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14. ABSTRACT Prevention trials demonstrated that selenium is a promising chemopreventive agent for prostate cancer. Selenium inhibited human prostate cancer cell growth, blocked cell cycle progression, and induced apoptotic cell death. We have demonstrated a novel mechanism of selenium anticancer action in which selenium markedly reduces androgen receptor (AR) expression and AR-mediated gene expression including prostate-specific antigen (PSA) in human prostate cancer cells in vitro and in vivo. Based on our novel finding that selenium disrupts AR signaling by reducing AR expression, it is conceivable that selenium (reducing AR expression) might improve the efficacy of androgen deprivation therapy. In this application, we will test the effects of selenium on prostate cancer therapy.					
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Introduction

The goal of this application is to elucidate the importance of down regulation of AR signaling by multiple selenium compounds and select the best leading selenium compound for prostate cancer chemoprevention and therapy. In this application, we will further study the mechanisms of AR downregulation by multiple selenium compounds and functional significance of this down regulation in prostate cancer chemoprevention and therapy. Prevention trials demonstrated that selenium reduced prostate cancer incidence by 50%, establishing selenium as a promising chemopreventive agent for prostate cancer. Selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced apoptotic cell death. We have demonstrated a novel mechanism of selenium anticancer action in which selenium markedly reduces androgen receptor (AR) expression and AR-mediated gene expression including prostate-specific antigen (PSA) in human prostate cancer cells in vitro and in vivo. Androgen signaling through androgen receptor (AR) plays an important role not only in maintaining the function of the prostate, but also in promoting the development of androgen-independent prostate cancer. AR signaling is often hyperactive in androgen-independent prostate cancer. A common treatment for prostate cancer is androgen deprivation. Although most men respond to androgen deprivation therapy initially, almost all relapse due to the growth of androgen-independent cancer cells. Most of the androgen deprivation treatments are either blocking androgen-AR binding or reducing the levels of androgen. Based on our novel finding that selenium disrupts AR signaling by reducing AR expression, a completely different mechanism from the current androgen deprivation therapy, it is conceivable that targeting AR signaling by a combination of androgen-deprivation therapy and selenium (reducing AR expression) might improve the efficacy of current androgen deprivation therapy. This concept was validated in vitro in which the combination of selenium and anti-androgen (Casodex) synergistically inhibited clonogenic ability of human prostate cancer cells, providing a rationale for in vivo validation of the combination of selenium and anti-androgen therapy for prostate cancer. The hypothesis is that anticancer effects of multiple selenium compounds are mediated, in part, by inhibition of AR activity and that decreased AR signaling may reduce the incident of prostate cancer and prevent or delay relapses after androgen deprivation therapy. The goal of this application is to elucidate the importance of down regulation of AR signaling by multiple selenium compounds and determine the best leading selenium compound for prostate cancer chemoprevention and therapy.

Body

We have made significant progress of task 1 (i.e., To compare the effect of multiple selenium compounds and determine the molecular basis of the effects of multiple selenium compounds on AR expression (Months 1-8). (Appendix 1)

MSA decreases AR mRNA stability Our results suggest that while MSA decreased AR mRNA levels at the transcriptional level, AR mRNA expression can also be regulated at post-transcriptional level. To examine whether MSA affects AR mRNA stability, LNCaP cells that express functional AR were treated with or without 5 μ M of

MSA in the presence of actinomycin D (5 $\mu\text{g/ml}$) to stop de novo mRNA synthesis. The total RNA was isolated at different time points and AR mRNA levels were measured by Northern blot analysis. The half-life of AR mRNA was determined by comparison of mRNA levels over time between cells treated with or without actinomycin D, either in the presence or absence of MSA. Since actinomycin D is capable of inducing cell death, we monitored cell growth for a period of 24 h and did not observe cell death or growth inhibition with the concentration of actinomycin D used (5 $\mu\text{g/ml}$). We did not observe significant cell death or growth inhibition at 5 μM MSA over a period of 24 h in LNCaP cells. MSA treatment initially enhanced AR mRNA levels within 6 h. However, AR mRNA levels were significantly decreased by MSA compared to the control at 8 h. Figure 1 shows on the semi-log plot, the mean values of percentage of AR mRNA levels over time relative to respective time zero AR mRNA value as 100%. In MSA treated cells, AR half-life was reduced to about 7 h from 12 h in the control cells, suggesting that AR mRNA degradation was greatly accelerated in the presence of MSA after 6 h.

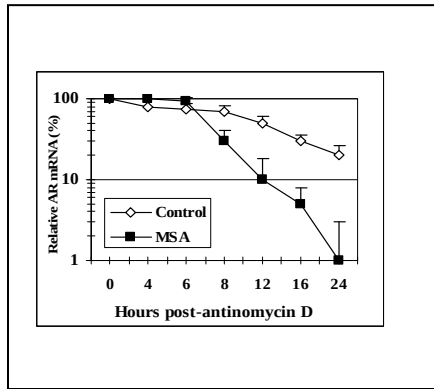


Figure 1 Effect of MSA on AR mRNA stability in LNCaP cells. The mRNA synthesis inhibitor actinomycin D (5 $\mu\text{g/ml}$) was added with or without 5 μM MSA at time 0. At specific time points, cells were harvested and total RNA as isolated by Northern blots. Points, means of three independent experiments plotted on semi-log scale relative to respective time zero AR mRNA value as 100%; bar, SD.

MSA increases AR protein turnover We have demonstrated that MSA decreased the levels of AR mRNA and protein in LNCaP cells. We next examined the effect of MSA on AR protein degradation after new protein synthesis was blocked by cycloheximide as a potential mechanism for downregulation of AR protein level. The protein synthesis inhibitor cycloheximide (50 $\mu\text{g/ml}$) was added with or without 5 μM MSA at time 0. At specified time points, cells were harvested and the levels of AR protein were measured by Western blot using anti-AR antibody. In MSA-treated cells, the half-life of AR protein was reduced to 6 h from 21 h in the control cells (Fig. 2A), suggesting that AR protein degradation was greatly enhanced in the presence of MSA. Systematic protein degradation by the ubiquitin-proteasome system plays an important role in the maintenance of protein stability. Protein ubiquitination provides the recognition signal for the 26S proteasome, leading to protein degradation. Studies demonstrated that AR protein level in cells is regulated by systemic protein degradation pathways. To examine whether selenium induced AR protein degradation via ubiquitin-proteasome system, the 26S proteasome inhibitor MG132 was added to the cells treated with MSA. MG132 was able to retard MSA effect on AR protein levels (Fig. 2B), suggesting that MSA induced AR degradation via a proteasome-dependent pathway.

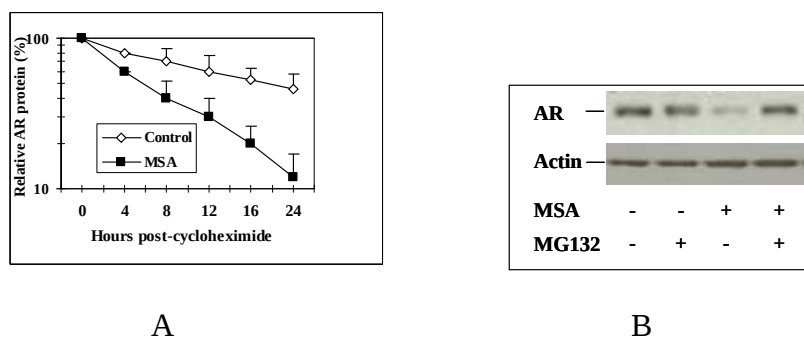


Figure 2. A. Effect of MSA on AR protein turnover in LNCaP cells. The protein synthesis inhibitor cycloheximide (50 μ g/ml) was added with or without 5 μ M MSA at time 0. At specific time points, cells were harvested and cell lysates were prepared. AR protein levels were determined by Western blot analysis using antibody specifically against AR and normalized to α -actin control. Points, means of three independent experiments plotted on semi-log scale relative to respective time zero AR value as 100%; bars, SD. **B.** Effect of MG132 on MSA induced AR protein degradation. MG132 (5 μ M) was added to LNCaP cells together with cycloheximide (50 μ g/ml) in the presence and absence of 5 μ M of MSA. The cell lysates were prepared at 24 h. AR protein levels were determined by Western blot analysis using antibodies specifically against AR and α -actin as a control.

Selenium inhibits AR nuclear translocation AR typically translocates to the nucleus to exert its function on gene expression. To examine whether selenium affects the translocation of AR, Western blot analysis was performed using cell extracts from either cytosolic or nuclear extracts. LNCaP cells were cultured in charcoal stripped FBS for 3 days before adding 10 nM of DHT in the absence or presence of 10 μ M MSA for 2 h. Nuclear and cytosolic fractions were prepared and used for Western blot analysis using the anti-AR antibody. DHT treatment increased the levels of AR protein expression in the nucleus which were reduced by the treatment with MSA (Fig. 3). In contrast, MSA had little effect on AR protein expression in the cytosol. The expression of RNA polymerase II (Pol II) and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively. These results suggest that MSA suppresses AR signaling in part via interruption of AR nuclear translocation.

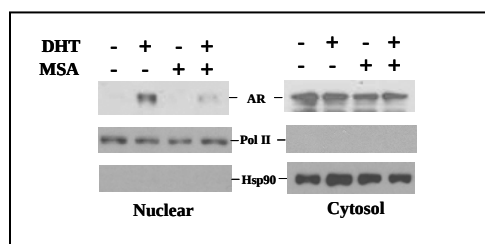


Figure 3. The effect of MSA on AR nuclear translocation. LNCaP cells were cultured in charcoal stripped FBS for 3 days and treated with 10 nM DHT with or without 10 μ M MSA for 2 h. The cells were harvested for preparation of cytosolic and nuclear fractions and analyzed by Western blotting using antibodies against AR, Pol II, or Hsp90. The expression of Pol II and Hsp90

were used as markers for the integrity of the nuclear and cytosolic fractions, respectively.

Selenium inhibits the recruitment of coactivators and enhances the recruitment of corepressors to AR target genes AR interacts with coregulators to achieve maximal transactivation activity. To examine the effects of selenium on the recruitment of coregulators to the promoters of AR target genes, chromatin immunoprecipitation (ChIP) analysis was performed. DHT increased the recruitment of AR and TIF-2, SRC-1 to the promoter of PSA gene in the absence of MSA and this recruitment was greatly diminished in the presence of 5 μ M MSA (Fig. 4). On the other hand, MSA treatment prevented the nuclear translocation of AR in the presence of hormone, thus the corepressors including SMRT and NCoR remain bound to the promoter of the PSA gene (Fig. 4). These results suggest that MSA-mediated reduction of AR activation may be due, at least in part, to a decrease in the recruitment of AR and its coactivators to the promoter of the AR target gene PSA, while maintains corepressors bound to the promoter.

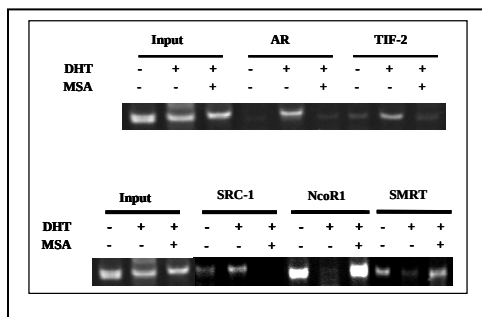


Figure 4. Effect of MSA on the recruitment of AR and coregulators to the promoter of an endogenous AR target gene, PSA. The *in vivo* binding of AR and coregulators to the PSA promoter was examined by the ChIP assay. LNCaP cells were cultured in charcoal stripped condition for 3 days. Soluble chromatin was prepared from cells treated with 10 nM DHT for 4 h (+) or untreated (-) in the presence (+) or absence (-) of 10 μ M MSA and immunoprecipitated with antibodies against AR, TIF-2, SRC-1, SMRT, and NCoR1. Co-precipitated DNA was amplified by PCR using primers that flank the ARE in the PSA promoter region. The presence of total PSA promoter DNA in the soluble chromatin prior to immunoprecipitation was included as input.

