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# Estrogen receptor activation and estrogen-regulated gene expression are unaffected by methylseleninic acid in LNCaP prostate cancer cells☆

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**Abstract**

Prostate cancer is the most frequently diagnosed cancer and a leading cause of cancer deaths in American men. High dietary intake and status of the essential trace element selenium (Se) have been consistently correlated with reduced risk for prostate cancer. One molecular mechanism by which Se may reduce prostate cancer risk is by catalyzing disulfide bond formation or, otherwise, complexing with reactive sulfhydryl groups in transcription factors, thus altering their binding to DNA and regulation of gene expression. Estrogen plays a role in the etiology of prostate cancer. Estrogen receptors contain cysteines in zinc fingers that are susceptible to oxidation and internal disulfide bond formation, which can prevent DNA binding. We hypothesized that Se alteration of estrogen receptor (ER) binding to DNA and estrogen-regulated gene expression may be one mechanism by which it exerts its chemopreventive effects. LNCaP human prostate cancer cells were treated with 0.05 μmol/L (control) or 5.0 μmol/L (high) Se as methylseleninic acid (MSA). Electrophoretic mobility shift assays showed that binding of ER-beta to the estrogen response element was a nonsignificant 14% lower in cells treated with high MSA. Run-on transcription assays showed no significant changes in transcription rates for estrogen-regulated genes, and steady-state mRNA levels for those genes, assayed by reverse transcription-polymerase chair reaction, were likewise unaffected by MSA. These results suggest that the well-documented chemopreventive effects of Se against prostate cancer may be mediated by mechanisms other than inhibition by monomethylated Se compounds of ER-beta activation or estrogen-regulated gene expression.


*Keywords:* Selenium; Methylseleninic acid; Estrogen; Gene regulation; Prostate cancer cells

## 1. Introduction

The American Cancer Society estimates that prostate cancer will account for 33% of new cancers in men, totaling over 234,000 new cases, with 27,350 deaths in 2006, making it the most frequently diagnosed cancer in American men and the third leading cause of cancer deaths [1]. The landmark study of Clark et al. [2,3] showed that a supplement of 200 μg of selenium (Se) significantly reduced the incidence of prostate cancer in patients with carcinoma of the skin. Since that time, numerous prospective cohort and case-control studies have confirmed that high Se intake or status is associated with reduced prostate cancer risk [4–8]. A recent review of diet in the development and progression of prostate cancer [9] found the evidence more convincing for a protective effect of Se than for any other dietary component. A randomized, placebo-controlled trial of Se and vitamin E as prostate cancer chemopreventive agents in 32,400 men (Selenium and Vitamin E Cancer Prevention Trial [SELECT]) is currently ongoing [10].

Many possible molecular targets for Se have been identified [4,11–13] to account for its chemoprotective effects. Ganther [14,15] proposed that one molecular mechanism by which monomethylated Se compounds may affect cancer risk is by catalyzing disulfide bond formation or, otherwise, complexing with reactive sulfhydryl groups in cellular proteins. Formation of such structures has been demonstrated in catalytic proteins [16]. A transcription factor whose activation may be regulated by disulfide bond formation is the estrogen receptor (ER). The ER contains

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cysteines in zinc (Zn) fingers that are susceptible to oxidation and internal disulfide formation, which can prevent DNA binding [17,18]. Whittal et al. [18] showed that Zn is expelled from the Zn finger previous to disulfide formation when treated with $H_2O_2$ or diamide. Jacob et al. [19] detailed the conditions under which Se compounds can catalyze the release of Zn from Zn fingers in metallothionein (MT). More recent results have confirmed that Se can damage or distort Zn finger domains [20].

The ER mediates the effects of estrogen. Animal models and human epidemiological studies have implicated estrogen as a promoter of prostate cancer in its early stages [21–24]. We hypothesized that one mechanism by which Se may exert its chemoprotective effects against prostate cancer may be to displace Zn in ER Zn fingers and catalyze disulfide bond formation, thus decreasing ER binding to DNA and expression of ER-regulated genes. To test this hypothesis, we examined ER binding to its DNA response element and associated expression of ER-regulated genes in human prostate cancer cells (LNCaP) treated with control (0.05 μmol/L) or high (5.0 μmol/L) concentrations of Se as methylseleninic acid (MSA) [25].

