeMet and METase. Cells were treated with 3.0 μmol/L SeMet and 0.1 unit/mL METase with or without 3.0 μmol/L MnTMPyP, and superoxide was measured using a chemiluminescence assay. RLU, relative light units. Points, mean of three independent experiments; bars, SD. *, P < 0.05, compared with no METase (A), SeMet or METase alone (B), and control or SeMet or METase (E and F).
marker, phosphorylated histone H2AX at Ser139 (H2AX), was analyzed by Western blot analysis (27). As shown in Fig. 2A and B, there were no significant changes in this phosphorylated protein in cells treated with SeMet plus METase, indicating that DNA damage is not the major factor causing p53 activation in this study. Figure 2C shows that only cotreatment with SeMet and METase resulted in significant elevations of total p53, P-p53 Ser15, p21\(^{Waf1}\) and Bax, whereas pretreatment with MnTMPyP inhibited the effect of SeMet and METase on these proteins. These results suggest that treatment with SeMet and METase produces superoxide, which subsequently activates p53 via a non-DNA damage pathway.

We next determined the role of p53 in SeMet- and METase-induced cell death using RNA interference to reduce levels of p53 protein. As shown in Fig. 3A, transfection with p53 siRNA inhibited up-regulation of total p53, P-p53 Ser15, and p21\(^{Waf1}\) proteins by cotreatment with SeMet and METase in LNCaP cells, whereas transfection with the negative control siRNA did not affect the results of SeMet plus METase treatment. However, up-regulation of Bax was not affected by p53 siRNA transfection, suggesting that Bax regulation by SeMet and METase is not completely p53 dependent. Cells transfected with p53 siRNA showed decreased sensitivity to SeMet plus METase compared with SeMet or METase, whereas the sensitivity did not change in cells transfected with the control siRNA (Fig. 3B). These results show that cell death induced by SeMet plus METase is p53 dependent.

**Effect of p53 on Cellular Response to SeMet and METase in p53-Null PC3 Cells**

To further verify that cellular sensitivity to SeMet and METase is dependent on p53, we next tested the sensitivity of p53-null PC3 cells to SeMet and METase before and after reexpression of wt p53. Dose-dependent and time course studies showed that PC3 cells were much less sensitive to SeMet plus METase compared with SeMet or METase, whereas the sensitivity did not change in cells transfected with the control siRNA (Fig. 3B). These results show that cell death induced by SeMet plus METase is p53 dependent.

![Figure 2](image_url)

**Figure 2.** Western blot analysis of effects of SeMet and METase on the expression of p53, p21\(^{Waf1}\), and Bax and phosphorylation of p53 (Ser15) and histone (Ser139, H2AX) in LNCaP cells. A, dose-dependent effect of SeMet and METase. Cells were treated with SeMet + 0.1 unit/ml METase for 18 h. B, time-dependent effect of SeMet and METase. Cells were treated with 3.0 μM SeMet + 0.1 unit/ml METase for 18 h. C, suppression of effects of SeMet and METase on p53, p21\(^{Waf1}\), and Bax by SOD mimic MnTMPyP. Cells were treated for 18 h. Protein loading: 40 μg for p53, P-p53 Ser15, p21\(^{Waf1}\), Bax, and H2AX and 20 μg for β-actin.

![Figure 3](image_url)

