

# Distinct Effects of Methylselenenic Acid *versus* Selenite on Apoptosis, Cell Cycle, and Protein Kinase Pathways in DU145 Human Prostate Cancer Cells<sup>1</sup>

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## Abstract

Selenium has been implicated as a promising chemopreventive agent for prostate cancer. Whereas the anticancer mechanisms have not been clearly defined, one hypothesis relates to selenium metabolites, especially the monomethyl selenium pool, generated under supranutritional selenium supplementation. To explore potential molecular targets for mediating the chemopreventive activity, we contrasted the effects of methylselenenic acid (MSeA), a novel precursor of methylselenol, *versus* sodium selenite, a representative of the hydrogen selenide metabolite pool, on apoptosis execution, cell cycle distribution, and selected protein kinases in DU145 human prostate cancer cells. Exposure of DU145 cells to 3  $\mu$ M MSeA led to a profound G<sub>1</sub> arrest at 24 h, and exposure to greater concentrations led to not only G<sub>1</sub> arrest, but also to DNA fragmentation and caspase-mediated cleavage of poly(ADP-ribose) polymerase (PARP), two biochemical hallmarks of apoptosis. Immunoblot analyses indicated that G<sub>1</sub> arrest induced by the subapoptogenic doses of MSeA was associated with increased expression of p27kip1 and p21cip1, but apoptosis was accompanied by dose-dependent decreases of phosphorylation of protein kinase AKT and extracellular signal-regulated kinase (ERK1/2) in the absence of any phosphorylation change in p38 mitogen-activated protein kinase (p38MAPK) and c-Jun NH<sub>2</sub>-terminal kinase (JNK1/2). In contrast, selenite exposure caused S-phase arrest and caspase-independent apoptotic DNA fragmentation, which were associated with decreased expression of p27kip1 and p21cip1 and increased phosphorylation of AKT, JNK1/2, and p38MAPK. Although apoptosis induction by MSeA exposure was not sensitive to superoxide dismutase added into the cell culture medium, cell detachment and DNA nucleosomal fragmentation induced by selenite exposure were greatly attenuated

by this enzyme, supporting a chemical mediator role of superoxide for these processes. Despite a temporal relationship of AKT and ERK1/2 de-phosphorylation changes before the onset of PARP cleavage in MSeA-exposed cells, experiments with phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 did not show an enhancing effect of specific blocking of AKT on MSeA-induction of PARP cleavage. Taken together, exposure of DU145 cells to MSeA *versus* selenite induced differential patterns of cell cycle arrest and apoptosis execution as well as distinct patterns of effects on AKT, ERK1/2, JNK1/2, and p38MAPK phosphorylation and p27kip1 and p21cip1 expression. Multiple molecular pathways are likely differentially targeted by selenium metabolite pools to mediate cancer chemoprevention.

## Introduction

Human cancer prevention trials have shown an exciting promise of selenium supplement (approximately four times the recommended daily value of 55  $\mu$ g) as an effective chemopreventive agent for several major cancers including prostate, lung, and colon cancers in the United States (1) and liver cancer in China (2). However, the mechanisms underlying its cancer chemopreventive activity are poorly understood at the present. Studies using animal carcinogenesis models have strongly implicated a monomethyl selenium metabolite pool, perhaps methylselenol, as an active *in vivo* selenium species for the chemopreventive activity (3–5). We and others (6–10) have shown that methyl selenium compounds that are immediate precursors of methylselenol (e.g., MSeA<sup>4</sup>; Ref. 6; Fig. 1A) induce several biochemical and cellular responses that are distinct from those induced by selenium forms that initially enter the hydrogen selenide pool (e.g., sodium selenite; Fig. 1A), which can metabolically give rise to methylselenol *in vivo* by methylation (11). Distinguishing differences include the fact that the methylselenol precursors induce caspase-mediated apoptosis of DU145 prostate cancer cells (12) and HL-60 leukemia cells (13) without induction of DNA single-strand breaks (6–9, 13), whereas sodium selenite exposure induces DNA single-strand breaks (i.e., genotoxic; Refs. 6–9, 13) and apoptotic cell death with no involvement of caspases (12, 13). In mammary cancer cell lines (7–10) and vascular endothelial cells (14), exposure to methylselenol precursors arrests the cells in G<sub>1</sub> phase,