## 2. Materials and methods

### 2.1. Cell culture

LNCaP human prostate cancer cells (CRL-1740, ATCC, Manassas, VA, USA) were cultured at 37°C in an atmosphere of 5% $CO_2$ in RPMI 1640 with phenol red (30-2001, ATCC), with 10% heat-inactivated fetal bovine serum (SH30070, HyClone Laboratories, Logan, UT, USA) and 1% penicillin–streptomycin solution (P-4333, Sigma–Aldrich, St. Louis, MO, USA). This cell line expresses ER-β but not ER-α [26,27]. All media included 30 μmol/L α-tocopherol (T-3251, Sigma–Aldrich) and 0.05 μmol/L Se (see below), as recommended by Leist et al. [28].

To examine the effects of Se, LNCaP cells that were 90% confluent in 25 $cm^2$ flasks were passaged 1:2 and equilibrated for 48 h in phenol red-free RPMI 1640 medium (R-8755, Sigma–Aldrich), modified as above. In addition, β-estradiol (E-4389, Sigma–Aldrich) was supplemented to bring the total estrogen content to 30 pg/ml to be within the range found in serum of men with and without prostate cancer [29–31]. Phenol red-free medium was used to eliminate estrogenic effects of phenol red [32]. After 48 h, medium was removed. Half of the flasks then received fresh medium containing 0.05 μmol/L Se as MSA ($CH_3SeO_2H$) and half received medium providing 5.0 μmol/L Se as MSA. Methylseleninic acid was synthesized by colleagues in the Department of Chemistry and Biochemistry at Brigham Young University from dimethyl diselenide, according to the published procedure of Kloc et al. [33], and its purity verified by elemental analysis. Cells were then incubated 72 h and harvested. This incubation time was chosen based on previous reports [34–36] in which effects of Se treatments in LNCaP and other cell lines were more pronounced at 72 h than at earlier time points. We previously used a 72-h incubation of LNCaP cells with 5.0 μmol/L MSA and showed a marked decrease in activation of the redox-regulated transcription factor nuclear factor κB (NF-κB) and in NF-κB-regulated gene expression [37].

### 2.2. Electrophoretic mobility shift assay

Nuclear extracts were prepared using a Panomics Nuclear Extraction Kit (AY2002, Panomics, Redwood City, CA), including dialysis (66335, Slide-A-Lyzer Dialysis cassettes; Pierce, Rockford, IL, USA) of the final extract, with the following modification: cells were trypsinized with 0.025% trypsin/0.265 mmol/L EDTA solution (CC-5012, Cambrex Bio Science Walkersville, Walkersville, MD, USA), removed from the flasks and washed twice with phosphate-buffered saline. Protein was quantified using Bradford reagent (B-6916, Sigma–Aldrich).

The forward and reverse sequences of the 46-mer double-stranded probe used in electrophoretic mobility shift assays (EMSAs) are shown in Table 1. This probe contains the

Table 1
Nucleotide sequences of EMSA probe (ERE) and PCR primers

| Probe or primer | Sequence (5′ to 3′) |
|---|---|
| ERE forward | TAACTGTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTTAATGTA |
| ERE reverse | TACATTAACTTTGATCAGGTCACTGTGACCTGACTTTGGACAGTTA |
| 18S rRNA forward | CGGCTTAATTTGACTCAACACG |
| 18S rRNA reverse | CTAAGAACGGCCATGCACC |
| IGFBP10 forward | GCCGCCTTGTGAAAGAAACC |
| IGFBP10 reverse | CTTGCCCTTTTTCAGGCTGC |
| Cdk2 forward | CAAGCCAGTACCCCATCTTCG |
| Cdk2 reverse | CAAATAGCCCAAGGCCAAGC |
| Cathepsin D forward | GCAAACTGCTGGACATCG |
| Cathepsin D reverse | ACCATTCTTCACGTAGGTGC |
| hpS2 forward | CAGACAGAGACGTGTACAGTGG |
| hpS2 reverse | AGCCCTTATTTGCACACTGG |

