Methyl selenium metabolites decrease prostate-specific antigen expression by inducing protein degradation and suppressing androgen-stimulated transcription

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Abstract

Prostate-specific antigen (PSA) is widely used clinically for prostate cancer diagnostics and as an indicator of therapeutic efficacy and recurrence. Several human chemoprevention trials are being conducted to validate the prostate cancer prevention efficacy of selenium and PSA is used in these trials as a biomarker of response. A better understanding of the effects of selenium metabolites on the kinetics of PSA turnover and secretion in prostate cancer cells treated with selenium at concentrations which are achievable physiologically will be important for interpreting the results of these trials. This study addresses whether the putative active anticancer selenium metabolite methylselenol or its precursor methylseleninic acid (MSeA) specifically inhibits PSA expression in the androgen-responsive LNCaP prostate cancer cell model. The results show that exposure to sub-apoptotic concentrations of MSeA and methylselenol inhibited PSA protein expression and secretion, whereas sodium selenite and selenomethionine lacked inhibitory effect. The inhibition was detectable at 3 h of exposure and required a threshold level of MSeA to sustain. Turnover experiments showed that MSeA caused rapid PSA degradation, which was partially blocked by lysosomal inhibitors, but not by a proteasomal inhibitor. Furthermore, MSeA treatment reduced PSA mRNA level, down-regulated androgen receptor protein expression, and inhibited androgen-stimulated PSA promoter transcription. In summary, methylselenol or MSeA specifically and rapidly inhibited PSA expression through two mechanisms of action: inducing PSA protein degradation and suppressing androgen-stimulated PSA transcription. These findings may have important mechanistic implications for the prostate specific cancer chemopreventive action of selenium. [Mol Cancer Ther 2004;3(5):605–11]

Introduction

Prostate-specific antigen (PSA) is an androgen-regulated serine protease produced by both normal prostate epithelial and prostate cancer cells (1). PSA is a member of the tissue kallikrein (hK) family, and is designated as hK3. It is a major protein in semen, and cleaves semenogelins in the seminal coagulum. The proteolytic activity of PSA in the tumor microenvironment could potentially cleave a number of proteins that may influence prostate cancer genesis and progression. One such protein is the insulin-like growth factor binding protein-3 (IGFBP-3), the major serum binding protein for insulin-like growth factor-1 (IGF-1) (2). IGF-1 is a growth factor for prostate cancer, and increased serum levels of IGF-1 have been shown to be a risk factor for this disease (3, 4). *In vitro* studies indicated that the cleavage of IGFBP-3 by PSA decreases IGF-1 binding, thereby leading to increased proliferation in response to added IGF-1 (5). PSA may also cleave proteins that affect prostate cancer cell migration and metastasis. For instance, PSA can cleave extracellular matrix proteins including fibronectin and laminin and the microinvasion of LNCaP cells can be inhibited by PSA-neutralizing antibodies (6). In spite of the above findings, the biological significance of the proteolytic activity of PSA in prostate carcinogenesis and progression has not been firmly established.

Clinically, circulating PSA is a well-accepted biomarker for monitoring prostate cancer progression, response to therapy, and recurrence. The value of PSA screening has recently been demonstrated in a cancer prevention setting with the 5α-reductase inhibitor finasteride (7). Finasteride is known to decrease the conversion of testosterone to dihydrotestosterone, the primary androgen in the prostate and for PSA expression regulation (1). To have some expectation of how blood PSA might be affected by a chemopreventive agent for which the impact on androgen level or signaling is not as clearly defined, it is critical to acquire a good understanding of the kinetics of PSA expression and turnover in cells treated with the chemopreventive agent and at concentrations which are achievable physiologically. The Selenium and Vitamin E

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Chemoprevention Trial (SELECT) is currently recruiting 32,000 healthy men to examine the efficacy of selenium alone or in combination with vitamin E in prostate cancer prevention (8). Several smaller trials are also being conducted to study the effect of selenium on high-grade prostatic intraepithelial neoplasia in at-risk patients or the development of prostate cancer in a watchful waiting cohort (9, 10). Serum PSA level is used in all these trials as a biomarker of disease progression and intervention efficacy. A sound knowledge of the relationship between selenium and PSA expression and turnover would be helpful to the interpretation of these studies. Methylseleninic acid (MSeA) has been reported to down-regulate PSA expression via disruption of androgen receptor (AR) signaling (11, 12). The present study focuses primarily on PSA by providing new information regarding the effects of selenium on PSA secretion and degradation. Additional data are also described to show that not all selenium compounds are alike in their ability to reduce PSA expression and that some threshold level of a key metabolite is required to sustain the inhibitory response.