We have made progression on task 2 (To determine the role of AR in selenium growth inhibition in prostate cancer). (Appendix 2)

We have demonstrated that overexpression of AR interferes with MSA-mediated growth inhibition. In an effort to evaluate the biological significance of MSA suppression of androgen receptor signaling, we transiently transfected LNCaP cells with a wild-type androgen receptor and assessed the response of the androgen receptor-overexpressing cells to MSA-induced growth inhibition. The MTT assay was conducted at 48 hours post-MSA, and the data are presented in Fig. 5A. In the absence of MSA, cell growth was not altered by the transfection of androgen receptor (data not shown), indicating that the endogenous level of androgen receptor is not a limiting factor for the growth of these cells. MSA treatment inhibited growth by 40% in the mock transfectants, as opposed to 27% in the androgen receptor transfectants. The difference is statistically significant ($P =$

0.003). Thus, androgen receptor overexpression was able to weaken the growth suppressive activity of MSA. One reason that the difference was seemingly compressed was due to the fact that only a fraction of cells was successfully transfected, and in this study, cell growth was assessed using the whole cell population. To address the last problem, we cotransfected cells with the androgen receptor expression vector and a membrane-GFP-encoding construct. The cells were then subjected to BrdUrd labeling, and the data were analyzed by gating just the GFP-positive cells. As shown in Fig. 5B, after selecting for the subset of GFP-positive cells, we found that MSA inhibited DNA synthesis by a very modest 16% in the androgen receptor transfectants, as opposed to 72% in the mock transfectants. Because the GFP and androgen receptor cDNAs are not located in the same plasmid construct, it is possible that not all the cells positive for GFP are also positive for the transfected androgen receptor. Thus, our selection process only led to an enrichment, rather than an exclusive selection, of double-positive cells. Therefore, the difference between the mock transfectants and the androgen receptor transfectants might have been even more pronounced if all the cells used in the experiment were successfully transfected with androgen receptor. Figure 5B also shows that when we did the BrdUrd labeling experiment with the nonenriched androgen receptor-transfected cells, the inhibition by MSA was about 45%, a value half-way between that achieved by the mock transfectants and the enriched androgen receptor transfectants.

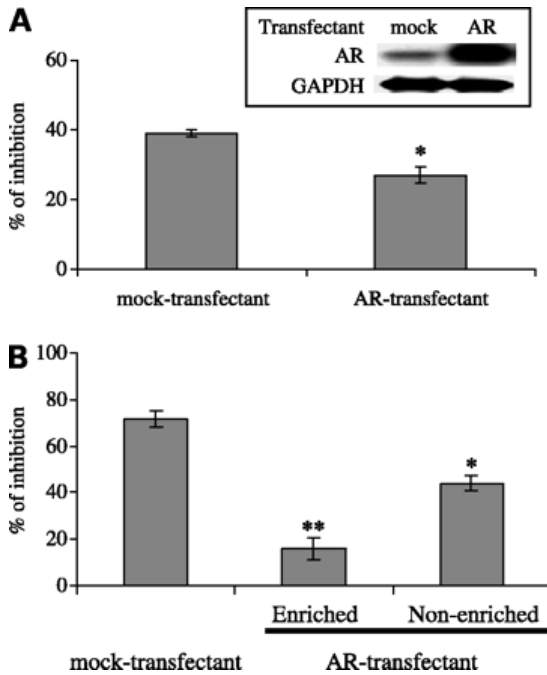


Figure 5. Effect of androgen receptor (AR) overexpression on MSA inhibition of cell growth. **A**, MTT cell growth assay in androgen receptor- or mock-transfected LNCaP cells treated with MSA. Western blot confirmation of androgen receptor protein level (*inset*). **B**, BrdUrd labeling of selected GFP-positive or nonselected androgen receptor-transfected LNCaP cells treated with MSA. *Columns*, % inhibition compared with untreated control. *, $P < 0.05$, statistically different from mock transfectant. **, $P < 0.05$, statistically different from mock transfectant and nonselected androgen receptor transfectant.

We have made progress on task 3 (To evaluate combination of selenium and anti-androgen therapies in mouse models of prostate carcinogenesis).

Combination of selenium and anti-androgen synergistically reduces AR transactivation

A common treatment modality for prostate cancer is androgen deprivation. The goal of these androgen deprivation treatments is either blocking androgen-AR binding or reducing the levels of androgen. Although anti-androgen treatment is effective, the anti-tumor effects can be temporary. Numerous studies have demonstrated that AR is expressed and AR signaling remains intact and is often hyperactive in androgen-independent prostate cancer. Selenium decreases AR expression and reduces AR activation provides a molecular basis for selenium chemoprevention and chemotherapy targeting AR signaling in prostate cancer. We hypothesize that an intervention strategy aimed at both blocking ligand binding (chemical or surgical castration) and dampening AR expression (selenium treatment) would be achieve better therapeutic effect than either alone. To test this hypothesis, we first examined whether combination of selenium with anti-androgen, flutamide (Flu), can synergistically inhibit AR transactivation in human prostate cancer cells *in vitro*. C4-2 cells were transfected with ARE-containing luciferase reporter and treated with either flutamide or MSA alone, or the combination of flutamide and MSA. The ARE luciferase activity was reduced by either flutamide or MSA alone, however, combination of flutamide and MSA achieved much greater inhibition of ARE luciferase activity than either flutamide or MSA alone (**Fig. 6**), suggesting that blocking AR ligand binding and reducing AR expression may synergistically inhibit AR transactivation in prostate cancer cells.

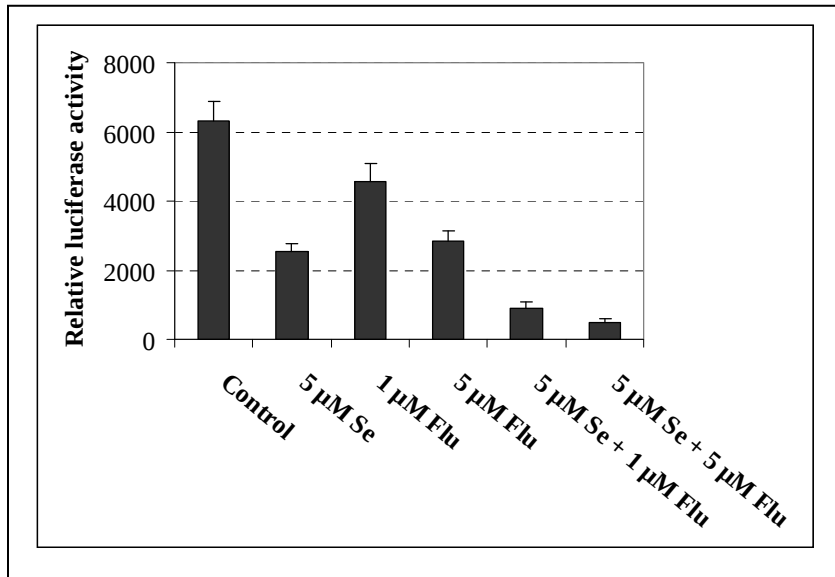


Fig. 6. The effect of combination of selenium (Se) and flutamide (Flu) on AR activation in C4-2 cells. C4-2 cells were transfected with plasmid containing androgen responsive element (ARE)-luc and treated with either Se or Flu alone or together as indicated. Luciferase activity was determined and normalized to the amount of protein.

The effects of combination of MSA and antiandrogen agents on clonogenic ability *in vitro* in C4-2 cells To test whether reduction of AR transactivation by the combination of flutamide with MSA affects cell clonogenic ability *in vitro*, C4-2 cells were treated with either flutamide or MSA alone or together, and clonogenic ability was determined. Treatment with flutamide or MSA alone reduced C4-2 clonogenic ability, combination of flutamide and MSA inhibited C4-2 clonogenic ability much greater than either one alone (**Fig. 7**), suggesting that combination of flutamide with MSA can synergistically inhibit C4-2 cell clonogenic ability *in vitro*.

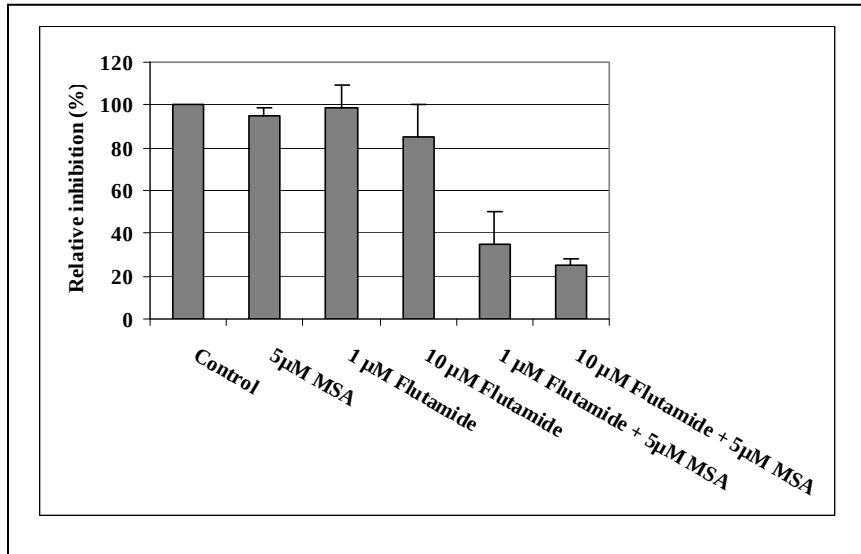


Fig. 7. The effects of combination of selenium (Se) and flutamide (Flu) treatment on clonogenic ability of C4-2 cells.

Effect of combination of selenium and anti-androgen on xenograft models of human prostate cancer

Having demonstrated that combination of selenium and anti-androgen agents can achieve better efficacy on inhibiting AR activation and cell clonogenic ability, we next test the effects of the combination on *in vivo* tumor growth. The effect of combination of methylselenocysteine (MSC) and hydroxyflutamide on tumor growth of C4-2 prostate cancer growth was demonstrated *in vivo*. The male nude mice were co-inoculated with 3×10^6 cells and Matrigel. When the tumors became palpable, the mice were divided into 4 groups with 8 mice in each group. One group was treated with 100 µg MSC, the rest groups were treated with 500 mcg of hydroxyflutamide, 500 mcg hydroxyflutamide plus 100 µg MSC, and vehicle control by i.p. injection daily for 12 days, respectively. Tumor volume was measured 3 times a week. As shown in **Fig. 8**, MSC + flutamide had a greater effect on suppression of tumor growth than flutamide alone.