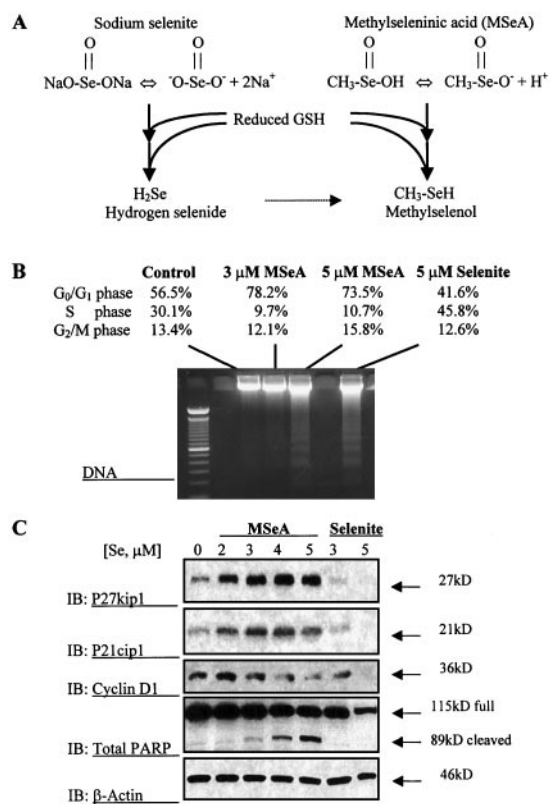
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<sup>4</sup> The abbreviations used are: MSeA, methylselenenic acid; ERK, extracellular signal-regulated kinase; JNK, Jun NH<sub>2</sub>-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase; SAPK, stress-activated protein kinase; SOD, superoxide dismutase.



**Fig. 1.** A, chemical structure of selenite and MSeA and their likely proximal metabolites on reaction with reduced glutathione (11, 27). *Dash arrow*, limited conversion of hydrogen selenide to methylselenol by methylation in cell culture model. B, flow cytometric analyses of cell cycle distribution and electrophoretic analysis of apoptotic nucleosomal DNA fragmentation of MSeA- and selenite-treated DU145 cells at 24-h exposure. The percentage distribution data for each treatment group were the average of two flasks. The data were representative of three independent experiments. C, immunoblot detection of expression changes of cell cycle regulator proteins p27kip1, p21cip1, and cyclin D and of PARP cleavage as an indicator of caspase-mediated apoptosis execution in MSeA- and selenite-treated DU145 cells at 24-h exposure.  $\beta$ -Actin was probed to indicate evenness of loading of protein extract from each treatment. *Arrows*, proteins of interest. *kD*, *M*, in thousands.

whereas exposure to selenite arrests cells in S phase of the cell cycle (7–10). More recently, we have reported that methylselenol precursors inhibit the expression of vascular endothelial growth factor, an angiogenic cytokine, by cancer epithelial cells and inhibit the expression of matrix metalloproteinase-2 expression by vascular endothelial cells, and that these effects are absent for selenite exposure (15). These findings, taken together, support the presence of at least two selenium metabolite pools that induce distinct types of antiangiogenesis, apoptosis, and cell cycle responses. The molecular targets and pathways underlying these differential responses remain to be defined.

To explore potential molecular targets and pathways concerning prostate cancer chemoprevention and therapy with selenium, we focused the present work on selected protein kinases of cell proliferation and apoptosis signal transduction in DU145 human prostate cancer cells. This cell line represents an aggressive and metastatic prostate cancer

phenotype without functional p53 and retinoblastoma tumor suppressor proteins and is independent of androgen for growth and survival (16). In earlier work, we have used this model to delineate the caspase-mediated apoptosis execution pathways induced by MSeA exposure (12).

Several protein kinase pathways are known to regulate cell proliferation and survival. The PI3K pathway mediates cell cycle progression and cell survival through downstream effector proteins, one of which is the protein kinase AKT (17, 18). Inhibition of PI3K in some cell lines is sufficient to induce apoptosis, and ectopic expression of constitutively active mutants of PI3K or AKT inhibits apoptosis. Regarding selenium action, our earlier work has implicated an inhibition of the PI3K pathway activity as a likely mechanism for inhibiting cell cycle G<sub>1</sub> to S phase progression in vascular endothelial cells by either MSeA (14) or methylselenol (19). The significance of PI3K/AKT pathway as a potential target for methyl selenium-induced cell cycle arrest and apoptosis signaling in prostate cancer cells is not known.

In addition to the PI3K-AKT pathway, a family of closely related serine-threonine protein kinases, known as MAPKs, have been implicated in cell proliferation and apoptosis signaling in diverse model systems (20–22). Mammalian MAPKs include three broad families: the classic ERKs (ERK1/2), JNKs (also termed SAPK1), and p38MAPK (also known as SAPK2a). In general, ERK1/2 are activated by mitogens and survival factors (20–22), leading to cell cycle entry and progression through modulating expression of G<sub>1</sub> cyclins and the activity of cyclin-dependent kinases, which are, in part, regulated by the cyclin-dependent kinase-inhibitory proteins p27kip1 and p21cip1 (23, 24). JNK1/2 and p38MAPK are stimulated by stress and apoptotic signals; and, in many instances, either or both mediate cell death signaling (21, 22). Therefore, the balance of signals among the various protein kinase pathways may be critical for cell proliferation and apoptosis (25). To our knowledge, there had been no extensive published work on the PI3K-AKT and the various MAPK pathways in relationship to selenium induction of prostate cancer apoptosis and cell cycle arrest.

In this report, we document distinct effects of MSeA versus selenite exposure on the phosphorylation states of AKT, ERK1/2, JNK1/2, and p38MAPK and the expression of p27kip1 and p21cip1 in DU145 prostate cancer cells and their associations with distinct patterns of cell cycle arrest and apoptosis execution. We present data supporting a differential involvement of superoxide generation for apoptosis induction by the two forms of selenium in DU145 cells. Furthermore, we show results from experiments with PI3K inhibitors to probe the significance of the PI3K-AKT pathway for methyl selenium induction of apoptosis signaling.