Materials and Methods

Materials

Sodium selenite pentahydrate was purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Selenite is nonenzymatically reduced by glutathione to generate hydrogen selenide, which is thought to be primarily responsible for induction of DNA single-strand breaks (13) (see Fig. 1). MSeA was synthesized and kindly provided by Dr. Howard Ganther, University of Wisconsin (14). MSeA is believed to quickly convert to methylselenol (CH_3SeH) nonenzymatically by reacting with reduced glutathione intracellularly (see Fig. 1). Methylselenol is the putative active selenium metabolite for cancer prevention (15, 16). Pure methylselenol cannot be used as is, because it is highly reactive and difficult to prepare chemically. Seleno-L-methionine (SeMet) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

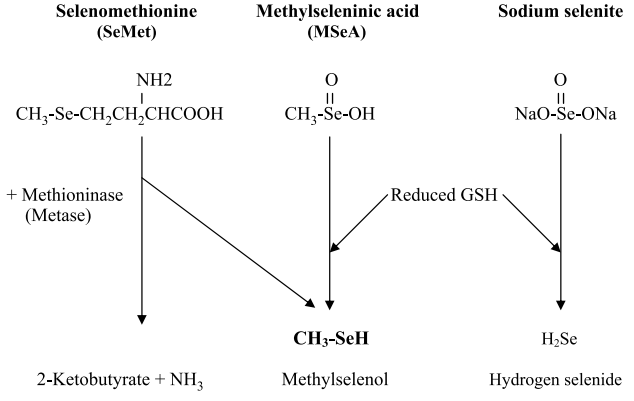

Figure 1. Selenium compounds used in this study and their likely conversion into the active selenium metabolite for suppressing PSA expression.

Methods

Methylselenol Generation from SeMet. As described in our earlier publication (17), methioninase (Metase) was used to release methylselenol from SeMet through the following reaction: $CH_3SeCH_2CH_2CH(NH_2)COOH + H_2O \rightarrow CH_3SeH + CH_3CH_2C(O)COOH + NH_3$ (see Fig. 1). Purified recombinant Metase was purchased from Wako Pure Chemical Industries, Ltd. (Richmond, VA). The enzyme preparation was reconstituted in PBS and added to the culture medium to initiate methylselenol release.

Cell Culture and Treatments. LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics. When cells were 60–70% confluent (usually 48 h after plating), the medium was changed and treatment with selenium was started. To standardize the condition of selenium exposure, cells were cultured in a volume to surface area ratio of 0.2 ml/cm^2 (15 ml for a T75 flask and 5 ml for a T25 flask).

Apoptosis Evaluation. At 24 h of selenium treatment, the spent medium was collected and spun at 200 × *g* for 5 min to collect detached floaters. These were combined with the adherent cells for the detection of DNA nucleosomal fragmentation by agarose gel electrophoresis as previously described (13). Immunoblot of cleaved poly(ADP-ribose)-polymerase (PARP) was used as a marker of caspase-mediated apoptosis as described previously (18). The antibody for cleaved PARP was purchased from Cell Signaling Technology (Beverly, MA).

ELISA for PSA. An ELISA kit from United Biotech Inc. (Mountain View, CA) was used for measurement of PSA in both conditioned media (secreted) and cell lysate (cellular). Before selenium treatment began, LNCaP cells were gently washed 3 times with PBS to reduce background PSA. Fresh medium containing selenium was then added. Conditioned media samples were collected and centrifuged to remove detached cells. Cell lysate was prepared in PBS by freezing on dry ice and thawing for three cycles and sonicated for 15 s. The supernatant obtained after centrifugation (16,000 × *g*, 20 min, 4°C) was collected and stored at −20°C. Protein content of the lysate was determined by the Lowry method for normalization of results.

Immunoblot Assay for PSA and Androgen Receptor. Cell lysate was prepared in RIPA buffer. Supernatants after centrifugation (14,000 × *g*, 20 min, 4°C) were recovered and the protein content was quantified by the Lowry assay. Proteins were size-separated by electrophoresis on SDS-polyacrylamide gels under non-reducing conditions and were electroblotted onto nitrocellulose membranes. An antibody for PSA was purchased from DAKO (Glostrup, Denmark), and an antibody for AR was purchased from BD PharMingen (San Diego, CA).