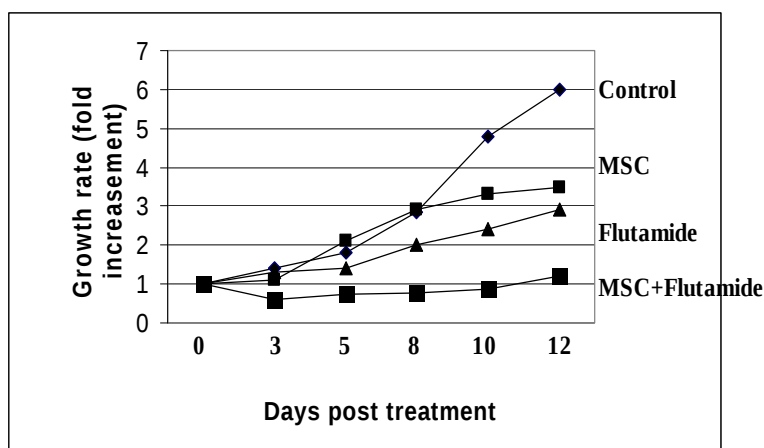


Figure 8. The effect of combination of MSC and hydroxyflutamide on tumor growth of C4-2 prostate cancer growth *in vivo*. The male nude mice were co-inoculated with 3×10^6 cells and Matrigel. When the tumor became palpable, the mice were divided into 4 groups with 8 mice in each group. One group was treated with 100 mcg MSC, the rest groups were treated with 500 mcg of hydroxyflutamide, 500 mcg hydroxyflutamide plus 100 mcg MSC, and vehicle control by i.p. injection daily for 12 days, respectively. Tumor volume was measured 3 times a week.

Effect of combination of MSC and flutamide treatment on AR, Akt expression

We previously demonstrated that selenium significantly suppressed AR expression and AR-regulated gene PSA expression in LNCaP cells *in vitro*. To determine whether selenium affects AR expression *in vivo*, we determined AR protein expression in the C4-2 bearing tumor tissues using Western blot. The levels of AR protein expression were considerably decreased by the treatment with either MSC or flutamide alone and by the combination of MSC and flutamide (**Fig. 9**). The expression of phosphorylate Akt was also decreased by the treatment with either MSC or flutamide alone, and the expression was further decreased by the combination of MSC and flutamide (**Fig. 9**).

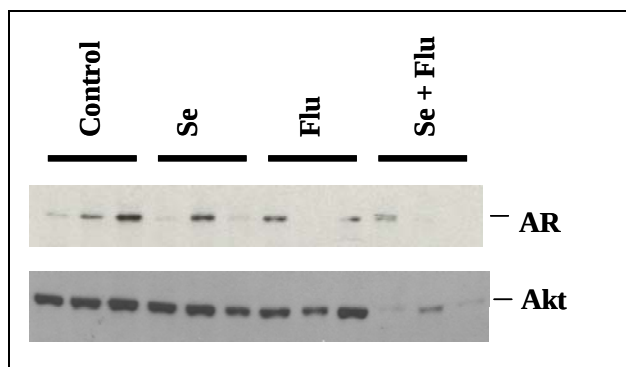


Fig. 9. AR and Akt protein expression in tumors by Western blot analysis. Nuclear extracts were isolated from tumors of individual mice and indicated and subjected to Western blot analysis.

MSA regulates HSP90 protein levels

We also found that selenium down regulate Hsp90 protein expression. LNCaP cells were treated with different concentrations of MSA (1, 2.5, 5, 10 μ M). Western blot was performed where protein was run on an 8% SDS-polyacrylamide gel and blocked overnight with HSP90 monoclonal antibody (SC-4F10). Hsp90 protein levels start to decrease after 5 μ M MSA treatment (Fig 10). Since Hsp90 is a AR chaperone protein, it is possible that selenium down-regulates AR by modulating Hsp90 chaperone function. We are currently performing experiments to further understanding the effects of selenium on Hsp90 and AR regulation in prostate cancer cells.

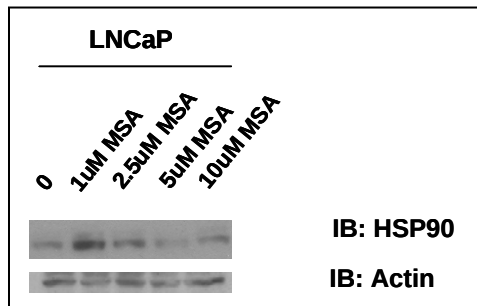


Fig. 10. LNCaP (1×10^6) was plated in 60mm plates and treated with different concentrations (1, 2.5, 5, 10 μ M) of MSA after 24 hours. Plates were maintained for an additional 24 hours before protein was extracted.

Key research accomplishments

- We demonstrated that selenium downregulates AR signaling in prostate cancer cells.
- MSA decreases AR mRNA stability.
- MSA increases AR protein turnover.
- Selenium inhibits AR nuclear translocation.
- Selenium inhibits the recruitment of coactivators and enhances the recruitment of corepressors to AR target genes.
- Combination of selenium with antiandrogen significantly reduced the number of colony formation than either selenium or antiandrogen alone.
- Combination of selenium with antiandrogen has a greater effect on suppression of tumor growth than either flutamide or selenium alone.
- Combination of selenium with antiandrogen significantly reduced the levels of AR and Akt protein expression in tumors.
- Selenium prevents SP1 protein binding to AR promoter, which could potentially decrease AR mRNA transcription.
- Selenium down regulates Hsp90 protein expression. Since Hsp90 is a AR chaperone protein, it is possible that selenium down-regulates AR by modulating Hsp90 chaperone function.

Reportable outcome

Publications:

1. Dong Y, Lee SO, Zhang H, Marshall J, Gao AC and Ip C. Prostate specific antigen (PSA) expression is down-regulated by selenium through disruption of androgen receptor signaling. *Cancer Res.* 64 (1): 19-22, 2004, (Gao, AC, corresponding author).
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3. Dong Y, Zhang HT, Gao AC, Marshall JR, and Ip C. Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. *Molecular Cancer Therapeutics* 4: 1047-1055, 2005.
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Abstract:

1. Lee SO, Chun JY, Nadiminty, N, and Gao AC. Selenium inhibits growth of LNCaP human prostate tumor accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). Innovative Minds in Prostate Cancer Today (IMPACT) meeting. Proceedings P18-14, p206, Atlanta, GA, September 5-8, 2007.

Conclusions

- We demonstrated that selenium downregulates AR signaling via multiple pathways including decreases AR mRNA and protein expression, decreases AR mRNA stability, increases AR protein turnover, inhibits AR nuclear translocation, and affects the recruitment of coregulators to the androgen responsive genes.
- Combination of selenium and anti-androgen therapies has better antitumor effect than either selenium or anti-androgen alone.

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1. Dong Y, Zhang HT, Gao AC, Marshall JR, and Ip C. Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. *Molecular Cancer Therapeutics* 4: 1047-1055, 2005.
2. Dong Y, Lee SO, Zhang H, Marshall J, Gao AC and Ip C. Prostate specific antigen (PSA) expression is down-regulated by selenium through disruption of androgen receptor signaling. *Cancer Res.* 64 (1): 19-22, 2004.
3. Chun JY, Nadiminty N, Lee SO, Onate SA, Lou W, and Gao AC. Mechanisms of selenium down regulation of androgen receptor signaling in prostate cancer. *Molecular Cancer Therapeutics*, 5 (4): 913-918, 2006.
4. Lee SO, Chun JY, Nadiminty N, Trump DL, Ip C, Dong Y, and Gao AC. Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). *Prostate* 66: 1070-1075, 2006.

List of personnel receiving pay from the research effort

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Mechanisms of selenium down-regulation of androgen receptor signaling in prostate cancer

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Abstract

Prevention trials showed that selenium reduced prostate cancer incidence by 50%, establishing selenium as a promising chemopreventive agent for prostate cancer. Selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced apoptotic cell death. Previous studies showed a novel mechanism of selenium anticancer action in which selenium markedly reduces androgen signaling and androgen receptor (AR)-mediated gene expression, including prostate-specific antigen (PSA), in human prostate cancer cells. The molecular mechanisms of selenium-mediated down-regulation of AR signaling are not clear. In this study, a systemic approach was taken to examine the modification of androgen signaling by selenium in human prostate cancer cells. In addition to reduced AR mRNA expression, selenium was found to initially increase the stability of AR mRNA within 6 hours while decreasing the stability of AR mRNA after 8 hours. Selenium increased AR protein degradation and reduced AR nuclear localization. Scatchard analysis indicated that selenium did not affect ligand binding to AR in LNCaP cells. Chromatin immunoprecipitation analyses showed that DHT increased the recruitment of AR and coactivators, such as SRC-1 and TIF-2, to the promoter of the *PSA* gene, and that recruitment was greatly diminished in the presence of 5 $\mu\text{mol/L}$ selenium. On the other hand, selenium enhanced the recruitment of corepressors, such as SMRT, to the promoter of the *PSA* gene. Taken

together, these results suggest that selenium disrupts AR signaling at multiple stages, including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and recruitment of coregulators. [Mol Cancer Ther 2006;5(4):913–8]

Introduction

The growth of prostate epithelial cells requires physiologic levels of androgen, both to stimulate proliferation and inhibit apoptotic death (1). Androgen binds to the androgen receptor (AR), which causes AR to bind to androgen-responsive elements in the promoters of androgen-regulated genes. This interaction is affected by many other transcription coregulators. These complex interactions among AR, androgen-responsive elements, and coregulators facilitate the activation or repression of genes regulating development, differentiation, and proliferation of target cells. Several androgen-responsive genes have been identified, including prostate-specific antigen (*PSA*) and *human glandular kallikrein 2* (2).

Selenium is an essential nutrient that has a chemopreventive effect against a variety of malignancies, including prostate cancer. A number of case-controlled epidemiologic studies have shown an inverse relationship between selenium status and prostate cancer risk (3–7). One of the most important studies of selenium as a chemopreventive agent is the Nutritional Prevention of Cancer study initiated by Clark et al. (8). Supplementation of people with selenized yeast was capable of reducing the overall cancer morbidity by nearly 50% (8). Although selenium treatment did not significantly affect the incidence of nonmelanoma skin cancers, patients receiving the supplement showed a significantly lower prevalence of developing lung (relative risk, 0.54), colon (relative risk, 0.42), or prostate cancer (relative risk, 0.37). Further analysis (9) reaffirmed the significant reduction in prostate cancer incidence by selenium (relative risk, 0.48; 95% confidence interval, = 0.28–0.80). The promising epidemiologic and prevention studies on selenium in prostate cancer provide the basis for the current Selenium and Vitamin E Chemoprevention Trial (10).

The biological activity of selenium is dependent on its chemical form. In general, inorganic selenium compounds, such as selenate or selenite, are known to produce genotoxic effects. Organic selenium-containing compounds, such as selenomethionine and methylselenocysteine, are better tolerated and exhibit anticarcinogenic activity. Methylseleninic acid ($\text{CH}_3\text{SeO}_2\text{H}$) was developed specifically for *in vitro* studies (11) because cultured cells respond poorly to selenomethionine (a commonly used selenium reagent) due to very low levels of β -lyase activity,

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which is required for conversion of selenomethionine to the active methylselenol (12). The effect of physiologic concentrations of methylseleninic acid on cultured cells has been documented in several studies (11, 13–15).

Cell culture studies showed that selenium inhibited the growth of prostate cancer cell lines, including androgen-sensitive LNCaP and androgen-insensitive DU145 and PC3 cells (14–17). *In vivo* studies also support the antitumorigenic role of selenium in prostate cancer. Dietary supplementation of selenium resulted in reduction of tumor growth in PC3 tumors in mice (18). There are a number of potential mechanisms proposed for the antiproliferative effects of selenium, including antioxidant effects, enhancement of immune function, stimulation of apoptosis, and induction of cell cycle arrest (16). We recently showed that methylseleninic acid is able to decrease markedly AR transcript and protein levels (14). The expression of *PSA*, a well-known androgen-regulated gene, is also inhibited by methylseleninic acid (13, 14).