The same PCR primers were used to determine transcription rates in run-on transcription assays, and to quantitate steady state mRNA levels in real-time RT-PCR analysis.

estrogen response element (ERE) from the Xenopus vitellogenin A2 gene and has been used in previous studies of protein-ERE interactions [38]. The double-stranded probe was end-labeled with $P^{32}$ using T4 polynucleotide kinase and incubated with nuclear extracts according to the method of García Pedrero et al. [39]. Mixtures were electrophoresed through a 5% nondenaturing polyacrylamide gel (polyacrylamide/bisacrylamide ratio, 39:1) in 0.5× TBE (tris-borate-EDTA buffer) at 100 V for 2–3 h. Gels were dried under vacuum at 80°C for 60 min and exposed to X-ray film (BioMax MS, Eastman Kodak, Rochester, NY, USA) at −80°C for 1–18 h and developed. Multiple exposures were used to obtain the strongest signals within the linear range of the film. Densitometric analysis (AlphaEase software, Alpha Innotech, San Leandro, CA, USA) was performed on multiple exposures of three separate EMSA gels.

### 2.3. Selection of ER-regulated genes

The genes chosen for study in the run-on transcription assay and steady-state mRNA experiments are regulated by estrogen in the prostate and are expressed in LNCaP cells. IGFBP10 [40], Cdk2 [41,42], cathepsin D [43,44] and HpS2 [26,45], also known as trefoil factor 1 [46], are all involved in growth and/or tumor progression.

### 2.4. Nuclear run-on transcription assay

Transcription rates for ER-regulated genes were determined in run-on transcription assays, as we previously described [47,48], but using biotinylated (rather than radioactive) uridine triphosphate, as described by Patrone et al. [49]. Biotinylated nascent transcripts were isolated using streptavidin coated DynaBeads (M-280, Dynal Biotech, Oslo, Norway) according to the manufacturer's directions.

In a novel modification, quantitation of biotin-labeled nascent transcripts was performed using real time reverse transcriptase-polymerase chain reaction (RT-PCR). First-strand cDNA synthesis was primed by random hexamers. PCR amplification was followed in real time using a LightCycler (Roche Molecular Biochemicals, Indianapolis, IN, USA), as we have previously described [50] to amplify transcripts for IGFBP10, Cdk2 and cathepsin D. PCR primers were designed for published sequences (GenBank) using OMIGA 2.0 software (Genetics Computer Group, Madison, WI, USA). The sequences of primers used are shown in Table 1. For each gene, six LightCycler runs were performed. Each run included three replicates for both 0.05 μmol/L and 5.0 μmol/L Se treatments. Transcription rates for 18S rRNA were also quantified and used for normalization.

### 2.5. Steady-state mRNA quantitation

Steady-state mRNA levels for ER-regulated genes were determined by real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized using, as primer, a mixture of single base-anchored oligo dTs (oligo dT-A, oligo dT-C, oligo dT-G). Real-time PCR amplification was followed to determine steady state mRNA levels for the same genes examined in run-on transcription assays, plus hpS2. Three runs of three replicates each of both 0.05- and 5.0-μmol/L Se treatments were performed, except for hpS2, for which a fourth run was completed.

### 2.6. Statistical analysis

For each replicate exposure of each EMSA gel, the density of the high Se and control Se bands were added to give a total density. The density of each band was then expressed as a percentage of that total. For statistical analysis, *t* tests were used to compare the mean band densities, as a percentage of the total, for control and high Se-treated samples.

To calculate relative gene expression in assays of transcription rates and steady-state mRNA levels, the mean of the replicates of the gene of interest was normalized by dividing by the mean of the replicates for 18S rRNA. For each gene the, normalized mean for the high Se group was divided by the normalized mean for the adequate Se group to give a ratio for relative gene expression.