Measurement of PSA Turnover. In estimating PSA protein turnover, LNCaP cells were treated with 20 μg/ml of cycloheximide to block new protein synthesis in the absence or presence of MSeA for 1, 2, 3, or 4 h. PSA in cellular lysate was measured by ELISA for the determination of half-life. In additional experiments, inhibitors of

26S proteasomal degradation (lactacystin) and lysosomal degradation (chloroquine and ammonium chloride) were used to determine the role of these intracellular degradation processes in PSA turnover. The inhibitory efficacy of lactacystin on proteasomal degradation was confirmed by immunoblotting of cyclin D1, which is known to be degraded through proteasome pathway (19).

RNA Extraction and PSA mRNA Detection. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). PSA cDNA clone was obtained from Invitrogen, and used to prepare probe for hybridization. The data were normalized to 18S rRNA for loading correction. Northern blotting and hybridization were performed using a standard procedure as described (12).

PSA Promoter-Luciferase Assay. To assess the possible inhibitory effect of MSeA on PSA transcription, LNCaP cells were co-transfected with a luciferase reporter plasmid driven by a 6-kb PSA promoter and a CMV-β-galactosidase (β-gal) vector and incubated in serum-free, phenol red-free medium for 24 h to reduce AR signaling as described previously (20). The cells were treated with different levels of MSeA with or without 1 nM mibolerone, a non-metabolizable androgen analogue, for 24 h in 5% charcoal stripped serum-added medium. Cell extracts were prepared for luciferase and β-gal assays. Luciferase activities were normalized to β-gal activities to correct for differences in transfection efficiency.

Results

Cell Death Response of LNCaP Cells to MSeA or Selenite

In view of the cytotoxicity of high levels of selenium, we first performed a dose range finding experiment to make sure that the PSA studies would be done without complication from cell death effects. MSeA exposure at 7 μM for 24 h caused minimal DNA fragmentation (Fig. 2A) or cleavage of PARP, a key substrate of caspase-3 (Fig. 2B). Exposure to 2 μM selenite for the same duration induced comparable DNA nucleosomal fragmentation and PARP cleavage. In subsequent experiments, we chose sub-apoptotic concentrations of MSeA (7 μM or lower) and selenite (2 μM) to investigate their effects on PSA expression.

MSeA, but not Selenite, Decreased Cellular and Secreted PSA

Treatment of cells with MSeA for 6 h resulted in a concentration-dependent decrease in cellular PSA level (Figure 3A). The results are expressed as percentage of the untreated control. The control value for cellular PSA was about 100 ng/mg protein based on the ELISA. MSeA decreased cellular PSA in a dose-dependent manner, with IC_{50} less than 3 μM. There was little change in cell number by MSeA treatment during this period (data not shown). Fig. 3A also shows that MSeA decreased the amount of secretory PSA recovered in the culture medium. Untreated control cells secreted about 90 ng PSA per T25 flask during this 6-h period. MSeA at a concentration of 3, 5, or 7 μM reduced this amount to about 82%, 55%, or 30% of the control value, respectively. Thus, it appeared that cellular

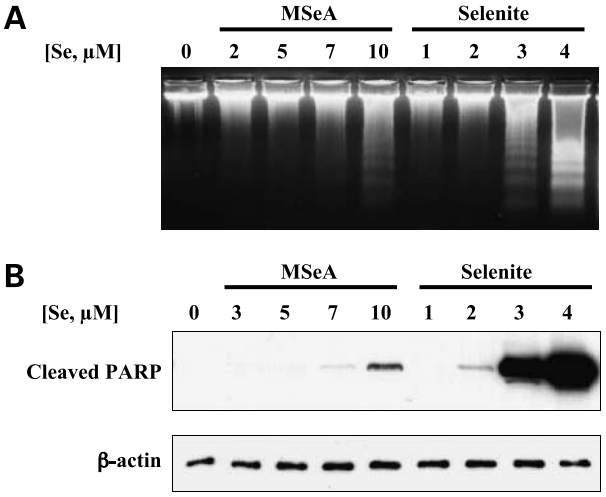

Figure 2. **A,** agarose gel (1.5%) electrophoretic detection of apoptotic nucleosomal DNA fragmentation at 24 h of exposure to increasing concentrations of MSeA or selenite in LNCaP cells. **B,** immunoblot detection of cleaved PARP (*M*, 89,000) as a biochemical marker of caspase-mediated apoptosis. β-Actin was probed to indicate evenness of loading of protein extract from each treatment.