The down-regulation of AR signaling by selenium provides an important mechanism for selenium prostate cancer chemoprevention. However, the molecular mechanisms of selenium-mediated down-regulation of AR signaling are not clear. AR is a ligand-dependent transcription factor. The activation of AR requires binding to its ligand, translocation to the nucleus, and interaction with coregulators, including coactivators and corepressors, in the AR target genes. In this study, a systemic approach was taken to examine the modification of androgen signaling by selenium in human prostate cancer cells. The results suggest that selenium affects AR signaling at multiple levels, including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and recruitment of coregulators.

Materials and Methods

Selenium Reagent and Cell Culture

Methylseleninic acid was synthesized as described previously (11). Human LNCaP prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were grown at 37°C in 5% CO₂ and 95% air.

Cytosolic and Nuclear Protein Preparation

LNCaP cells were cultured in charcoal-stripped fetal bovine serum for 3 days. The cells were treated with 10 nmol/L DHT in the absence or presence of 10 μmol/L methylseleninic acid for 2 hours. Cells were harvested, washed with PBS twice, and resuspended in a hypotonic buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.1% NP40] and incubated on ice for 10 minutes. Nuclei were precipitated by 3,000 × g centrifugation at 4°C for 10 minutes. The supernatant was collected as the cytosolic fraction. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% Triton X-100] and incubated on ice for 30 minutes. The nuclear

lysate was precleared by 10,000 rpm centrifugation at 4°C for 15 minutes. Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

Northern Blot Analysis

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample were electrophoresed on 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 500-bp fragment of AR cDNA was labeled with [α -³²P]dCTP (3,000 Ci/mmol; ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out during 3 hours at 65°C in Rapid-hyb buffer (Amersham, Arlington Heights, IL). Membranes were washed for 15 minutes at 65°C in 2× SSC, 0.1% SDS (twice), 0.5× SSC, 0.1% SDS and 0.1× SSC, and 0.1% SDS. Radioactivity in the membranes was analyzed with a Storm Phosphorimager System.

Western Blot Analysis

The protein extracts were resolved on 12.5% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS/0.1% Tween 20, membranes were incubated for 1 hour at room temperature with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti- α -actin (Sigma, St. Louis, MO), anti-RNA polymerase II (Promega, Madison, WI), or anti-Hsp90 (Sigma) diluted in 1% milk in PBS/Tween 20. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

In vitro AR Ligand-Binding Assay

Ligand-binding assay was done as previously described (19). LNCaP prostate cancer cells were plated at 1×10^6 per plate in 10-cm plates and allowed to grow to confluence for 3 days. Cells were treated with 10 μmol/L methylseleninic acid for 4 hours before harvesting and homogenization in TEDG buffer [10 mmol/L Tris (pH 7.4), 1.5 mmol/L EDTA, 10% glycerol, and 1 mmol/L DTT added immediately before use]. The cell suspension was passed through a 26-gauge needle (10–15 times) to homogenize. The homogenate was incubated on ice for 10 minutes and centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatant was collected and used as the cytosolic fraction. Total protein was estimated in the extracts from both untreated and methylseleninic acid-treated cells, and equal amounts of protein were used in the subsequent assay. The extracts were incubated with 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, and 1 nmol/L ³H-R1881 either in the presence or absence of 100 nmol/L (excess) unlabeled (cold) R1881 in a total reaction volume of 250 μL (made up with TEDG buffer). The reaction mixtures were incubated on ice throughout the assay. Dextran-coated charcoal suspension (500 μL; 0.25% charcoal, 0.025% dextran in 1× PBS) was added to each sample and incubated at 4°C with vigorous shaking for 10 minutes. The samples were centrifuged at 3,000 rpm

for 10 minutes at 4°C. The supernatant (500 µL) was added to 5 mL of scintillation fluid and counted in a liquid scintillation counter. The amount of the radio-labeled ligand bound to the receptor in the presence and absence of competing unlabeled ligand was calculated and expressed as fmol/mg protein. The difference between count per minute with ³H-R1881 only and count per minute with ³H-R1881 + cold R1881 was calculated and taken as the amount of bound ³H-R1881. The data were analyzed by Scatchard analysis as described previously (19).

AR mRNA Stability Assay

Equal numbers of LNCaP cells were plated in 10-cm plates and incubated at 37°C until they reached 70% confluence. Cells were either pretreated with 5 µg/mL actinomycin D before treatment with 10 µmol/L methylseleninic acid, or they were treated with 5 µg/mL actinomycin D and 10 µmol/L methylseleninic acid together for 0, 4, 8, 12, 24, 36, and 48 hours. Total RNA was extracted with TRIzol reagent (Invitrogen, San Diego, CA), and 20 µg of total RNA from each sample were run on a 1.2% formaldehyde-agarose gel. The membrane was hybridized with the AR cDNA probe labeled with ³²P-dCTP. After hybridization and washing, radioactivity in the membranes was analyzed with a Storm Phosphorimager System, and the levels of AR mRNA were quantified by Phosphorimager. The turnover of AR mRNA was determined by comparing mRNA levels over time in cells treated with or without methylseleninic acid.

AR Protein Stability Assay

Equal numbers of LNCaP cells were plated in 60-mm plates and incubated at 37°C until they reached 70% confluence. Cells were either pretreated with 50 µg/mL cycloheximide before treatment with 10 µmol/L methylseleninic acid, or they were treated with 50 µg/mL cycloheximide and 10 µmol/L methylseleninic acid simultaneously for 0, 4, 8, 12, 24, 36, and 48 hours. Cells were homogenized in high salt buffer [10 mmol/L HEPES (pH 7.5), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1% NP40], and the supernatants were used as the whole-cell lysates. Equal amounts of protein were run on 10% SDS-PAGE and probed with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-α-actin antibody (Sigma). To determine if the proteasomal degradation pathway played a role in the degradation of AR protein in cells treated with methylseleninic acid, cells were treated with 5 µmol/L MG-132 (a proteasome inhibitor) in addition to cycloheximide. The levels of AR protein were quantified and normalized to the amount of actin. The AR protein turnover was determined by comparing AR protein levels over time in cells treated with or without methylseleninic acid.

Chromatin Immunoprecipitation Assay

LNCaP cells were cultured in phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum for 3 days. Cells were treated with or without 10 µmol/L methylseleninic acid for 4 hours in the absence or presence of 10 nmol/L DHT. The AR and

coregulator complexes were cross-linked inside the cells by the addition of formaldehyde (1% final concentration) to the cells in culture. Whole-cell extracts were prepared using sonication and an aliquot of the cross-linked receptor protein complexes were immunoprecipitated by incubation with either the AR specific antibody (AR441, Santa Cruz Biotechnology) or antibody that specifically recognize coactivator TIF-2, SRC-1, or corepressors, such as SMRT or NCoR (Santa Cruz Biotechnology), overnight at 4°C with rotation. Chromatin-antibody complexes were isolated from solution by incubation with protein G-Sepharose beads for 1 hour at 4°C with rotation. The Sepharose-bound immune complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 mol/L NaHCO₃), and DNA was extracted. DNA samples from chromatin immunoprecipitation preparations were analyzed by PCR using primers spanning the PSA gene in the region of promoter (forward, 5'-CCTAGATGAA-GTCTCCATGAGCTACA; reverse, 5'-GGGAGGGAGAGC-TAGCACTTG).

Results

Methylseleninic Acid Decreases AR mRNA Stability

Our results suggest that whereas methylseleninic acid decreased AR mRNA levels at the transcriptional level (14), AR mRNA expression can also be regulated at post-transcriptional level. To examine whether methylseleninic acid affects AR mRNA stability, LNCaP cells that express functional AR were treated with or without 5 µmol/L methylseleninic acid in the presence of actinomycin D (5 µg/mL) to stop *de novo* mRNA synthesis. The total RNA was isolated at different time points, and AR mRNA levels were measured by Northern blot analysis. The half-life of AR mRNA was determined by comparison of mRNA levels over time between cells treated with or without actinomycin D, either in the presence or absence of methylseleninic acid. Because actinomycin D is capable of inducing cell death, we monitored cell growth for a period of 24 hours and did not observe cell death or growth inhibition with the concentration of actinomycin D used (5 µg/mL). We did not observe significant cell death or growth inhibition at 5 µmol/L methylseleninic acid over a period of 24 hours in LNCaP cells (14). Methylseleninic acid treatment initially enhanced AR mRNA levels within 6 hours. However, AR mRNA levels were significantly decreased by methylseleninic acid compared with the control at 8 hours. Figure 1 shows on the semilog plot, the mean values of percentage of AR mRNA levels over time relative to respective time 0 AR mRNA value as 100%. In methylseleninic acid-treated cells, AR half-life was reduced to about 7 hours from 12 hours in the control cells, suggesting that AR mRNA degradation was greatly accelerated in the presence of methylseleninic acid after 6 hours.

Methylseleninic Acid Increases AR Protein Turnover

We have shown that methylseleninic acid decreased the levels of AR mRNA and protein in LNCaP cells (14). We

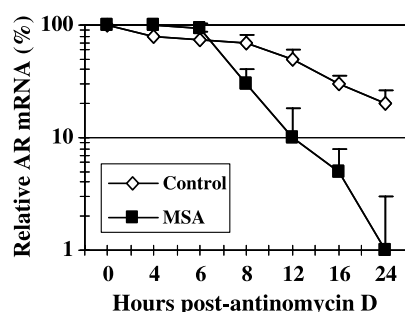


Figure 1. Effect of MSA on AR mRNA stability in LNCaP cells. The mRNA synthesis inhibitor antimycin D (5 μ g/mL) was added with or without 5 μ mol/L methylseleninic acid (MSA) at time 0. At specific time points, cells were harvested, and total RNA was isolated by Northern blots. Points, means of three independent experiments plotted on semilog scale relative to respective time 0 AR mRNA value as 100%; bar, SD.

next examined the effect of methylseleninic acid on AR protein degradation after new protein synthesis was blocked by cycloheximide as a potential mechanism for down-regulation of AR protein level. The protein synthesis inhibitor cycloheximide (50 μ g/mL) was added with or without 5 μ mol/L methylseleninic acid at time 0. At specified time points, cells were harvested, and the levels of AR protein were measured by Western blot using anti-AR antibody. In methylseleninic acid-treated cells, the half-life of AR protein was reduced to 6 hours from 21 hours in the control cells (Fig. 2A), suggesting that AR protein degradation was greatly enhanced in the presence of methylseleninic acid. Systematic protein degradation by the ubiquitin-proteasome system plays an important role in the maintenance of protein stability. Protein ubiquitination provides the recognition signal for the 26S proteasome, leading to protein degradation (20, 21). Studies showed that AR protein level in cells is regulated by systemic protein degradation pathways (22, 23). To examine whether selenium induced AR protein degradation via ubiquitin-proteasome system, the 26S proteasome inhibitor MG132 was added to the cells treated with methylseleninic acid. MG132 was able to retard methylseleninic acid effect on AR protein levels (Fig. 2B), suggesting that methylseleninic acid induced AR degradation via a proteasome-dependent pathway.

Selenium Inhibits AR Nuclear Translocation

AR typically translocates to the nucleus to exert its function on gene expression. To examine whether selenium affects the translocation of AR, Western blot analysis was done using cell extracts from either cytosolic or nuclear extracts. LNCaP cells were cultured in charcoal-stripped fetal bovine serum for 3 days before adding 10 nmol/L of DHT in the absence or presence of 10 μ mol/L methylseleninic acid for 2 hours. Nuclear and cytosolic fractions were prepared and used for Western blot analysis using the anti-AR antibody. DHT treatment increased the levels of AR protein expression in the nucleus, which were reduced by the treatment with methylseleninic acid (Fig. 3). In contrast, methylseleninic acid had little effect on AR

protein expression in the cytosol. The expression of RNA polymerase II and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively. These results suggest that methylseleninic acid suppresses AR signaling in part via interruption of AR nuclear translocation.