To apply standard statistical methods to these data would have required that each replicate for a gene of interest be normalized to 18S rRNA. This would have required that those two species be quantitated in the same LightCycler capillary (multiplex). However, in this work, each replicate for each gene was assayed in a separate capillary. Thus, individual replicates of the gene of interest could not be matched with individual replicates of 18S rRNA for normalization, and standard statistical methods could not be used. Instead, normalization was done using the means of all the replicates for each gene, and the statistical significance of the ratios for relative gene expression was calculated using a permutation test (1000 replications of randomized gene/18S rRNA ratios), as described by Ramsey and Shafer [51].

Permutation tests find *P* values as the proportion of regroupings that lead to test statistics as extreme as the observed one. After calculating relative gene expression as described above, the permutation test (sometimes called a randomization test) was performed as follows: for each Se treatment, the 6–9 replicates for the gene of interest and the 6–9 replicates for 18S rRNA were combined into a single group then randomly assigned to the "gene of interest" or "18S rRNA" group. The mean of the randomly assigned gene of interest values was then normalized by dividing by the mean of the randomly assigned 18S rRNA values. This was done for both Se treatments, and the ratio of the normalized high to normalized control was again calculated. This randomization and recalculation was repeated 1000 times, and the number of recalculated ratios greater than and less than the original calculated ratio were tallied to determine the probability (*P* value) of obtaining the original ratio simply by chance.

## 3. Results

Fig. 1 shows typical results from an electrophoretic mobility shift assay. Densitometric analysis was performed on multiple exposures of three separate EMSA gels. These analyses showed that the band in the control Se lane accounted for 53.8±6.9% (mean±S.D.) of the total density of the control and high Se bands combined, while the high Se band accounted for 46.2±6.9% of the total. This difference was not statistically significant.

Fig. 2 shows the results of the run-on transcription assays. The ratios of high Se/control Se, adjusted for 18S, were 0.994 for IGFBP10, 0.880 for Cdk2 and 0.894 for cathepsin D. The probability that the transcription rate ratio for IGFBP10 was due strictly to random chance was 0.385; for Cdk2, 0.347 and for cathepsin D, 0.372.

Results from the analysis of steady-state mRNA levels for ER-regulated genes are shown in Fig. 3. The ratios, adjusted for 18S, of high Se/control Se for IGFBP10, Cdk2, Cathepsin D and hpS2 were 1.231, 1.108, 1.029 and 1.126, respectively. The probability that the 23% difference in steady state levels of mRNA between high and control Se samples for IGFBP10 occurred by random chance was only 0.034. For Cdk2, the probability was 0.119; for Cathepsin D, 0.413 and for hpS2, 0.196.