**Figure 3.** Suppressive effects of p53 siRNA transfection on cellular response to SeMet and METase in LNCaP cells. A, Western blot analysis of suppression of SeMet- and METase-induced up-regulation of p53, p21\(^{Waf1}\), and Bax by p53 siRNA transfection. Cells were transfected with 50 nmol/L p53 siRNA or control siRNA for 36 h and then treated with 3 μmol/L SeMet + 0.1 unit/ml METase for 18 h. Protein loading: 40 μg for p53, P-p53 Ser15, p21\(^{Waf1}\), and Bax and 20 μg for β-actin. B, MTT assay of viability of LNCaP cells with p53 siRNA transfection and treatment with 3.0 μmol/L SeMet and/or 0.1 unit/ml METase. Cells were transfected with 50 nmol/L p53 siRNA or control siRNA for 36 h and then treated with SeMet and/or METase for 5 d. Columns, mean of three independent experiments; bars, SD. *, P < 0.05, in comparison of control siRNA with SeMet or METase only or p53 siRNA with SeMet, METase, or SeMet + METase.
(Fig. 1B), whereas only 10% of PC3 cells died at 72 h and <20% of cells died at 120 h at the same dose of SeMet (Fig. 4B). Western blot analysis showed that PC3 cells had no detectable p53 and very low levels of p21 and Bax at the protein loading levels (40 μg) analyzed (Fig. 4C). Following transduction of p53 adenoviral constructs (p53-Ad), p53 was reexpressed and p21\textsuperscript{Waf1} and Bax were elevated in PC3 cells. SeMet plus METase treatment further increased levels of total p53 and P-p53 Ser15, p21\textsuperscript{Waf1} and Bax proteins in PC3 cells without p53-Ad transduction, indicating that p21\textsuperscript{Waf1} and Bax regulation can be both p53 dependent and p53 independent. Figure 4D shows that reexpression of p53 enhanced the sensitivity of PC3 cells to SeMet plus METase only, whereas transduction of control adenoviral constructs (control-Ad) did not alter the cellular sensitivity to SeMet plus METase. These results clearly show that cell death induced by SeMet plus METase is p53 dependent.

p53-Dependent Superoxide Production by SeMet plus METase Treatment in LNCaP and PC3 Cells

Because SeMet- and METase-induced cell apoptosis is dependent on p53, we further analyzed the role of superoxide in SeMet- plus METase-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 SeMet- plus METase-induced elevation of superoxide, although p53 siRNA transfection alone also increased levels of superoxide compared with the control. The latter was most likely due to superoxide production from RNAiFect and siRNA transfection, which was observed in our previous study (8). Conversely, reexpression of wt p53 in PC3 cells significantly increased superoxide production following SeMet and METase treatment (Fig. 5B). Overexpression of p53 or treatment with SeMet or METase only also increased superoxide levels in PC3 cells, but the magnitudes were much lower than their combination. These results show that superoxide production by SeMet and METase treatment can be enhanced by p53, suggesting that superoxide may act as a p53 activator and downstream mediator of p53-dependent apoptosis. It is well known that ROS can cause oxidative stress and cell apoptosis and that p53 can be activated by oxidative stress to regulate cell cycle arrest and apoptosis (28, 29). It has been reported that ROS were downstream effectors of p53 (30), which is consistent with our observation in this study.

p53-Dependent, Superoxide-Mediated Mitochondrial Pathways of Apoptosis Induced by SeMet plus METase Treatment

It has been known that p53 can execute apoptosis through mitochondria via transcription-dependent and transcription-independent pathways (31, 32). To explore mitochondrial-dependent apoptosis, mitochondria were isolated from the cytosol of LNCaP cells. Mitochondrial translocation of p53 and cytochrome \textit{c} release from mitochondria were assessed by Western blot analysis. As shown in Fig. 6A, protein levels of p53, P-p53 Ser15,
p21Waf1, and Bax increased in LNCaP cells following treatment with 3.0 μmol/L SeMet plus 0.1 unit/mL METase for 18 h. In addition, a substantial amount of p53 translocated to mitochondria. The levels of cytochrome c in mitochondria also dramatically increased with a substantial amount released into the cytosol. Levels of Bax were elevated in the cytosol, but no significant changes were observed in mitochondria. To further assess the mitochondrial pathway of apoptosis, MMP was studied by JC-1 fluorescent dye staining, a procedure that analyzes depolarization of mitochondrial membranes. As shown in Fig. 6B, a significant decrease in MMP occurred only in LNCaP cells treated with 3.0 μmol/L SeMet plus 0.1 unit/mL METase for 18 h. The depolarization of MMP by SeMet plus METase was suppressed by transfection with p53 siRNA. In contrast, PC3 cells showed no significant change in MMP following SeMet, METase, or combined treatment (Fig. 6C). After reexpression of wt p53 by adenoviral transduction, only SeMet plus METase treatment significantly decreased MMP in PC3 cells.