Selenium Inhibits the Recruitment of Coactivators and Enhances the Recruitment of Corepressors to AR Target Genes

AR interacts with coregulators to achieve maximal transactivation activity. To examine the effects of selenium on the recruitment of coregulators to the promoters of AR target genes, chromatin immunoprecipitation analysis was done. DHT increased the recruitment of AR and TIF-2 and SRC-1 to the promoter of the *PSA* gene in the absence of methylseleninic acid, and this recruitment was greatly diminished in the presence of 5 μ mol/L methylseleninic acid (Fig. 4). On the other hand, methylseleninic acid treatment prevented the nuclear translocation of AR in the presence of hormone; thus, the corepressors, including SMRT and NcoR, remain bound to the promoter of the *PSA* gene (Fig. 4). These results suggest that methylseleninic acid-mediated reduction of AR activation may be due, at least in part, to a decrease in the recruitment of AR and its coactivators to the promoter of the AR target gene *PSA*, while maintaining corepressors bound to the promoter.

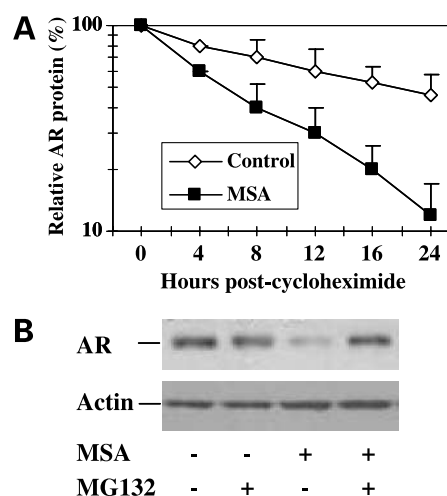


Figure 2. A, effect of methylseleninic acid (MSA) on AR protein turnover in LNCaP cells. The protein synthesis inhibitor cycloheximide (50 μ g/mL) was added with or without 5 μ mol/L methylseleninic acid at time 0. At specific time points, cells were harvested, and cell lysates were prepared. AR protein levels were determined by Western blot analysis using antibody specifically against AR and normalized to α -actin control. Points, means of three independent experiments plotted on semilog scale relative to respective time 0 AR value as 100%; bars, SD. B, effect of MG132 on methylseleninic acid-induced AR protein degradation. MG132 (5 μ mol/L) was added to LNCaP cells together with cycloheximide (50 μ g/mL) in the presence and absence of 5 μ mol/L methylseleninic acid. The cell lysates were prepared at 24 h. AR protein levels were determined by Western blot analysis using antibodies specifically against AR and α -actin as a control.

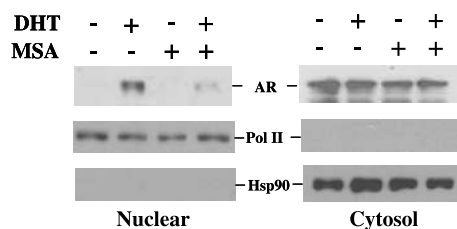


Figure 3. The effect of methylseleninic acid (MSA) on AR nuclear translocation. LNCaP cells were cultured in charcoal-stripped FBS for 3 d and treated with 10 nmol/L DHT with or without 10 μ mol/L methylseleninic acid for 2 h. The cells were harvested for preparation of cytosolic and nuclear fractions and analyzed by Western blotting using antibodies against AR, polymerase II, or Hsp90. The expression of polymerase II and Hsp90 were used as markers for the integrity of the nuclear and cytosolic fractions, respectively.

Discussion

Selenium is an important trace element exhibiting anticancer activity. There are a number of potential mechanisms proposed for the anticancer effects of selenium, including antioxidant effects, enhancement of immune function, stimulation of apoptosis, and induction of cell cycle arrest (16). We previously showed a novel mechanism of selenium action in which selenium disrupts androgen signaling by inhibiting AR mRNA and protein expression and reducing the expression of AR target genes (14). These studies provide an important molecular mechanism of selenium chemoprevention and potential therapy for prostate cancer. In the present study, the mechanisms of selenium-mediated AR signaling down-regulation were examined. Selenium decreased AR mRNA stability, accelerated AR protein degradation, and blocked AR nuclear translocation. In addition, selenium inhibited the recruitment of coactivators and maintained corepressors bound to the promoters of AR target genes.

AR is a ligand-dependent transcription factor whose activation is initiated by its binding to androgen and subsequent translocation to the nucleus, where it binds to the promoters and activates the transcription of AR target genes. Any interruption of this process may alter AR signaling and result in abnormal androgen action. To examine whether selenium affects AR ligand binding, *in vitro* AR binding activity was done using 3 H-labeled R1881 and was subjected to Scatchard analysis in the absence and presence of 5 μ mol/L methylseleninic acid in LNCaP cells. The results showed that selenium did not affect R1881 binding to AR (Fig. 5). Because LNCaP cells express a mutant AR, LAPC-4 cells containing a wild-type AR were used for AR ligand binding assay and selenium did not affect R1881 binding to AR in LAPC-4 cells (data not shown). The fact that selenium does not affect AR ligand binding suggests a different antiandrogen mechanism by selenium from flutamide or Casodex, which block ligand binding to AR (24).

AR transactivation may require cooperation with many other coregulators including coactivators and corepressors. It is known that androgen-AR may cooperate with various

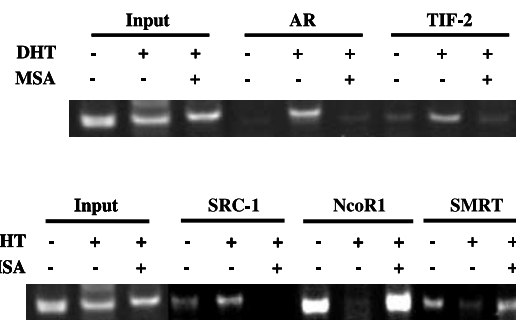


Figure 4. Effect of methylseleninic acid (MSA) on the recruitment of AR and coregulators to the promoter of an endogenous AR target gene *PSA*. The *in vivo* binding of AR and coregulators to the PSA promoter was examined by the chromatin immunoprecipitation assay. LNCaP cells were cultured in charcoal-stripped condition for 3 d. Soluble chromatin was prepared from cells treated with 10 nmol/L DHT for 4 h (+) or untreated (-) in the presence (+) or absence (-) of 10 μ mol/L methylseleninic acid and immunoprecipitated with antibodies against AR, TIF-2, SRC-1, SMRT, and NCoR1. Coprecipitated DNA was amplified by PCR using primers that flank the ARE in the PSA promoter region. The presence of total PSA promoter DNA in the soluble chromatin before immunoprecipitation was included as input.

coregulators to modulate their target genes for proper or maximal function. Coregulators such as TIF-2 and SRC-1 interact with AR to enhance ligand-dependent transactivation of AR. The expression of TIF-2 and SRC-1 is increased in cancer and recurrent prostate cancer after medical or surgical castration (25), suggesting that TIF2 and SRC-1 may be involved in the development and progression of prostate cancer. Our findings showed that selenium can interrupt the interaction between AR and coregulators by blocking the recruitment of coactivators (SRC-1 and TIF-2) while maintaining corepressors (SMRT and NCoR) bound to the promoters of AR target genes. These findings suggest that selenium not only disrupts AR signaling, but also interrupts the interaction of coregulators with AR to achieve maximal effect on androgen function.

A common treatment modality for prostate cancer is androgen deprivation, which can be achieved by surgical

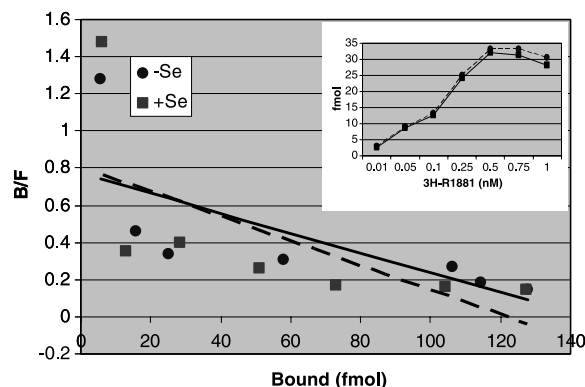


Figure 5. Scatchard analysis of specific R1881 binding to AR in LNCaP cells in the absence and presence of 5 μ mol/L methylseleninic acid (Se). Points, means from triplicate experiments; bars, SE. Inset, saturation binding results.

castration, chemical castration, or a combination of surgical and chemical castrations. The goal of these androgen deprivation treatments is either to block androgen-AR binding or to reduce the levels of androgen. Although antiandrogen treatment is effective, the antitumor effects may be temporary. Virtually, every patient will relapse due to the growth of androgen-independent prostate cancer cells. There is an urgent need for testing new therapies based on different mechanisms to target AR signaling for androgen-independent prostate cancer. AR signaling is often hyperactive in androgen-independent prostate cancer and plays a critical role in the growth and progression of prostate cancer. A treatment aims at reducing AR expression may represent an attractive approach to target androgen signaling in prostate cancer. Our findings show that selenium disrupts androgen signaling at multiple stages of AR signaling pathways, including AR mRNA expression, mRNA stability, protein degradation, nuclear translocation, and interaction with coregulators in prostate cancer (14). This unique antiandrogen activity suggests that selenium may serve as a therapeutic agent, in addition to a chemopreventive agent, for prostate cancer. Understanding the molecular mechanism of selenium-mediated down-regulation of AR signaling may aid in the development of effective treatments aimed at targeting AR signaling for prostate cancer. We are currently testing the combination treatment to more effectively target AR signaling in prostate cancer using antiandrogen agents (flutamide or Casodex, blocking ligand binding to AR) and selenium (reducing AR expression) based on our understanding of the mechanisms of their action.

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Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers

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Abstract

Our previous report showed that methylseleninic acid (MSA) significantly decreases the expression of androgen receptor and prostate-specific antigen (PSA) in LNCaP cells. The present study extended the above observations by showing the universality of this phenomenon and that the inhibitory effect of MSA on prostate cancer cell growth and cancer-specific biomarkers is mediated through androgen receptor down-regulation. First, MSA decreases the expression of androgen receptor and PSA in five human prostate cancer cell lines (LNCaP, LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3), irrespective of their androgen receptor genotype (wild type versus mutant) or sensitivity to androgen-stimulated growth. Second, by using the ARE-luciferase reporter gene assay, we found that MSA suppression of androgen receptor transactivation is accounted for primarily by the reduction of androgen receptor protein level. Third, MSA inhibition of five androgen receptor-regulated genes implicated in prostate carcinogenesis (*PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*) is significantly attenuated by androgen receptor overexpression. Fourth, transfection of androgen receptor in LNCaP cells weakened noticeably the inhibitory effect of MSA on cell growth and proliferation. Androgen receptor signaling has been documented extensively to play an important role in the development of both androgen-dependent and -independent prostate

cancer. Our finding that MSA reduces androgen receptor availability by blocking androgen receptor transcription provides justification for a mechanism-driven intervention strategy in using selenium to control prostate cancer progression. [Mol Cancer Ther 2005;4(7):1047–55]

Introduction

Prostate cancer is the second most common cancer and the second leading cause of cancer death in men in the United States. Androgen plays an important role not only in maintaining the function of the prostate but also in promoting the development of prostate cancer (1). Androgen binds to the androgen receptor, which subsequently translocates to the nucleus and interacts with specific androgen-responsive elements (ARE) on the promoters of target genes. The interaction leads to the activation or repression of genes involved in the proliferation and differentiation of the prostate cells (2). Prostate-specific antigen (PSA) and kallikrein 2 (KLK2) are two well-accepted targets of androgen receptor. PSA, also known as kallikrein 3, is an established serum marker for the diagnosis and prognosis of prostate cancer. Although KLK2 is not as widely used as PSA, it is increasingly recognized to provide added information to disease staging (3, 4).