## Materials and Methods

**Chemicals and Reagents.** Sodium selenite pentahydrate was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). MSeA ( $\text{CH}_3\text{SeO}_2\text{H}$ ) was synthesized as described elsewhere (26, 27). Intracellularly, MSeA most likely reacts with reduced glutathione to generate methylselenol  $\text{CH}_3\text{SeH}$  (27). Bovine liver catalase and bovine erythrocyte (Cu, Zn)-SOD, the PI3K

inhibitor wortmannin, and an antibody for  $\beta$ -actin were purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 was purchased from CalBiochem-Novabiochem Corp (La Jolla, CA). Antibodies for full-length human PARP or the cleaved PARP ( $M_r$  89,000 fragment), caspase-7 and those for protein kinases and their phosphorylated forms (AKT Ser473; ERK1/2 Thr202/Tyr204; JNK Thr183/Tyr185; p38MAPK Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA).

**Cell Culture and Treatments.** DU145 cells were originally obtained from the American Type Culture Collection. DU145 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics as described previously (12). Cells at 50–70% confluence, usually 24–48 h after plating, were given a medium change and treated with selenium or other agents. To standardize selenium exposure, cells were treated in a medium volume to culture surface area ratio of 0.2 ml/cm<sup>2</sup> (15 ml for a T75 flask and 5 ml for a T25 flask). Concentrated selenium stock solutions were diluted in PBS to 1-mM working solutions before the addition to culture medium. In experiments in which PI3K inhibitors wortmannin and LY294002 were used, these compounds were dissolved in DMSO as concentrated stocks. After necessary dilution, the inhibitor(s) and MSeA were mixed into treatment media first and then fed to cells. DMSO (2  $\mu$ l/ml or less) was added to groups that did not receive the inhibitor to control for solvent vehicle effects. DMSO at the concentration used did not by itself cause any observable adverse morphological responses.

**Cell Cycle Distribution and Apoptosis Evaluation.** After experimental treatments, spent media were collected and spun at 1000  $\times g$  for 5 min to collect detached cells or floaters, which were combined with respective adherent cells for cell cycle distribution analyses by flow cytometry at the University of Colorado Cancer Center flow cytometry core facility, Denver, CO. For the detection of DNA nucleosomal fragmentation as a biochemical indicator of apoptosis, DNA was extracted from adherent cells and floaters combined (unless stated otherwise) and analyzed by agarose gel electrophoresis as described previously (28). Cleavages of PARP and caspase-7 as markers of caspase-mediated apoptosis were detected by immunoblot analyses (12).

**Immunoblot Analyses.** After experimental treatments for the designated duration, DU145 cell lysates (floaters and adherent cells combined) were prepared as described previously (12). Supernatants after centrifugation (14,000  $\times g$  for 20 min) were recovered, and the protein content was quantified by the Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA). Twenty to 40  $\mu$ g of total protein were size-separated by electrophoresis on SDS-polyacrylamide gels under nonreducing conditions. The proteins were electroblotted onto nitrocellulose membranes and probed for the phosphorylated and/or the total forms of each kinase. For the detection of PARP and caspase cleavages, 80–100  $\mu$ g of total protein extract were used per treatment. Positive control samples obtained from the antibody suppliers were used whenever available.

## Results

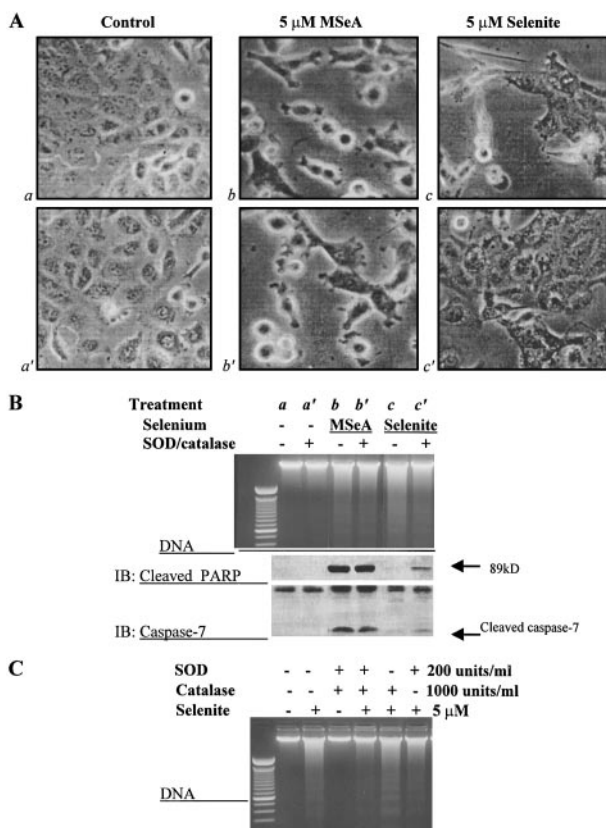
**Distinct Cell Cycle Arrest and Cell Death Responses Elicited by MSeA versus Selenite Exposure.** Flow cytometric analyses of cell cycle distribution of DU145 cells that had been exposed to 3 or 5  $\mu$ M MSeA for 24 h showed an  $\sim$ 2/3 reduction of S-phase cells and an enrichment of G<sub>1</sub> phase cells without significant changes of G<sub>2</sub>-M cells (Fig. 1B). At 3  $\mu$ M or lower concentration of exposure, G<sub>1</sub> arrest was achieved without apoptosis, as indicated by lack of nucleosomal DNA fragmentation (Fig. 1B) and caspase-mediated PARP cleavage (Fig. 1C). In time-course experiments, the G<sub>1</sub> arrest effects began to manifest at  $\sim$ 12 h of exposure to MSeA (not shown).