PSA was inhibited to a greater magnitude by MSeA than was the secretory PSA. Selenite, on the other hand, did not decrease cellular or secretory PSA within the same time frame. Figure 3B shows the immunoblotting of PSA expression level in cell lysate, confirming the inhibition by MSeA at 6 h and through 24 h. Immunoblot also confirmed the lack of an effect by 2 μM selenite treatment, from 6 to 24 h.

Methylselenol Generated from Selenomethionine *in Situ* Decreased PSA

To verify the specific effect of the monomethylated selenium metabolite, we tested the impact of methylselenol generated by reacting SeMet with Metase (see "Methods" and Fig. 1) on cellular PSA at 6 h of exposure. Neither Metase nor its substrate SeMet alone inhibited PSA expression (Fig. 3C). Metase plus SeMet, however, decreased PSA level in a concentration-dependent manner. The above findings show that not all chemical forms of selenium have similar efficacies in reducing PSA expression.

Changes in PSA Level in Response to MSeA as a Function of Time

The suppressive effect of MSeA on PSA level was detectable and highly significant by 3 h of exposure in cell lysate (Fig. 4A) and in conditioned media (data not shown). The persistence of this inhibitory response was determined after 6, 12, or 24 h of exposure to 3, 5, or 7 μM MSeA (Fig. 4B). In general, the higher MSeA doses produced a more sustained reduction of PSA during the 24-h period. With the 3-μM MSeA dose, the cellular level of PSA reached a nadir at 6 h but gradually rebounded to near control level by 24 h of exposure. The data suggest that MSeA, when present at a low concentration, is likely to be exhausted over time. In an attempt to verify the above interpretation, a second MSeA dose was introduced at 24 h. After the

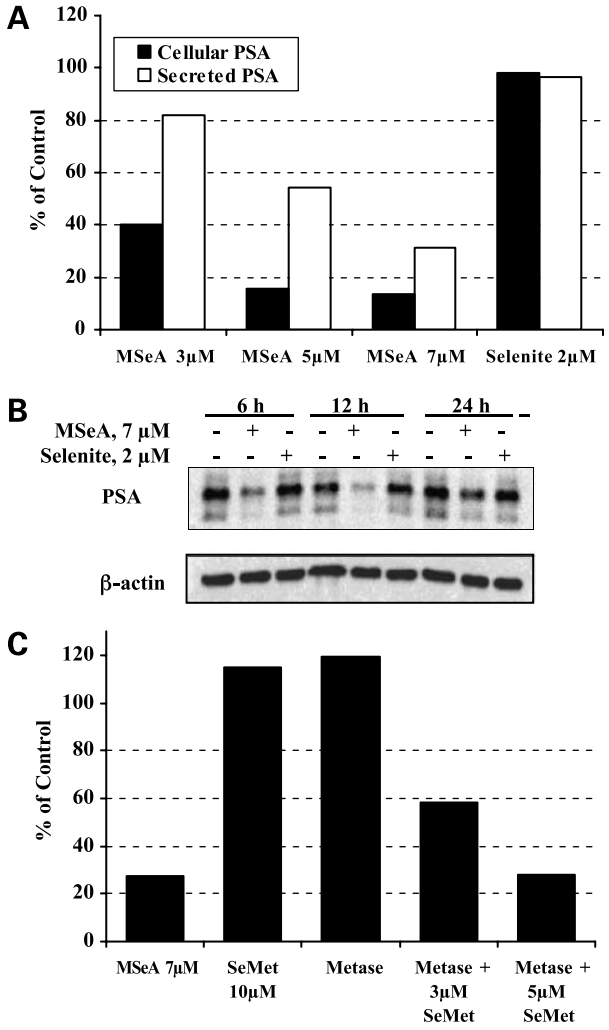

Figure 3. **A,** ELISA detection of cellular PSA (*solid bars*) and secreted PSA in conditioned medium (*empty bars*) after exposure of LNCaP cells for 6 h to indicated concentrations of MSeA or selenite. **B,** immunoblot confirmation of differential effect of MSeA *versus* selenite exposure for 6, 12, and 24 h on PSA expression. β-Actin was probed to indicate evenness of loading of protein extract from each treatment. **C,** effect of methylselenol generated by methioninase (*Metase*, 0.05 unit/ml) with selenomethionine (*SeMet*) substrate on cellular PSA expression in LNCaP cells at 6 h of exposure. For experiments in **A** and **C**, each treatment was done in two flasks and PSA measurement was done in duplicate or triplicate for each flask. The average value of two flasks is presented as percentage of untreated control, variability ~5%.