We next analyzed caspase-9 activity with a chemiluminescence assay for mitochondrial-dependent apoptosis. As shown in Fig. 6D, only treatment with 3.0 μmol/L SeMet plus 0.1 unit/mL METase increased the caspase-9 activity in LNCaP cells. Activation of caspase-9 by SeMet plus METase treatment was suppressed by p53 siRNA transfection. In contrast, PC3 cells showed no significant change in caspase-9 activity following SeMet plus METase treatment (Fig. 6E). After reexpression of wt p53 by adenoviral transduction, SeMet plus METase treatment significantly increased caspase-9 activity in PC3 cells. Figure 6D and E also shows that caspase-9 activation by H2O2 was only partially dependent on p53. These data show that apoptosis induced by SeMet plus METase is p53 dependent via mitochondrial pathway(s).

Discussion

Selenium is an essential trace element for human health and is an anticancer agent in animal and clinical studies (1–3). Maintenance of maximal levels of selenium-containing antioxidant enzymes requires only nutritional levels of selenium supplementation, whereas cancer chemoprevention requires supranutritional levels of selenium supplementation, indicating that other mechanism(s) may be involved in cancer chemoprevention by selenium in addition to its antioxidant effects. Combs and Gray (1) suggested that nutritional levels of selenium supplementation provide antioxidant protection against oxidative stress, whereas supranutritional levels may cause subtoxic effects to induce cell growth inhibition and/or apoptosis for cancer prevention.

Accumulating evidence from experimental studies indicates that active metabolites, particularly redox cycling ones, play an important role in inhibition of proliferation and induction of apoptosis by some selenium compounds (2, 14). ROS, particularly superoxide, are produced by several selenium compounds when they interact with reduced glutathione (GSH), and induction of apoptosis was associated with ROS production. Spallholz (14) suggested that the anticarcinogenic property of these selenium compounds is likely due to the toxicity of superoxide generated from redox cycling of certain metabolites. It has been well documented that metabolism of selenite is involved in oxidation of GSH and superoxide production in biological systems. Selenite reacts with GSH to form selenodiglutathione and glutathione disulfide. Selenodiglutathione reacts with NADPH or GSH to produce hydrogen selenide. Hydrogen selenide is oxidized by O2 to produce elemental selenium and superoxide. The intermediate metabolite selenotrisulfide generated from interaction of selenite with GSH may also produce superoxide and other ROS (14). One study reported that selenocystamine can interact with GSH to form reduced...
diselenide that interacts with O₂ to produce superoxide (33), suggesting that the selenopersulfide anion formed from selenite may react with O₂ to produce superoxide in a similar pathway. Therefore, superoxide is most likely to play a major role in the pro-oxidant effects by some selenium compounds. Studies also found that different chemical forms of selenium compounds have different efficacy in cancer prevention and selenium compounds with superoxide production generally have better anticancer activity (14, 25), suggesting that the subtoxic yet pro-oxidative effect of these selenium compounds may be the mechanism by which selenium induces cell growth inhibition and apoptosis. Selenite-induced cell death can be inhibited by treatment with a SOD mimic or by overexpression of MnSOD (8, 34). Recent studies showed that normal prostate epithelial cells had high levels of MnSOD and low sensitivity to selenite compared with prostate cancer cells (35, 36), suggesting that high levels of MnSOD protect normal epithelial cells against superoxide toxicity from selenium compounds. These data support the concept that superoxide is responsible for apoptosis induced by certain selenium compounds.

Unlike selenite, SeMet is very ineffective in vitro, although it can reduce cancer incidence in vivo, but has lower in vivo anticarcinogenic effect than selenite (3, 14). Recent studies showed that in vitro anticancer activity of SeMet was significantly enhanced in cancer cells with overexpression of METase or with METase treatment (20, 21). This enhanced activity of SeMet by METase was suppressed by SOD treatment. Spallholz et al. (25) showed that SeMet plus METase generated superoxide in an in vitro chemiluminescence assay. METase is an enzyme that can convert SeMet to methylselenol and has been found in bacteria and the protozoan T. vaginalis (37). Other studies...
have shown analogous enzymes to METase in tissues of humans and mice (37–41). These data suggest that superoxide production by SeMet may contribute to its anticancer action \textit{in vivo} because METase is present in tissues. Experimental evidence indicates that methylselenol is the selenium metabolite responsible for cancer chemoprevention (42). A recent study showed that methylselenol generated superoxide from the direct reduction of both dimethylselenide and methylseleninic acid in the presence of GSH (43). One study showed that the toxic pro-oxidant methylselenol was released from SeMet by cancer cells transformed with the adenoviral METase gene (20). Methylselenol damaged the mitochondria via oxidative stress and caused cytochrome c release into the cytosol, thereby activating caspases and promoting apoptosis. Accordingly, superoxide production from the catalysis of SeMet by METase is postulated to be associated with the reaction of methylselenol or other selenium radicals with oxygen (25). Our study clearly showed that superoxide was produced only in the presence of both SeMet and METase. Low activity of SeMet \textit{in vitro} is most likely due to lack or low activity of METase in cancer cells. Low anticancer activity of SeMet may also be due to its direct incorporation into proteins in place of methionine and therefore is unable to undergo redox cycling to produce active metabolites, including superoxide, in cancer cells.