In contrast to the G<sub>1</sub> arrest effect observed in MSeA exposed cells, cells exposed to 5  $\mu$ M selenite were arrested in S phase without any significant effect on G<sub>2</sub>-M cells (Fig. 1B). Exposure to 3  $\mu$ M selenite did not affect the distribution pattern in comparison with the control cells (not shown). The S-phase arrest induced by selenite exposure was associated with DNA nucleosomal fragmentation (Fig. 1B) with no involvement of PARP cleavage (Fig. 1C).

**Differential Effects of MSeA versus Selenite Exposure on Cell Cycle Regulatory Proteins.** Biochemically, G<sub>1</sub> versus S-phase arrest effects induced by these two types of selenium were differentially associated with the expression patterns of p27kip1 and p21cip1, two key inhibitory proteins for cyclin-dependent kinases for regulating G<sub>1</sub> cell cycle progression in many cell types (23, 24). After 24-h exposure to MSeA, a dose-dependent increase of p27kip1 protein level, starting with as little as 2  $\mu$ M MSeA, was evident (Fig. 1C). p21cip1 protein level showed a similar pattern of increase, albeit the magnitude of change was less dramatic (Fig. 1C). In contrast to the expression patterns in MSeA exposed cells, a reduction of p27kip1 and p21cip1 expression level was observed in cells exposed to 5  $\mu$ M selenite (Fig. 1C). The expression level of cyclin D1, an important G<sub>1</sub> cyclin for cell cycle progression (24), was decreased in cells exposed to an apoptogenic level of either MSeA or selenite (Fig. 1C). These data indicate that elevated expression levels of p27kip1 and p21cip1 proteins were associated with G<sub>1</sub> arrest in MSeA-exposed cells irrespective of apoptosis and that the decreased expression of these two proteins was associated with S-phase arrest and apoptosis induced by selenite exposure.

**Differential Involvement of Superoxide in MSeA versus Selenite Induction of Apoptosis.** As far as primary chemical mediators of apoptosis signaling induced by the two forms of selenium are concerned, our earlier work has implicated superoxide for the genotoxic and apoptotic actions of selenite in mouse leukemia cells using a SOD-mimetic agent (9). This hypothesis was further strengthened by a recent study in LNCaP prostate cancer cells with a different SOD mimetic agent that effectively blocked the apoptotic efficacy of selenite exposure (29).

In the present study, we assessed the impact of SOD (200 units/ml) and catalase (1000 units/ml) added to cell culture media on apoptosis induced by MSeA or selenite at 20-h exposure. The enzyme combination by itself did not affect cell morphology (Fig. 2A, *a' versus a*) or DNA nucleosomal



**Fig. 2.** Effects of SOD and/or catalase on MSeA- versus selenite-induced apoptosis of DU145 cells at 20 h of exposure. **A**, representative phase-contrast photomicrographs of DU145 cells after 20-h exposure to PBS as control (*panel a*), 5  $\mu$ M MSeA (*panel b*), or 5  $\mu$ M sodium selenite (*panel c*). *Panels a'–c'*, the respective groups with the addition of 200 units of SOD and 1000 units of catalase per ml of medium. Note the prominence of cytoplasmic vacuoles in adherent cells after selenite exposure regardless of SOD/catalase treatment.  $\times 200$ . **B**, agarose gel electrophoretic detection of nucleosomal DNA fragmentation and immunoblot detection of cleavage of PARP and caspase-7 for samples in **A**. The leftmost Lane on the agarose gel was loaded with 100-bp DNA size markers. **C**, comparison of SOD versus catalase effect on selenite-induction of DU145 apoptotic DNA fragmentation after 20-h exposure to 5  $\mu$ M selenite.

integrity (Fig. 2B, Lane *a'* versus *a*). SOD plus catalase did not alter the morphological (Fig. 2A, *panel b'* versus *b*) and DNA fragmentation (Fig. 2B, Lane *b'* versus *b*) responses of DU145 cells to MSeA exposure. Similarly, the extent of PARP cleavage in MSeA-exposed cells was not affected by SOD plus catalase, nor was the cleavage (indicative of activation) of caspase-7, one of the executioner caspases including caspase-3 that are responsible for PARP cleavage (Fig. 2B, Lane *b'* versus *b'*; Ref. 12).