challenge with fresh MSeA, the cellular level of PSA dropped significantly in the next 12-h period (Fig. 4C). The observation strongly implies that a threshold level of MSeA is needed to sustain the inhibitory effect on PSA expression.

MSeA Increased Cellular PSA Degradation

We examined the effect of MSeA on PSA turnover after new protein synthesis was blocked by cycloheximide as a potential mechanism for PSA suppression. Figure 5 shows, on the semi-log plot, the mean values of three independent experiments (each performed with duplicate flasks per time point) ± SD. In MSeA-treated cells, the half-life was reduced to 1.5 h from 3.9 h in the control cells, suggesting that PSA degradation was greatly enhanced in the presence of MSeA.

Because intracellular protein degradation is mediated principally by proteasomes (21) and lysosomes (22), we studied the effects of proteasomal or lysosomal inhibitor on PSA degradation. As shown in Fig. 6A, the inclusion of a proteasomal inhibitor, lactacystin, did not affect the turnover of PSA, either in the absence or presence of

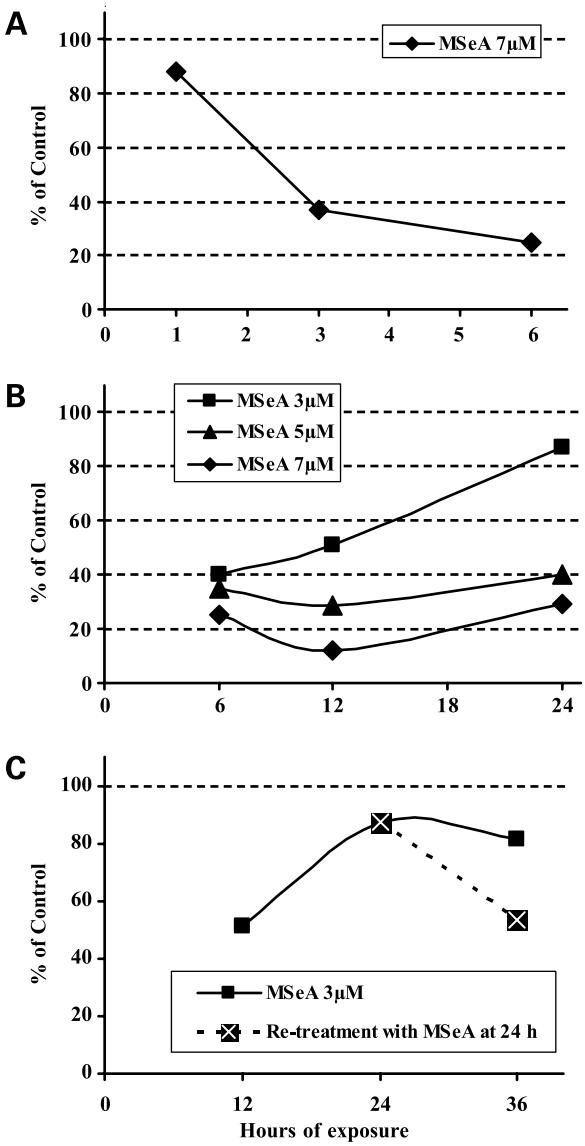

Figure 4. **A,** acute time course of effects of MSeA on cellular PSA protein expression. **B,** extended time course of effects of MSeA on cellular PSA expression. Three concentrations as indicated were used for treatment. **C,** effect of spiking with 3 μM MSeA at 24 h on cellular PSA level at 36 h. PSA was measured by ELISA and was normalized to total protein in cells exposed to extended treatment. *Points,* average of two flasks, variability ~5%. The data are presented as percentage relative to the respective untreated control at each time point for comparison across experiments.