The randomized, placebo-controlled Nutritional Prevention of Cancer trial showed that selenium supplementation reduced the incidence of prostate cancer by 50% (5, 6). This trial was designed initially to assess the effect of selenium on nonmelanoma skin cancer. Because men accounted for a sizable proportion of the cohort (974 of a total of 1,312), there was sufficient power to analyze the changes in prostate cancer risk. When the prostate cancer data were further stratified, there was evidence of a greater reduction in risk from selenium supplementation among men who had low baseline plasma PSA levels (6). Early-stage prostate cancer is mostly responsive to androgen stimulation. The inference that the protective effect of selenium might be more pronounced in early-stage prostate cancer, as reflected by low PSA secretion, lends credence to the idea that selenium might affect androgen signaling.

Recently, we reported that a selenium metabolite, in the form of methylseleninic acid (MSA), greatly down-regulates the expression of androgen receptor and PSA in the androgen-responsive LNCaP human prostate cancer cells (7, 8). The suppression of androgen receptor signaling occurs well before any significant growth inhibition, which is accompanied by correlative changes in numerous cell

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cycle and apoptosis regulatory molecules (9–13). Androgen receptor signaling involves multiple steps, the receptor itself is just one of many effectors that participate in the process. For example, heat shock proteins are known to modulate the stability of androgen receptor as well as its affinity to androgen (14, 15). The transactivating activity of androgen receptor can be affected markedly by a large number of coactivators and corepressors (16). Our microarray analysis suggests that MSA alters the expression of several heat shock proteins, coactivators, and corepressors of the superfamily of steroid hormone receptors (17). In view of these confounding effects, the present study was designed to determine the role of androgen receptor down-regulation per se in MSA interference of androgen receptor signaling. Our approach was to use the ARE-luciferase reporter gene assay to find out the extent to which selenium suppression of androgen receptor transactivation could be reversed when the luciferase activity is normalized based on androgen receptor protein level. We also investigated whether androgen receptor transfection might attenuate selenium-mediated down-regulation of five androgen receptor targets: *PSA*, *KLK2*, ATP-binding cassette C4 (*ABCC4*, also known as *MRP4*), 24-dehydrocholesterol reductase (*DHCR24*, also known as *seladin-1*), and soluble guanylate cyclase 1 α 3 (*GUCY1A3*). These five androgen-inducible genes were selected based on the criteria that they are consistently overexpressed in prostate cancer compared with normal prostate tissue (18). Finally, in an effort to evaluate the biological significance of the selenium-androgen receptor signaling axis, we investigated whether androgen receptor overexpression might block the growth inhibitory effect of selenium.

Materials and Methods

Selenium Reagent, Prostate Cancer Cell Lines, 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay, and Bromodeoxyuridine-Labeling Analysis

MSA was synthesized as previously described (19). The LNCaP and CWR22Rv1 human prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). The LAPC-4 cell line was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center. The two androgen-unresponsive LNCaP sublines, LNCaP-LN3 and LNCaP-C81, were obtained from Dr. Curtis A. Pettaway (University of Texas M.D. Anderson Cancer Center) and Dr. Ming-Fong Lin (University of Nebraska Medical Center), respectively. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L glutamine. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell growth and the bromodeoxyuridine (BrdUrd) labeling for DNA synthesis were done as described in our previous publication (17).

Transient Transfection of Androgen Receptor

The procedure was carried out using the Lipofect-AMINE Plus reagent (Invitrogen, Carlsbad, CA) per instruction of the manufacturer. At 24 hours before transfection, cells were plated in growth medium without antibiotics at a density to reach 90% to 95% confluency at transfection. The pSG5hAR androgen receptor expression vector (20) or the pSG5 mock plasmid (Stratagene, La Jolla, CA) was introduced into LNCaP cells with or without the cotransfection of the pEGFP-F membrane-GFP-encoding construct (BD Biosciences, San Jose, CA). The purpose of the green fluorescent protein (GFP) was to enable us to enrich for the subset of positively androgen receptor-transfected cells. During cotransfection, the two plasmids were added at 1:1 molar ratio. The amount of DNA transfected was 12 μ g per 10-cm culture dish. The DNA/liposome mixture was removed at 3 hours after transfection. For the MTT assay, the cells were trypsinized 16 hours later and plated in triplicate onto a 96-well plate. Cells were allowed to recover for an additional 24 hours before exposure to 10 μ mol/L MSA. The MTT assay was conducted at 48 hours post-MSA treatment. For the BrdUrd-labeling analysis, the cells were subjected to MSA treatment at 24 hours posttransfection and labeled with BrdUrd after 24 hours of MSA treatment.

Reporter Gene Assay

The ARE-luciferase reporter plasmid, containing three repeats of the ARE region ligated in tandem to the luciferase reporter (20), was transiently transfected into cells at a concentration of 9 μ g per 10-cm culture dish. After incubating with the transfection mixture for 3 hours, the cells were trypsinized, resuspended in medium containing charcoal-stripped serum and 10 nmol/L dihydrotestosterone (Sigma, St. Louis, MO), and plated in triplicate onto 6-well plates. Cells were allowed to recover for an additional 24 hours before exposure to 10 μ mol/L MSA. After 6 or 16 hours of MSA treatment, cells were lysed in reporter lysis buffer (Promega, Madison WI), and the luciferase activity was assayed using the Luciferase Assay System (Promega). Protein concentration in cell extracts was determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized by the protein concentration of the sample. The transfection experiments were repeated thrice.

Western Blot Analysis

Details of the procedure for Western blot analysis were described previously (17). Immunoreactive bands were quantitated by volume densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase. The following monoclonal antibodies were used in this study (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti-androgen receptor (BD Biosciences), and anti-PSA (Lab Vision, Fremont, CA).

Real-time Reverse Transcription-PCR

Real-time reverse transcription-PCR analysis was done as described previously (21). The PCR primers and

Taqman probes for β -actin, androgen receptor, PSA, KLK2, ABCC4, DHCR24, and GUCY1A3 were Assays-on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 minutes, then a denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantitation of gene expression was done using the comparative C_T ($\Delta\Delta C_T$) method (22).

Androgen Receptor mRNA Stability Assay

Actinomycin D (5 μ g/mL) was added to the cultures to stop new RNA synthesis at the time of MSA treatment, and androgen receptor mRNA levels were measured by real-time reverse transcription-PCR at hourly intervals for the next 6 hours. The turnover of androgen receptor mRNA was determined by comparing mRNA levels over time in cells treated with or without MSA.

Statistical Analysis

The Student's two-tailed *t* test was used to determine significant differences between treatment and control values, and *P* < 0.05 was considered statistically significant.

Results

MSA Depresses Androgen Receptor Transcription

Figure 1 shows the effect of MSA on androgen receptor transcript and protein levels as well as androgen receptor mRNA stability in LNCaP cells. The decrease in androgen receptor transcript, as determined by real-time reverse transcription-PCR, occurred very quickly (Fig. 1A). On the average, there was about a 50% reduction in the first three hours after treatment with 10 μ mol/L MSA; by 6 hours, the magnitude of inhibition rose to 80%. At the protein level, there was no change in androgen receptor in the first two hours (Fig. 1B). A modest decrease began to appear at 3 hours, and the inhibition became very pronounced at 6 hours (Fig. 1B). The observation is consistent with the time-dependent sequence of reduced mRNA leading to decreased protein expression. To determine whether the down-regulation of androgen receptor mRNA was due to decreased transcription or increased mRNA degradation, we did an mRNA stability assay under the condition in which new RNA synthesis was blocked. Actinomycin D was added to the culture at the time of MSA treatment, and androgen receptor mRNA levels were followed in a 6-hour time course experiment. Because actinomycin D could be cytotoxic, we also monitored cell growth for up to 8 hours and did not observe cell death or significant growth inhibition during this period. Our results showed that treatment with MSA actually increased the stability of androgen receptor mRNA (Fig. 1C). This observation rules out increased mRNA degradation as a contributing factor. Therefore, the decrease in androgen receptor mRNA level by MSA is likely to be accounted for by a vigorous block of androgen receptor transcription.

We next examined the effect of MSA on the expression of androgen receptor and PSA in four additional human

prostate cancer cell lines: LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3. The LAPC-4 cells are androgen responsive and express a wild-type androgen receptor (23), as opposed to LNCaP cells that are also androgen responsive but express a mutant, although functional, androgen receptor. The other three cell lines are all androgen-unresponsive and express a mutant but functional androgen receptor (24–27). As shown in Fig. 2 (left), MSA decreased androgen receptor and PSA transcript levels progressively as a function of time in all four cell lines examined. The reduction in androgen receptor and PSA proteins (right) paralleled the drop in the transcripts. In LAPC-4, CWR22Rv1, and LNCaP-C81 cells, a decrease in PSA transcript was already detectable as early as 3 hours, at a time when there was no apparent loss of the androgen receptor protein. The data suggest that MSA disrupts androgen receptor signaling through additional mechanism(s) beyond reducing the availability of the androgen receptor protein.

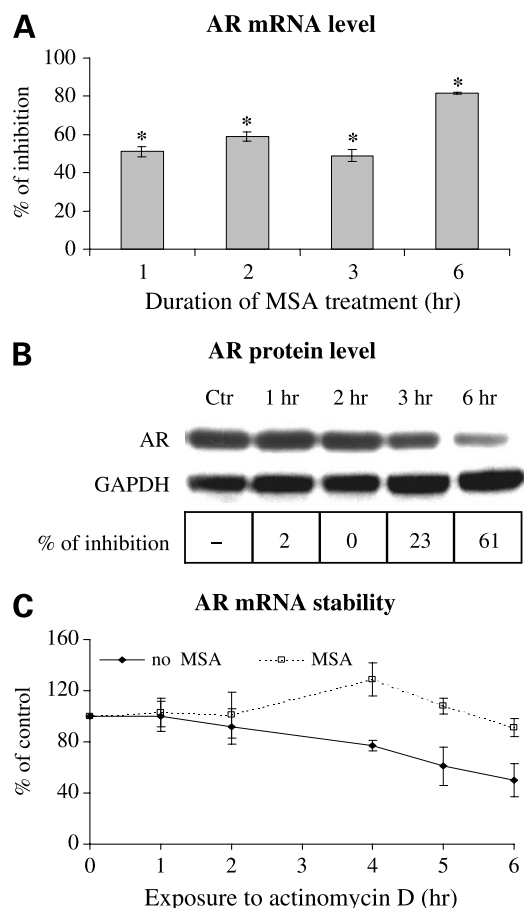


Figure 1. Effect of MSA on androgen receptor (AR) expression in LNCaP cells. **A**, inhibition of androgen receptor mRNA level as determined by real-time RT-PCR. **B**, inhibition of androgen receptor protein level as determined by Western blot analysis. **C**, androgen receptor mRNA stability in the presence or absence of MSA. Bars, SE. *, *P* < 0.05, statistically different compared with untreated control.