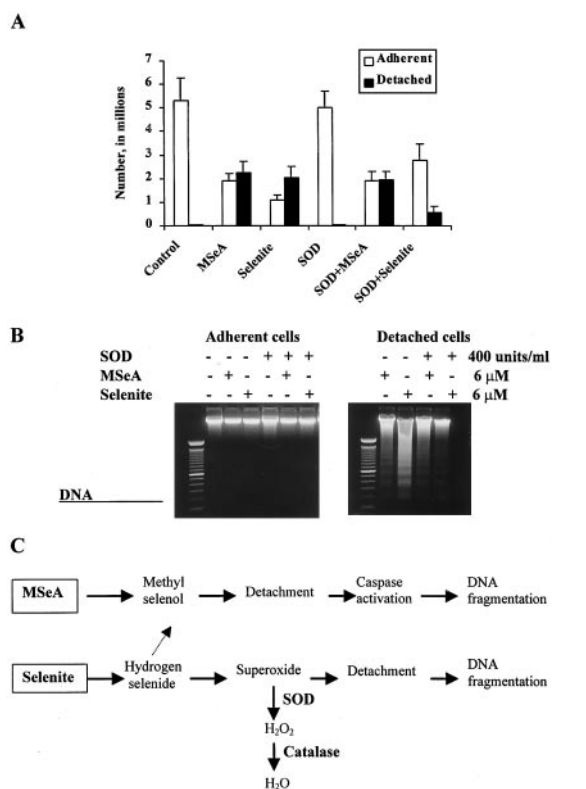
On the other hand, SOD plus catalase increased the number of adherent cells (Fig. 2A, *panel c'* versus *c*) and decreased the extent of DNA fragmentation (Fig. 2B, Lane *c'* versus *c*). These enzymes did not block the selenite-induced formation of cytoplasmic vacuoles in adherent cells (Fig. 2A, *panel c'* versus *c*). Cytoplasmic vacuoles have recently been shown as swollen mitochondria in LNCaP prostate cancer cells (29). Cotreatment of SOD plus catalase with selenite had a negligible effect on the low level of PARP cleavage or

caspase-7 cleavage (Fig. 2B, Lane *c'* versus *c*). Taken together, the results show that selenite-induced DNA apoptotic fragmentation was independent of caspase-mediated PARP cleavage and was at least in part mediated through superoxide and/or hydrogen peroxide generation.

To further distinguish the reactive oxygen species involved, we compared the effect of SOD or catalase alone on the extent of DNA fragmentation induced by selenite exposure. Catalase did not provide any protection against selenite-induced DNA fragmentation, whereas SOD provided the same level of protection afforded by SOD and catalase combined (Fig. 2C). Therefore, the generation of superoxide, rather than hydrogen peroxide, was primarily responsible for apoptosis induction by selenite exposure in DU145 cells.

Because our earlier work has shown the loss of cell attachment as a prerequisite for caspase activation and DNA nucleosomal fragmentation induced by MSeA exposure of DU145 cells, resembling detachment-activated apoptosis, *i.e.*, anoikis (12), we next examined the effect of SOD cotreatment with MSeA versus selenite on this cellular event. The detached cells (floaters) in conditioned media after selenite exposure for 24 h were collected by centrifugation and analyzed separately from the respective adherent cells. SOD cotreatment with MSeA did not change the number of adherent cells and that of floaters; in contrast, SOD cotreatment with selenite significantly increased the number of adherent cells and decreased that for the floaters (Fig. 3A). DNA nucleosomal fragmentation was detected exclusively in the floaters but absent in the adherent cells, regardless of selenite or MSeA exposure (Fig. 3B). SOD cotreatment with selenite not only significantly decreased the number of floaters (Fig. 1A) but also inhibited the extent of DNA nucleosomal fragmentation in the floaters (Fig. 3B). These data indicate that cell detachment was a necessary step for caspase-independent cell death execution induced by selenite in DU145 cells. Distinct from MSeA-induced cell detachment, the selenite-induced cell detachment and death execution were mediated in major part by superoxide (see scheme in Fig. 3C). However, cytoplasmic vacuoles induced by selenite exposure appeared to be insensitive to SOD and were not likely caused by superoxide generation.

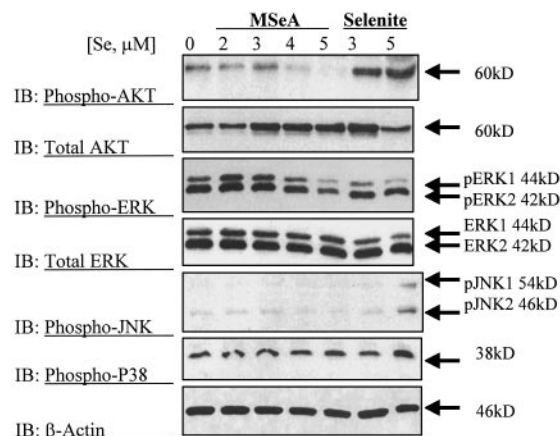
**Differential Effects of MSeA versus Selenite on AKT, ERK1/2, JNK1/2, and p38MAPK.** To determine the potential involvement of these protein kinases in cell cycle arrest and apoptosis in DU145 cells, we surveyed their phosphorylation status after 24-h exposure in dose-response experiments. As shown in Fig. 4, MSeA exposure at subapoptotic doses (up to 3  $\mu$ M) did not decrease AKT or ERK1/2 phosphorylation levels. At doses that led to apoptotic cell death, MSeA-exposed cells contained decreased phosphorylation levels of these two protein kinases in dose-dependent manners. The level of their respective total enzyme proteins was not decreased by MSeA exposure. There was no detectable effect of MSeA exposure in the entire dose range on JNK1/2 or p38MAPK phosphorylation levels by 24 h of exposure. These data indicated that decreases in AKT and/or ERK phosphorylation were associated with apoptosis, but not with G<sub>1</sub> arrest, induced by MSeA exposure in the absence of induction of SAPKs, JNK1/2 and p38MAPK.