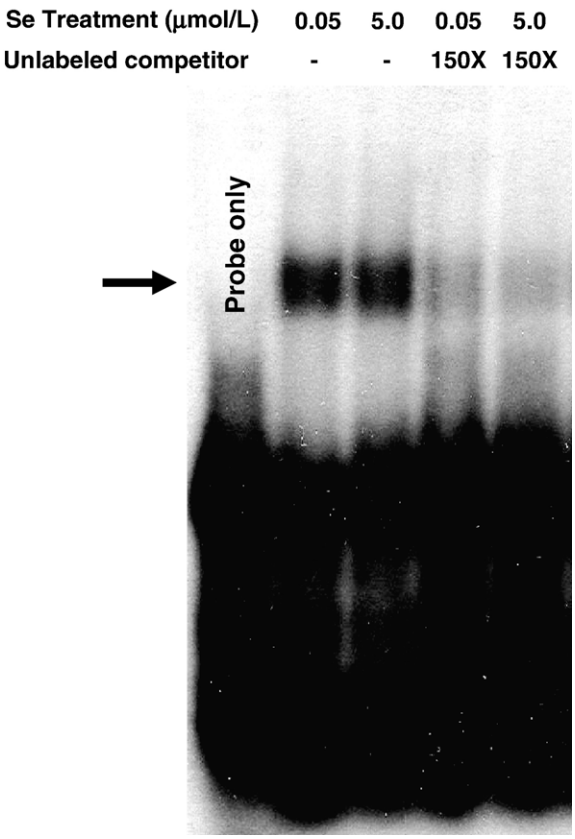


Fig. 1. Binding of ER to its DNA response element. LNCaP cells were grown in medium supplying 0.05 or 5.0 µmol/L Se as MSA. Nuclear extracts were prepared and used in electrophoretic mobility shift assays to examine binding of ER to its DNA response element (ERE). Arrow indicates ER/ERE complex. Densitometric analysis of multiple exposures for three independent gels showed that the average density of the band in the 5.0 µmol/L lane (without unlabeled competitor) was a nonsignificant 14% lower than that of the corresponding band in the 0.05 µmol/L lane. Disappearance of the band resulting from the addition of unlabeled competitor oligonucleotide probe to the incubation confirmed the specificity of binding to this probe.

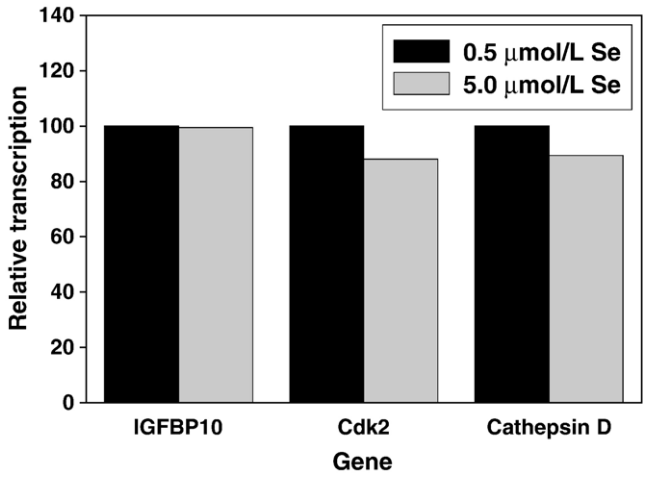


Fig. 2. Transcription rates of ER-regulated genes. LNCaP cells were grown in medium supplying 0.05 or 5.0 µmol/L Se as MSA. Nuclei were harvested and used in run-on transcription assays to quantitate relative rates of transcription of ER-regulated genes. Compared to cells treated with 0.05 µmol/L Se, transcription rates in cells treated with 5.0 µmol/L Se were 99% for IGFBP10, 88% for Cdk2 and 89% for cathepsin D. The probability that these differences were due strictly to random chance was 0.385, 0.347 and 0.372 for IGFBP10, Cdk2 and cathepsin D, respectively.

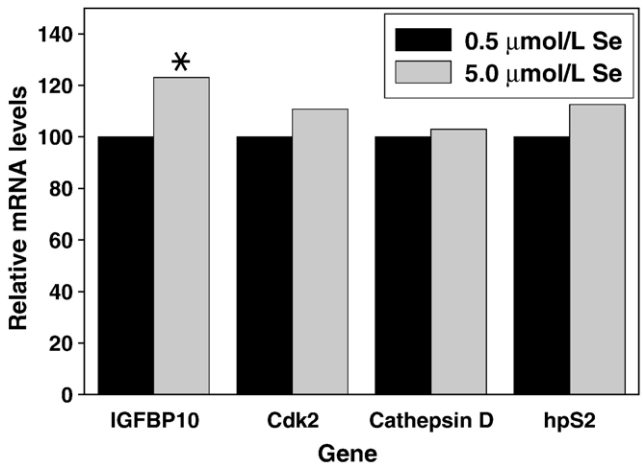


Fig. 3. Steady-state levels of mRNA for ER-regulated genes. LNCaP cells were grown in medium supplying 0.05 or 5.0 µmol/L Se as MSA. Total RNA was harvested and used in real-time RT-PCR assays to determine steady-state levels of mRNA for ER-regulated genes. Compared to cells treated with 0.05 µmol/L Se, steady-state mRNA levels in cells treated with 5.0 µmol/L Se were 123% for IGFBP10, 111% for Cdk2, 103% for cathepsin D and 113% for hpS2. *The probability that the 23% difference in steady-state levels of mRNA between high and control Se samples for IGFBP10 occurred by random chance was 0.034. For Cdk2, cathepsin D and hpS2, the probabilities that differences were due to random chance were 0.119, 0.413 and 0.196, respectively.