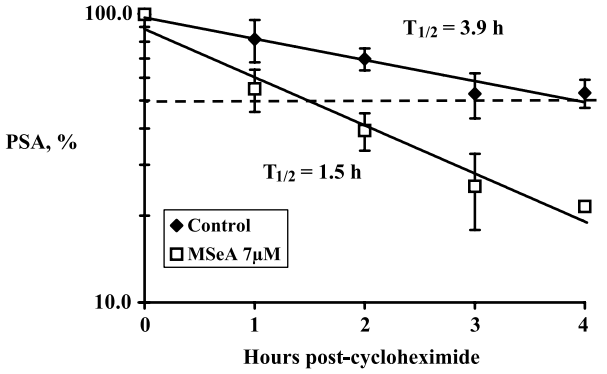

Figure 5. Effect of MSeA on PSA protein turnover in LNCaP cells. The protein synthesis inhibitor cycloheximide (20 μg/ml) was added with or without 7 μM MSeA at time 0. At specified time points, cells were harvested and cell lysate PSA was measured by ELISA. *Points,* mean of three independent experiments plotted on semi-log scale relative to respective time 0 PSA value as 100%; *bars,* SD.

MSeA. This level of lactacystin abolished proteasomal-mediated cyclin D1 turnover in LNCaP cells as shown here by immunoblot, confirming this effective dose described in a recent paper (19), The above finding suggests that PSA is probably not degraded by proteasomes in LNCaP cells.

Inhibiting lysosomal degradation by lysosomotropic agent chloroquine or ammonium chloride in the absence of MSeA resulted in an enhanced cellular PSA retention (Fig. 6B). When these inhibitors were used along with MSeA, a partial reversal of the MSeA effect on PSA degradation was observed. These results are consistent with an induction of lysosomal degradation in the turnover of PSA.

MSeA Decreased PSA mRNA Abundance and Androgen-Stimulated Transcription

To assess gene transcription-related effects, we next evaluated PSA mRNA level by Northern blot (Fig. 7A) in MSeA-treated cells. The results showed a rapid and marked decrease at 3 h and a partial recovery by 24 h. This pattern was consistent with the change in PSA level over the same time period as shown in Figs. 3B and 4. Immunoblot analysis of AR expression showed that MSeA decreased AR expression level by 12 h of exposure, but not at 6 h (Fig. 7B). To determine the impact of MSeA on AR-mediated transcription of PSA, we evaluated the PSA-luciferase promoter activity in a transient transfection assay. As shown in Fig. 7C, MSeA treatment for 24 h inhibited androgen-stimulated PSA transcription by over 60%, but did not affect basal PSA promoter activity without androgen stimulation. The data show that the MSeA suppressive effect on PSA level was, at least in part, caused by decreased PSA synthesis due to lower transcript abundance and decreased androgen-mediated gene transcription.

Discussion

Our results showed that MSeA rapidly decreased cellular PSA level in LNCaP cells and the secretion of PSA into the culture medium (Figs. 3 and 4). The suppressive effect of MSeA was observed at concentrations that were ineffective at causing apoptosis (Fig. 2). Because we chose to use sub-apoptotic selenium exposure concentrations in these studies, the down-regulation of PSA was not associated with a general shutdown of gene transcription and protein synthesis due to cell death. Under the same cell culture conditions, a sub-apoptotic dose of sodium selenite did not inhibit PSA expression (Fig. 3). In agreement with two previous reports (23, 24), we did not observe any inhibitory effect of selenomethionine on PSA expression (Fig. 3C). However, we show a dose-dependent inhibition on PSA by selenomethionine only in the presence of Metase to release methylselenol enzymatically from the parent compound (Fig. 3C). Taken together, the data