**Fig. 3.** A, effects of SOD (400 units/ml) coinubation on MSeA- versus selenite-induced cell detachment after 24 h of exposure. The concentration of each selenium agent was 6  $\mu\text{M}$  in this experiment. Conditioned media were collected and spun to pellet the detached cells. Adherent cells were trypsinized off T75 cell culture flasks. The number of adherent cells and detached cells was counted manually with a hemocytometer. Vertical bar, SD of replicate counting of 5–10 fields per sample. The data were representative of three similar experiments. B, DNA nucleosomal fragmentation status of MSeA- versus selenite-exposed adherent cells and detached cells from the experiment in A. C, schema for involvement of superoxide in apoptosis induction pathways in DU145 cells by MSeA versus selenite exposure.

In contrast to the suppression effects of MSeA exposure on AKT phosphorylation, exposure to selenite for 24 h at both a subapoptotic (3- $\mu\text{M}$ ) and apoptotic dose (5- $\mu\text{M}$ ) increased the phosphorylation level of AKT (Fig. 4). Contrary to the lack of effects by MSeA exposure on JNK1/2 or p38MAPK phosphorylation, selenite exposure at 5  $\mu\text{M}$  led to increased phosphorylation of these stress- and apoptosis-related protein kinases (Fig. 4). There was no appreciable selenite effect on the phosphorylation level of ERK2, although a minor inhibitory action on the phosphorylation of ERK1 might be associated with the apoptotic dose of selenite (Fig. 4). These results, therefore, confirm and extend the differential associations of these major protein kinases with apoptosis (12) and cell cycle arrest of DU145 prostate cancer cells induced by the two types of selenium.

**Temporal Patterns of MSeA-induced Phosphorylation Changes of Protein Kinases.** To probe the connection among protein kinase phosphorylation status and apoptosis induced by MSeA exposure, we next investigated the temporal patterns of AKT and ERK1/2 changes and p27kip1



**Fig. 4.** Effects of MSeA versus selenite exposure for 24 h on the phosphorylation status and expression level of selected protein kinases in a dose-response experiment (same as in Fig. 1C).  $\beta$ -Actin expression was probed to indicate evenness of loading of protein extract from each treatment. Arrows, proteins of interest.

expression in relationship to apoptosis execution in DU145 cells (12 h and beyond; Fig. 5A). Exposure to 5  $\mu\text{M}$  MSeA led to an accelerated PARP cleavage between 12 and 16 h. Hypophosphorylation of AKT as well as of ERK1/2 was detected at 12 h. p27kip1 expression was elevated in the same time frame as the accelerated cleavage of PARP. The phosphorylation status of p38MAPK was not affected during the exposure period examined.

In an experiment designed to examine the more immediate responses (4–12 h; Fig. 5B), MSeA exposure increased AKT phosphorylation between 4 h and 8 h and then led to a sharp decrease of AKT phosphorylation at 10 h, which preceded the occurrence of accelerated PARP cleavage at 12 h. In contrast to AKT, the phosphorylation level of ERK1/2 was not increased during the acute MSeA exposure phase (up to 8 h). A reduction of ERK1/2 phosphorylation level was observed at 10 h of exposure, coinciding with the sudden switching of AKT phosphorylation status between 8 h and 10 h. These time course experiments indicated that the dephosphorylation of AKT and ERK1/2 preceded the occurrence of caspase-mediated apoptosis execution and of the onset of increased expression of p27kip1 and further ruled out an involvement of p38MAPK phosphorylation in cell cycle arrest and apoptosis execution induced by MSeA.

**Minimal Contribution of AKT Phosphorylation Changes to MSeA Induction of Apoptosis.** The temporal patterns of AKT and ERK phosphorylation changes in relation to caspase-mediated apoptosis suggested potential contributions to apoptosis signaling induced by MSeA exposure. Because of the known significance of PI3K/AKT pathway for supporting cell survival in other models (17, 18), we next examined the interaction of MSeA with PI3K inhibitors. As shown in Fig. 6A, treatment of DU145 cells with wortmannin for 4 h and 20 h significantly inhibited AKT phosphorylation but did not, by itself, lead to apoptosis. Wortmannin cotreatment with MSeA did not significantly alter the extent of PARP cleavage at 20 h when compared with that of MSeA exposure alone.