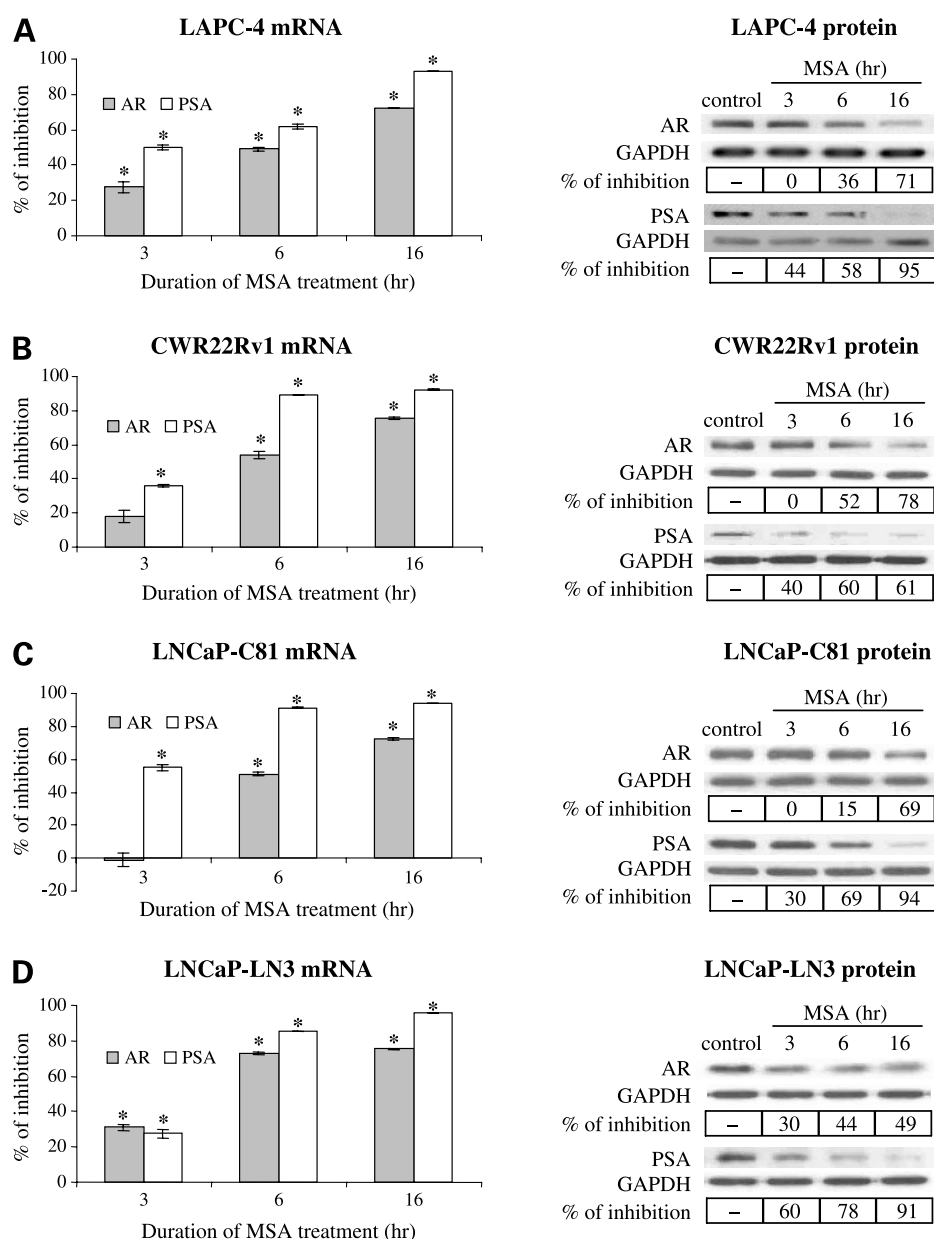


Figure 2. Effect of MSA on androgen receptor (AR) and PSA expression in LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3 cells. *Left*, mRNA levels as determined by real-time RT-PCR; *right*, protein levels as determined by Western blot analysis. *Columns*, % inhibition; *bars*, SE. *, $P < 0.05$, statistically different compared with untreated control.

MSA-Mediated Androgen Receptor Down-Regulation Leads to a Reduction of Androgen Receptor Transactivating Activity

The transactivation of androgen receptor is an indicator of androgen receptor signaling and can be quantified readily by a reporter gene assay. To investigate whether the reduced availability of androgen receptor by MSA is a major factor in modulating androgen receptor transcriptional activity, we transiently transfected LNCaP cells with the ARE-luciferase reporter plasmid and normalized the luciferase activity based on the level of the androgen receptor protein. This normalization step eliminates the level of androgen receptor expression as a determinant of androgen receptor transactivation. The luciferase reporter assay was carried out at

6 and 16 hours after treatment with 10 $\mu\text{mol/L}$ MSA. At these two time points, androgen receptor protein level was inhibited by 60% and 77%, respectively (Fig. 3B, *inset*). As can be seen in Fig. 3A, without normalizing for the difference in androgen receptor protein level between the MSA-treated and -untreated samples, the ARE-promoter activity was decreased by 65% or 75%, respectively, after 6 or 16 hours of MSA treatment. However, after normalization, the ARE-promoter activity was inhibited by a meager 15% at the 6-hour time point, and the inhibition disappeared completely at 16 hours (Fig. 3B). These findings suggest that the reduced availability of the androgen receptor protein is the major factor in contributing to MSA disruption of androgen receptor signaling.

Overexpression of Androgen Receptor Attenuates the Effect of MSA on the Down-Regulation of Androgen Receptor – Regulated Genes

To delineate the role of low androgen receptor abundance as a cause of reduced PSA expression by selenium, we transiently transfected LNCaP cells with a wild-type androgen receptor construct and determined the response of PSA to MSA. After 3 hours of MSA exposure, PSA transcript was depressed by about 75% in the mock-transfected cells but only by about 45% in the androgen receptor-transfected cells (Fig. 4A). Based on our routine experience of a 40% transfection efficiency as determined by GFP cotransfection analysis (described below), we believe that the inhibitory effect of MSA on PSA mRNA might have been reversed completely in positive androgen receptor transfectants. Our conclusion was derived from the following theoretical calculation: 40% of $(1 - x) + 60\%$ of $(1 - a) = 1 - b$, where $x = \%$ inhibition in positive androgen receptor transfectants, $a = 75\%$ inhibition in mock transfectants, and $b = 45\%$ inhibition in the mixed population of androgen receptor-transfected cells. Solving for x in the above equation gave a value of 0% inhibition. In

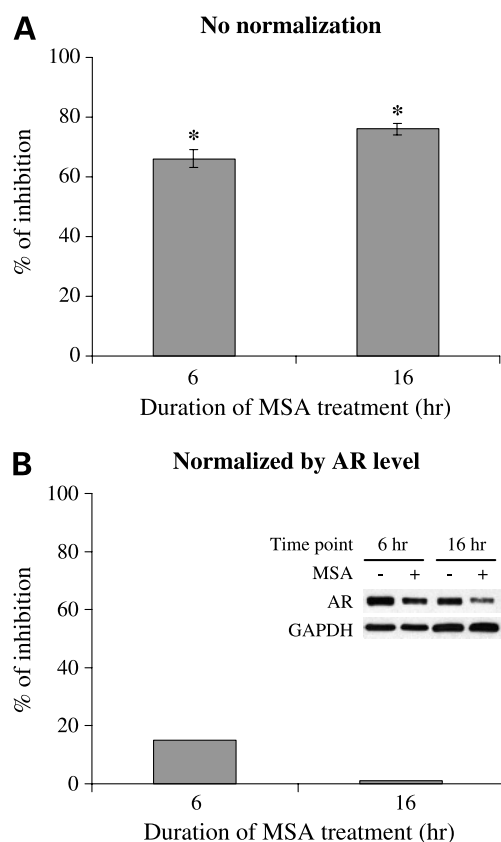


Figure 3. Effect of MSA on ARE-promoter activity before (**A**) and after (**B**) normalizing by androgen receptor (AR) protein level. **B**, representative Western blot analysis of androgen receptor protein level in the cell extracts (*inset*). Androgen receptor protein level was depressed by 60% or 77% at 6 or 16 h, respectively. *Columns*, % inhibition; *bars*, SE. *, $P < 0.05$, statistically different compared with untreated control.

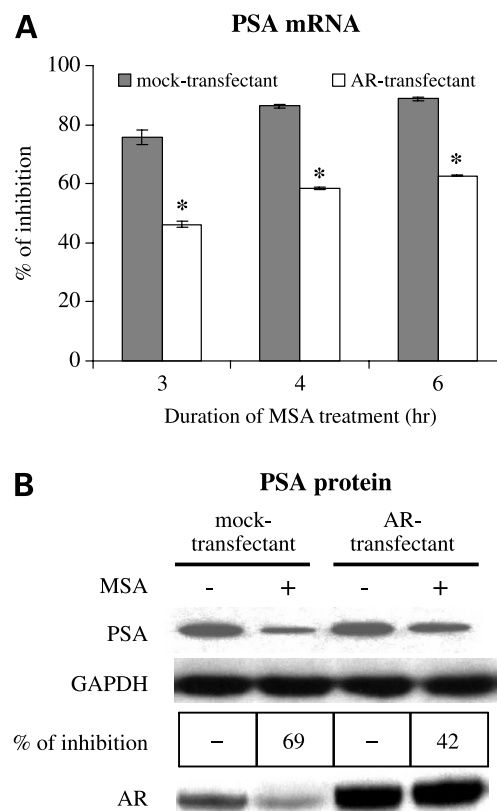


Figure 4. Effect of androgen receptor (AR) overexpression on MSA down-regulation of PSA. **A**, PSA mRNA expression as determined by real-time RT-PCR, in androgen receptor- or mock-transfected LNCaP cells treated with MSA. *Columns*, % inhibition. *, $P < 0.05$, statistically different compared with the value from the mock transfectant. **B**, PSA Western blot analysis in androgen receptor- or mock-transfected LNCaP cells treated with MSA.

other words, there was no inhibition of PSA expression by MSA in the positive androgen receptor transfectants (i.e., complete reversal). The difference between the mock- and androgen receptor-transfected cells, although still apparent, was not as great at 4 and 6 hours compared with that at 3 hours. The fact that a robust androgen receptor presence was not sufficient to completely counteract the suppressive effect of MSA on the transcription of PSA at the later time points suggests that there could be a delay in the recruitment of additional mechanisms by which MSA might diminish androgen receptor signaling. We also studied the protein level of PSA by Western blotting. The Western analysis was done at 24 hours after MSA treatment. As shown in Fig. 4B, PSA protein was depressed by about 70% in the mock-transfected cells but only by about 40% in the androgen receptor-transfected cells. The protein levels of androgen receptor in the mock- and androgen receptor-transfected cells are also shown in Fig. 4B for confirmation purposes.

Using a bioinformatic data mining approach, we recently identified five additional androgen-inducible genes that are expressed at a higher level in prostate cancer compared

with normal prostate tissue; furthermore, their expression is repressed by MSA (18). These genes are *KLK2*, *ABCC4* (also known as *MRP4*), *DHCR24* (also known as *seladin-1*), *GUCY1A3*, and long-chain fatty acid CoA ligase 3 (*FACL3*). MSA down-regulation of their expression only occurs in LNCaP cells but not in the androgen-unresponsive PC-3 cells that express an extremely low level of androgen receptor (18). To verify that the decreased expression of these genes is a direct consequence of MSA suppression of androgen receptor signaling, we applied the same androgen receptor overexpression protocol as described above and used real-time reverse transcription-PCR to quantitate their transcript levels. The *FACL3* gene was not included in this study as no Assays-on-Demand primers and probes are available for this gene. The results are shown in Fig. 5. Androgen receptor transfection significantly muted the inhibition of gene expression by MSA. In general, the difference in % inhibition between the mock and androgen receptor transfectants was greatest at 3 hours and narrowed gradually with time. The overall pattern was very similar for *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. The data thus show a key role of androgen receptor down-regulation in mediating the inhibitory effects of MSA on the expression of androgen receptor-regulated genes.