The tumor suppressor p53 protein plays an important role in apoptosis (44, 45). Induction of apoptosis is considered to be central to the tumor-suppressive function of p53. p53 can translocate to mitochondria in response to DNA damage or other stressors, resulting in apoptosis via alteration of the MMP and cytochrome c release into the cytosol with resultant caspase activation (31, 46, 47). p53-dependent apoptosis has also been shown to be mediated by ROS (28). Apoptosis triggered by p53 has been reported to be dependent on an increase in ROS and the release of apoptotic factors from mitochondrial damage (47). 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. When cells are exposed to oxidative stress, p53 is expressed at high levels by posttranslational modifications, including phosphorylation, acetylation, and glycosylation (48, 49). These modifications occur rapidly and lead to the activation of p53, resulting in cell cycle arrest or apoptosis. Therefore, ROS can function as p53 activators or p53 downstream effectors.

Our data showed that wt p53-expressing LNCaP cells were more sensitive to SeMet plus METase treatment than p53-null PC3 cells. SeMet plus METase treatment resulted in increased intracellular superoxide, p53 activation, and cell apoptosis. SeMet and METase treatment also resulted in translocation of p53 to mitochondria, cytochrome c release into the cytosol, and activation of caspase-9. The effects in LNCaP cells were suppressed by the SOD mimic MnTMPyP or by knockdown of p53 via RNA interference. On the other hand, the effects of SeMet plus METase were enhanced by restoration of wt p53 expression in p53-null PC3 cells. In addition, our study showed that superoxide production by SeMet and METase treatment was enhanced by restoration of p53 expression in PC3 cells and decreased by knockdown of p53 in LNCaP cells. These results indicate that induction of apoptosis by SeMet plus METase treatment is superoxide mediated and p53 dependent via mitochondrial pathway(s) in association with translocation of p53 to mitochondria. The results also suggest that superoxide is a p53 activator and a downstream mediator of p53-dependent apoptosis. These effects of SeMet and METase are identical to those of selenium observed in our previous study (8). One should note that selenium may prevent cancer via multiple mechanisms and cancer cell response to selenium may also depend on other factors, such as androgen dependence. In addition to the difference of the p53 status between LNCaP and PC3 cells, LNCaP cells express androgen receptor and respond to androgen treatment. Recent studies have shown that the selenium compounds can suppress the androgen receptor and its signaling in LNCaP and LAPC-4 cells (50, 51). Thus, we believe that superoxide-mediated, p53-dependent apoptosis is only one of the mechanisms by which selenium exerts its anticancer activity.

In summary, results from this study and others indicate that superoxide production from SeMet catalysis by METase plays a role in induction of cancer cell apoptosis, suggesting that production of superoxide from SeMet metabolism may be responsible, at least in part, for anticancer action \textit{in vivo}. The results from our previous and current studies show that superoxide and p53 play an important role in selenium- and SeMet-induced apoptosis, and apoptosis induced by these two selenium compounds is triggered via mitochondrial pathway(s). Our studies suggest that superoxide generated from redox metabolism of selenite and SeMet may account, at least in part, for the mechanism of anticancer action of selenium. Our results also suggest that anticancer efficacy depends not only in the dose and form of selenium but also in the metabolism of selenium, superoxide production from selenium metabolites, and the antioxidant capacity and p53 status of cancer cells.

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Apoptosis induced by selenomethionine and methioninase is superoxide mediated and p53 dependent in human prostate cancer cells

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