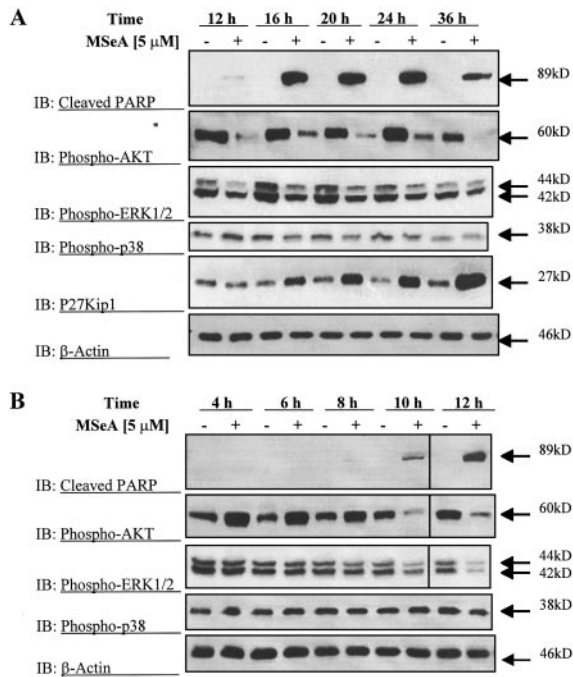


Fig. 5. Time course (A, 12–36 h; B, 4–12 h) of effects of MSeA exposure on protein kinase phosphorylation status and expression of p27kip1 in relationship to MSeA-induced cleavage of PARP in DU145 cells. These two experiments were done several months apart, which may account for the slight different timing of onset of PARP cleavage.

Because of a concern over the instability of wortmannin in aqueous solution (30), we then examined the effect of a more stable PI3K inhibitor LY294002 (31). LY294002 was introduced at 8 h of MSeA exposure, a time frame just before AKT undertaking a dramatic switch from hyper- to hypophosphorylation. Although LY294002 specifically inhibited AKT phosphorylation without affecting ERK1/2 phosphorylation, its cotreatment with MSeA exposure did not increase the extent of caspase-mediated PARP cleavage over that of MSeA exposure alone (Fig. 6B). Taken together, these results indicated that MSeA induction of AKT dephosphorylation might be one of many contributory signals for apoptosis signaling, but, by itself, it is not sufficient to account for apoptosis induction in this cell line.

**Discussion**

Prostate cancer is the most common histological malignancy and second leading cause of cancer deaths among North American men (32). Prostate cancer chemoprevention with dietary supplements holds strong promise for reducing the risk of this deadly disease. The prostate appears to be a responsive organ site for the cancer chemopreventive activity of selenium in American men (1). Although the normal prostate epithelial cells *in vivo* have very slow growth kinetics, increased mitogenic activity, cell cycle dysregulation, and increased cell survival as a result of autocrine and paracrine activation loops are common features of malignant prostate cancer cells (16, 33, 34). Antiproliferative activity through cell cycle arrest and induction of apoptosis are ex-

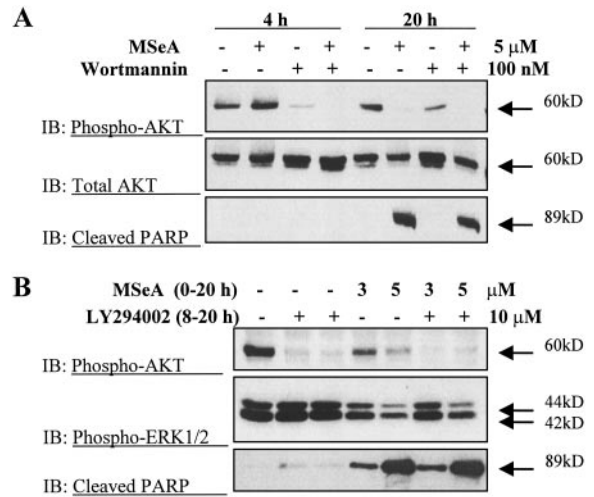


Fig. 6. A, effect of PI3K inhibitor wortmannin on AKT phosphorylation and MSeA-induced PARP cleavage in DU145 cells. B, effect of PI3K inhibitor LY294002 introduced 8 h after initiation of MSeA exposure on AKT and ERK1/2 phosphorylation and PARP cleavage at 20 h in DU145 cells. Arrows, proteins of interest.

pected to contribute to the direct actions of selenium on target epithelial cells to inhibit the growth of prostate cancerous lesions in the context of cancer chemoprevention. The present work was undertaken within the framework of selenium metabolites and their potential molecular targets for mediating cell cycle arrest and apoptosis.

We documented and characterized the differential effects of MSeA and selenite in DU145 prostate cancer cells on apoptosis signaling and cell cycle arrest with respect to PI3K/AKT, ERK1/2, p38MAPK, and JNK1/2 kinases. Pertaining to cell cycle action, MSeA exposure led to a profound G<sub>1</sub> arrest, irrespective of apoptosis induction; in contrast, selenite exposure led to cell cycle arrest in S phase (Fig. 1B). These patterns were in agreement with data obtained in mammary cancer cell lines (7–10). The cell cycle arrest effects were associated with differential patterns of expression of p21cip1 and p27kip1 (Fig. 1C), in that MSeA-arrested cells contained much elevated p27kip1 and p21cip1, whereas these proteins were down-regulated in the selenite-exposed cells. In a time course experiment, p27kip1 induction by exposure to an apoptogenic level of MSeA occurred concomitantly with PARP cleavage (Fig. 5A). Because cell cycle G<sub>1</sub> arrest and p27kip1 and p21cip1 induction occurred at subapoptotic doses of MSeA exposure, it will be important in future work to determine whether G<sub>1</sub> arrest is a prerequisite for apoptosis signaling, and what role P27/kip1 and p21cip1 play in methyl selenium induction of G<sub>1</sub> arrest.