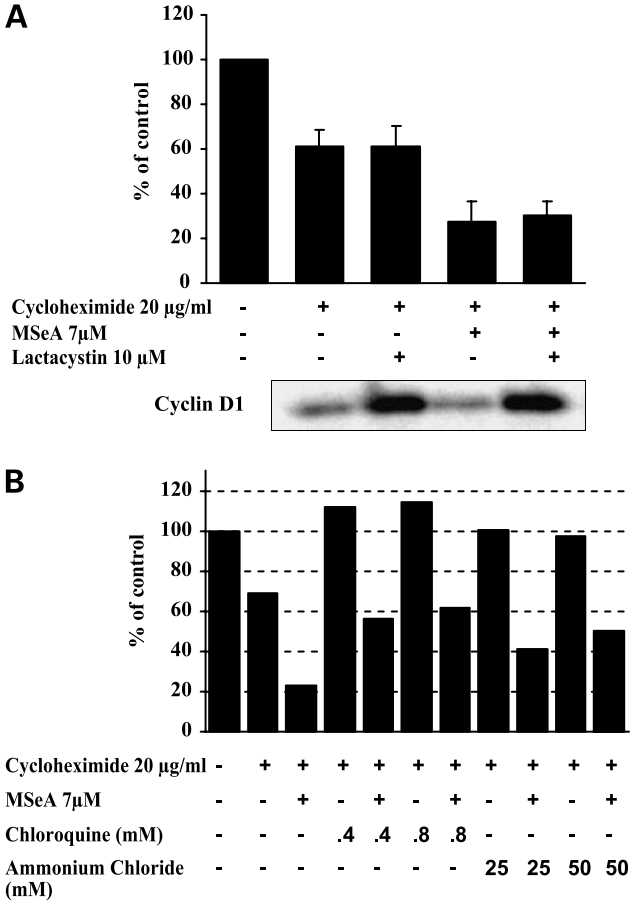

Figure 6. **A,** lack of effects of proteasomal inhibitor lactacystin on MSeA-induced degradation of PSA. Results were from three independent experiments. Lactacystin was either pretreated for 2 h or co-treated with MSeA with the same results. The efficacy of lactacystin for blocking proteasomal degradation was confirmed by immunoblotting for cyclin D1 accumulation. **B,** effects of lysosomal inhibitors chloroquine and ammonium chloride on PSA turnover. Each inhibitor was added alone or with MSeA in simultaneous treatments. Pretreatment with these inhibitors for 2 h before MSeA treatment showed same patterns of effects. For experiments in *both panels*, cells were harvested after 3 h of MSeA exposure and cell lysate PSA was measured by ELISA. Each treatment was done in duplicate flasks, variability ~5%.

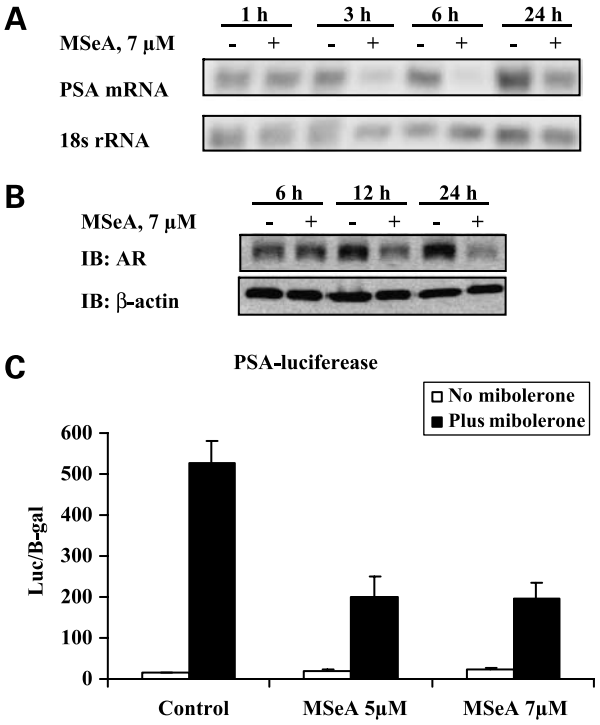

Figure 7. **A,** Northern blot detection of PSA mRNA during MSeA exposure of 1 – 24 h. 18S rRNA was reprobed to serve as loading control. **B,** immunoblot analysis of AR protein expression during MSeA exposure of 6 – 24 h. β-Actin was probed to indicate evenness of loading of protein extract from each treatment. **C,** effect of MSeA on androgen-stimulated PSA promoter activity. LNCaP cells were co-transfected with PSA promoter-luciferase and CMV-β-gal vectors and incubated in serum-free, phenol red-free medium for 24 h. The cells were treated with MSeA ± 1 nM mibolerone (a non-metabolizable androgen analogue) for 24 h in 5% charcoal stripped serum-added medium. Cell extracts were assayed for luciferase and β-gal activities (n = 3 wells). Luciferase activities were normalized to β-gal activities for correcting transfection efficiency differences.

strongly suggest that monomethyl selenium metabolite(s), either in the form of MSeA (added directly to the culture) or methylselenol (generated *in situ* from selenomethionine), is the active species for down-regulating PSA expression in LNCaP cells.