Overexpression of Androgen Receptor Interferes with MSA-Mediated Growth Inhibition

In an effort to evaluate the biological significance of MSA suppression of androgen receptor signaling, we transiently transfected LNCaP cells with a wild-type androgen receptor and assessed the response of the androgen

receptor-overexpressing cells to MSA-induced growth inhibition. The MTT assay was conducted at 48 hours post-MSA, and the data are presented in Fig. 6A. In the absence of MSA, cell growth was not altered by the transfection of androgen receptor (data not shown), indicating that the endogenous level of androgen receptor is not a limiting factor for the growth of these cells. MSA treatment inhibited growth by 40% in the mock transfectants, as opposed to 27% in the androgen receptor transfectants. The difference is statistically significant ($P = 0.003$). Thus, androgen receptor overexpression was able to weaken the growth suppressive activity of MSA. One reason that the difference was seemingly compressed was due to the fact that only a fraction of cells was successfully transfected, and in this study, cell growth was assessed using the whole cell population. To address the last problem, we cotransfected cells with the androgen receptor expression vector and a membrane-GFP-encoding construct. The cells were then subjected to BrdUrd labeling, and the data were analyzed by gating just the GFP-positive cells. As shown in Fig. 6B, after selecting for the subset of GFP-positive cells, we found that MSA inhibited DNA synthesis by a very modest 16% in the androgen receptor transfectants, as opposed to 72% in the mock transfectants. Because the GFP and androgen receptor cDNAs are not located in the same plasmid construct, it is possible that not all the cells positive for GFP are also positive for the transfected androgen receptor. Thus, our selection process only led to an enrichment, rather than an exclusive selection, of double-positive cells. Therefore, the difference

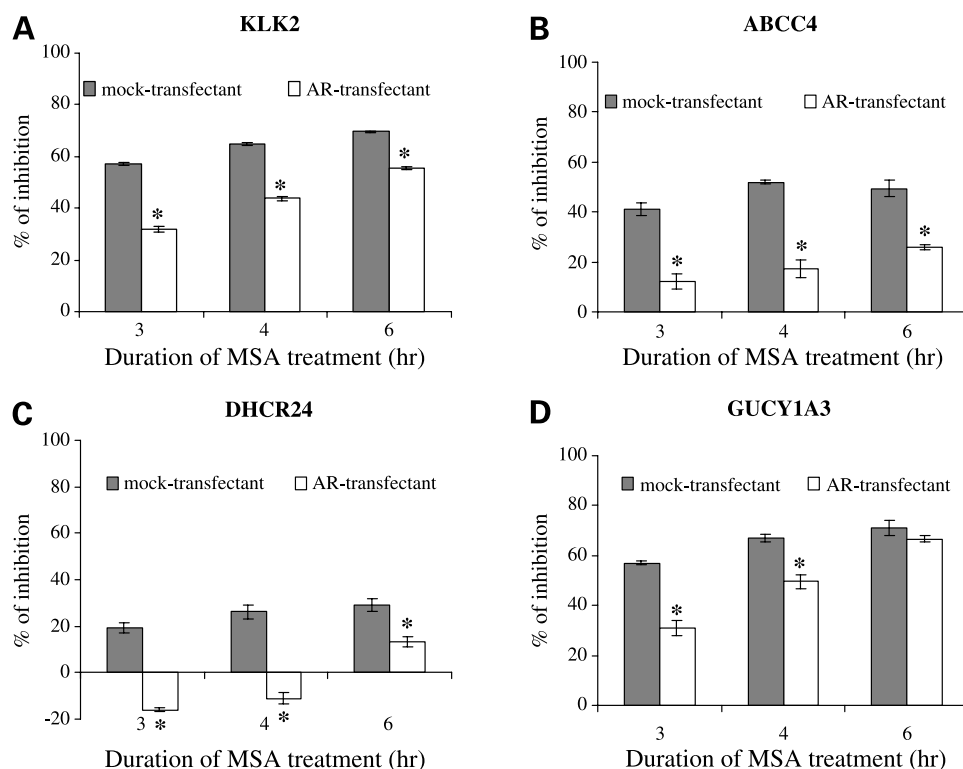


Figure 5. Effect of androgen receptor (AR) overexpression on MSA down-regulation of *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3* mRNA expression as determined by real-time RT-PCR, in androgen receptor- or mock-transfected LNCaP cells treated with MSA. Columns, % inhibition. *, $P < 0.05$, statistically different compared with the value from the mock transfectant.

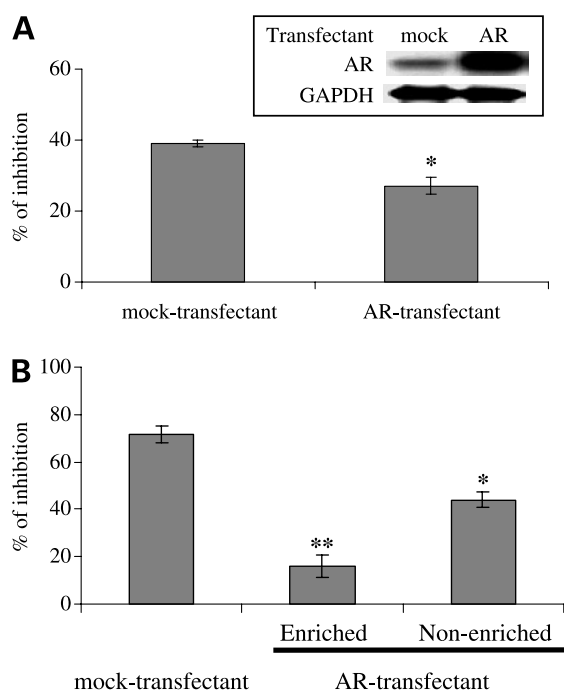


Figure 6. Effect of androgen receptor (AR) overexpression on MSA inhibition of cell growth. **A**, MTT cell growth assay in androgen receptor- or mock-transfected LNCaP cells treated with MSA. Western blot confirmation of androgen receptor protein level (inset). **B**, BrdUrd labeling of selected GFP-positive or nonselected androgen receptor-transfected LNCaP cells treated with MSA. Columns, % inhibition compared with untreated control. *, $P < 0.05$, statistically different from mock transfectant. **, $P < 0.05$, statistically different from mock transfectant and nonselected androgen receptor transfectant.

between the mock transfectants and the androgen receptor transfectants might have been even more pronounced if all the cells used in the experiment were successfully transfected with androgen receptor. Figure 6B also shows that when we did the BrdUrd labeling experiment with the nonenriched androgen receptor-transfected cells, the inhibition by MSA was about 45%, a value half-way between that achieved by the mock transfectants and the enriched androgen receptor transfectants.

Discussion

Our previous report showed that selenium significantly decreases the expression and the transactivating activity of androgen receptor in LNCaP cells (21). The present study extended the above observations by showing the universality of this phenomenon and a key role of androgen receptor down-regulation in mediating the inhibitory effects of selenium on prostate cancer cell growth and the expression of cancer-specific biomarkers. First, selenium decreases the expression of androgen receptor and PSA in five human prostate cancer cell lines, irrespective of their androgen receptor genotype (wild type versus mutant) or sensitivity to androgen-stimulated growth. Second, a reporter gene assay with the ARE-luciferase construct indicated that depletion of the androgen receptor protein

is a major factor for selenium depression of androgen receptor transactivating activity. Third, overexpression of androgen receptor greatly weakens the inhibitory effects of selenium on prostate cancer cell proliferation as well as the expression of five androgen receptor-regulated genes implicated in prostate carcinogenesis: *PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. These findings, however, do not necessarily exclude additional mechanisms by which selenium diminishes androgen receptor signaling (e.g., via modulation of ligand binding, androgen receptor dimerization, nuclear translocation, and the interaction of androgen receptor with its coregulators). In fact, our previous report provided some evidence that selenium is able to inhibit the binding of androgen receptor to the ARE in the absence of a drop in the androgen receptor level (21).

A selenium intervention strategy aimed at diminishing the expression of androgen receptor could be helpful not only for reducing prostate cancer incidence but also for preventing relapses after endocrine therapy. Almost all patients with advanced prostate cancer respond initially to treatments that interfere with the androgen receptor-signaling process. However, these treatments often fail after prolonged use and recurrence becomes a major clinical issue (28). The development of hormone refractory prostate cancer is not associated with loss of androgen receptor (29, 30). Instead, the appearance of several molecular alterations frequently leads to a lower threshold requirement of androgens for the proliferation and survival of prostate cancer cells. Androgen receptor gene mutations could result in a promiscuous receptor with a broad ligand-binding and transactivation spectrum (31). Amplification and/or overexpression of androgen receptor may hypersensitize cells to subphysiologic levels of androgens (32–35). A recent report by Chen et al. (35) claimed that increased androgen receptor expression is both necessary and sufficient to convert prostate cancer growth from androgen-dependent to -independent and that androgen receptor antagonists may display agonistic activity in cells with elevated androgen receptor expression. On the other hand, several studies showed that knocking down the expression of androgen receptor inhibits the growth of prostate cancer cells, both *in vitro* and *in vivo*, and induces apoptosis (36–39). Because selenium blocks the transcription of androgen receptor (see Fig. 1), this treatment modality may prove to be effective in prostate cancer intervention.

The down-regulation of androgen receptor targets by selenium has important clinical implication. We have studied *PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. All these genes are expressed at a higher level in prostate cancer compared with normal prostate tissue (18). *PSA* and *KLK2* are prostate-specific differentiation markers. They belong to the serine protease family and are both secretory proteins. *PSA* is the most useful serum marker for the diagnosis and prognosis of prostate cancer. The combined use of *PSA* and *KLK2* has been shown to improve the specificity of biochemical detection of prostate cancer (40–44) and the accuracy in predicting tumor grade and

stage (3, 4). ABCC4 (also known as MRP4) is a member of the multidrug resistance-associated protein family of transporters. Overexpression of ABCC4 in neuroblastoma is associated with poor prognosis and resistance to the topoisomerase I poison irinotecan and its active metabolite SN-38 (45). Thus, the down-regulation of MRP4 by selenium might represent a potential mechanism by which selenium enhances the therapeutic efficacy of a number of anticancer drugs, including irinotecan (46). DHCR24 (also known as seladin-1) is an antiapoptotic protein, it inhibits the activity of caspase 3 (47). The overexpression of this gene has also been reported in adrenocortical adenoma cells compared with adjacent nontumor cells (48). GUCY1A3 catalyzes the conversion of GTP to the second messenger cyclic guanosine 3',5'-monophosphate, which regulates the activity of protein kinases, phosphodiesterases, and ion channels (49). Future selenium intervention trial may consider monitoring androgen receptor, PSA, KLK2, ABCC4, DHCR24, and GUCY1A3 in biopsied prostate samples, to obtain a more comprehensive picture of an individual's responsiveness to selenium. Recent data also showed that cellular PSA is more sensitive than secretory PSA to selenium intervention (50). This is one more reason why it is preferable to do the analysis in biopsied prostate tissue.

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