Concerning the potential chemical mediators of apoptosis signaling induced by these two types of selenium, experiments with SOD and catalase indicated that selenite induction of caspase-independent apoptosis was sensitive to and inhibited by the addition of SOD in the culture media, but not catalase alone (Figs. 2 and 3). Furthermore, the data indicated that cell detachment and DNA fragmentation within the floaters, but not cytoplasmic vacuole formation in the adher-

ent cells (Fig. 2A), were SOD-sensitive cellular events with selenite exposure (Fig. 3). These data together support superoxide generation as a primary mediator for death signaling induced by selenite exposure. Because cytoplasmic vacuole formation precedes cell detachment on selenite exposure, its significance for death signaling remains to be established in light of the recent finding that cytoplasmic vacuoles represent swollen mitochondria in LNCaP prostate cancer cells (29). Likewise, the relationships among superoxide generation, DNA single-strand breaks, and caspase-independent apoptosis execution require further investigation.

Our data did not show a significant effect of SOD/catalase or SOD alone on MSeA induction of caspase-mediated apoptosis (Figs. 2 and 3), indicating that MSeA action was not likely mediated by either superoxide or hydrogen peroxide. However, it has been reported that intracellular methylselenol generated by methionine- $\gamma$ -lyase using selenomethionine as a substrate leads to massive apoptosis and production of superoxide (35). However, the measurement of superoxide production in that study was done with a selenomethionine substrate level at least a magnitude higher than that necessary to induce apoptotic response (35). The discrepancy between our results and this cited work may relate to the vastly different levels of cell apoptosis in the two studies or to the MSeA exposure in our own, which may produce methylselenol as well as additional selenium metabolites that contribute to apoptosis signaling. More work will be needed to clarify this issue.

With respect to protein kinase pathways and selenium induction of apoptosis signaling and cell cycle arrest, a number of points were noteworthy. First, MSeA exposure led to changes of AKT and ERK phosphorylation without affecting JNK1/2 or p38MAPK, whereas selenite exposure led to increased phosphorylation of AKT, JNK1/2, and p38MAPK (Fig. 4). Second, these changes in protein kinase phosphorylation were observed in closer association with apoptosis than with cell cycle arrests (Figs. 1 and 4). The latter observation cast doubt on the hypothesis that phosphorylation/dephosphorylation of these protein kinases serves as primary signaling for the cell cycle-inhibitory action of either selenium in this cell line. However, it should be recognized that the possibility exists for selenium to modulate the activities of protein kinases and other enzymes through phosphorylation-independent mechanisms such as redox modification (36, 37).

To further explore the connection between AKT and ERK phosphorylation changes and MSeA-induced apoptosis signaling, we examined the time course of their changes in relationship to caspase-mediated apoptosis execution. The results (Fig. 5) provided a rough temporal sequence of events during MSeA induction of apoptosis. Exposure to MSeA led to an acute elevation of AKT phosphorylation that lasted through 8 h. Because no enhancing effect by MSeA was observed for ERK1/2 or p38MAPK or JNK1/2 within the same time frame, the acute hyperphosphorylation action by MSeA exposure is likely specific for AKT itself or for the PI3K-AKT pathway. The onset of caspase-mediated apoptosis execution as revealed by the appearance of cleaved

PARP was observed several hours after the sudden onset of dephosphorylation of AKT and ERK1/2. These temporal patterns suggested the potential significance of either one or both protein kinase pathways for apoptosis signaling induced by MSeA. The data from experiments with PI3K inhibitors (Fig. 6) showed that the specific inhibition of the PI3K-AKT axis alone in this cell line was not sufficient to trigger apoptosis within the time frame studied. It is, therefore, likely that MSeA induction of dephosphorylation of AKT is only one of many contributory signals, but not in itself sufficient, for apoptosis. Further work is needed to dissect the contribution of the PI3K-AKT and ERK1/2 pathways to apoptosis signaling using genetic approaches with constitutively active mutants of these pathway constituents.

In summary, the data showed distinct patterns of modulation of PI3K-AKT, ERK1/2, JNK1/2, and p38MAPK by MSeA *versus* selenite exposure, in close association with apoptosis execution through caspase-dependent and -independent pathways, respectively. These differences, plus the distinct cell cycle actions and differential involvement of superoxide as a chemical mediator of apoptosis, further contrasted the actions of two selenium metabolite pools in the DU145 prostate cancer cell line. An understanding of the biochemical and molecular details for these distinct actions of the two pools of selenium will be important for designing more effective selenium agents to specifically target desirable molecular pathways and cellular processes for prostate cancer prevention.

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