When present at low concentrations, MSeA could be used up over time. It is therefore not surprising to see that at a concentration of 3 μM, its effectiveness gradually weakened when the culture was maintained past 12 h (Fig. 4B). By spiking the medium with a fresh supply of MSeA, the inhibition of PSA was promptly restored (Fig. 4C). The finding suggests that a persistent down-regulation of PSA requires the cells to be exposed continuously to some threshold level of the monomethyl selenium metabolite. It is noteworthy that in US adults, the average plasma selenium concentration is 1.5 μM and supplementation with selenized yeast in a landmark clinical trial that showed a reduction of prostate cancer risk by more than half brought the selenium concentration to 2.4 μM (25). Because the IC_{50} for suppressing cellular PSA expression in our study is less than 3 μM MSeA (Fig. 3A), there is good probability for achieving the selenium enrichment that is needed to inhibit androgen signaling and PSA expression through pharmacological means of selenium delivery.

We have identified two potential mechanisms by which MSeA decreased cellular PSA expression. The first is at the level of protein degradation, and to our knowledge, this is novel. MSeA induced a rapid degradation of PSA detectable by 1 h (Fig. 5), ahead of a decrease of transcript abundance (3 h) (Fig. 7). Our data support lysosomal, but not proteasomal, degradation of intracellular PSA in LNCaP cells and a possible induction of lysosomal activation by MSeA (Fig. 6). Microarray gene expression analysis showed that MSeA is capable of greatly enhancing the transcription of lysosomal cathepsin B and cathepsin L (http://www.roswellpark.org/Dong_Ip_Se_microarray/). This is consistent with our observation that lysosomal inhibitors, such as chloroquine and ammonium chloride, could partially reverse the effect of MSeA on PSA degradation. However, our data did not rule out additional subcellular sites of PSA degradation inducible by MSeA exposure.

Our earlier work has shown that methylselenol precursors, but not selenite or selenide salts, rapidly decreased the expression of vascular endothelial growth factor (VEGF) by prostate cancer cells and matrix metalloproteinase (MMP)-2 by vascular endothelial cells, with significant implications for angiogenic switch regulation (26). Both are secretory proteins and contain intrachain or intermolecular disulfide bonds (26). PSA has five intrachain disulfide bonds (1). On the basis of these commonalities, it could be insightful to determine whether MSeA induces a rapid degradation of VEGF and MMP-2 proteins as well, and if so, whether a common pathway (*e.g.*, lysosomal) is triggered by methyl selenium in the degradation of all three secretory proteins.

The second mechanism is at the gene transcription level. PSA is a known target of AR activation by androgens (1). Treatment with MSeA also decreased the abundance of PSA mRNA (Fig. 7A) that was accompanied by a delayed reduction of AR protein expression (Fig. 7B). Our data showed that MSeA decreased androgen-stimulated PSA promoter transcription without affecting its basal activity in the absence of androgen (Fig. 7C). Our data confirm that in addition to inducing PSA protein degradation, MSeA also decreases androgen signaling, as has been reported recently (11, 12).

Prostate cancer is the most common noncutaneous cancer and the second leading cause of cancer death in American men (27). Androgen and AR are required for the development of both the normal prostate gland and prostate cancer. In the early stages, almost all cancer cells are androgen dependent and highly sensitive to androgen ablation and anti-androgen therapies. Therefore, the methyl selenium-metabolite specific inhibition on PSA expression and AR-mediated signaling may be a unique mechanism to account for the chemopreventive action of selenium against prostate cancer (25, 28). If PSA secreted into the microenvironment of the prostate plays a role in

prostate carcinogenesis and progression, as alluded to in the ''Introduction,'' even small decreases in secretory PSA may be beneficial over time. A recent pilot study has documented that selenized yeast (predominantly as selenomethionine form) supplementation to healthy men with low baseline PSA was found to produce small, but significant, decreases in circulating PSA (29). As much as the cell culture model looks encouraging for methyl selenium intervention of PSA expression and secretion, it is critical to validate these findings in animal models and ultimately in human intervention trials. The methylselenol precursors are attractive candidate agents for future trials.

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