## 4. Discussion

To have relevance to human dietary chemoprevention, experiments in animals and cultured cells must use nutritionally relevant forms of Se provided at physiologically relevant concentrations. In addition, cell lines or animal models chosen must be reasonably representative of some characteristic of the human disease. In this work, a nutritionally relevant form of Se was used at a physiological, serum-achievable concentration of total Se. The contribution of different chemical forms to total plasma Se varies with the forms and quantity of dietary Se intake [52,53], and the availability of various plasma Se compounds for uptake by body tissues and conversion to metabolically active forms is unknown. Naturally occurring food forms of Se, such as selenocysteine or Se-methylselenocysteine, can be converted to methylselenol ($CH_3SeH$), which appears to be the common metabolite derived from many anticarcinogenic Se compounds [15,54]. However, cultured cells have low activity of the lyase necessary to convert larger organic Se molecules to the monomethylated form [55]. Supplying MSA to cultured cells, as done in this work and other studies [56–58], directly provides a monomethylated form of Se that can be easily converted to $CH_3SeH$. A concentration of 5.0 μmol/L MSA is commonly used in cell culture studies [56,57,59]. This concentration of total Se is well below the blood level associated with frank toxicity in humans [60] and is achievable in humans by consuming a high Se diet or by supplementation. To mimic, as closely as possible, the conditions that could be found in a human male with high dietary Se intake, we chose not to further vary our MSA doses and estrogen levels or add competitors. While these decisions limit the scope of our conclusions, they are more relevant in application to human males.

Recent results from other laboratories have revealed multiple mechanisms by which MSA may inhibit growth of prostate cancer cells. These mechanisms include decreased androgen receptor (AR) expression and reduced AR signaling [61,62], global thiol redox modification of proteins [63], induction of apoptosis [64] and potentiation of the effects of other antitumorigenic agents [65,66].

The LNCaP cell line used in this study is AR-positive and androgen-sensitive, representing an earlier form of the disease. Other commonly used prostate cancer cell lines, such as PC-3 or DU-145, are AR-negative and androgen-insensitive. Almost all prostate tumors, regardless of their sensitivity to androgen, express the AR [67]. Thus, any cell line lacking an AR is of uncertain relevance as a model of human disease.

Previous studies clearly demonstrated that (1) Zn finger oxidation in purified ERs reduced their binding to DNA [18]; (2) Se interacts oxidatively with protein sulfhydryl clusters [16] and (3) Se may displace Zn from MT in vitro [19]. However, our results suggest that such alterations of ER-β by MSA do not readily take place in prostate cancer cells. There are several differences in experimental conditions between those studies and the present experiments, which likely account for the differences in results.

Jacob et al. [19] displaced Zn from MT using phenyl derivatives of Se, which are more stable than their aliphatic counterparts, but are not as biologically relevant. Whittal et al. [18] treated purified full-length ER or a recombinant ER-DNA binding domain with diamide or hydrogen peroxide to induce disulfide bond formation and showed reduced binding of the treated, purified proteins to DNA. However, those experiments did not include the cofactors known to be required in vivo for ER binding to its DNA response element [68,69]. If cofactors are unaffected in vivo by MSA, a modest effect of MSA on ER-β Zn fingers may not be sufficient to significantly alter ER-β DNA binding. Likewise, cofactors or other regulators of transcription rates or mRNA turnover for ER-regulated genes may be unaffected by MSA, thus minimizing effects of any slight structural alteration of the ER-β protein on downstream gene expression.

Quantitatively, the concentration of ERs is minor, compared to other Zn finger-containing proteins, which may account for as much as 1% of all human gene products [17]. LNCaP cells express ER-β at a low level and express no ER-α [26,27]. It is possible that at the concentration used, the MSA in our experiments interacted primarily with other more abundant Zn fingers present in the cell, diminishing interaction with ER-β.

Our results are internally consistent, showing no significant effects on ER-β DNA binding nor on associated transcription rates and steady state mRNA levels for ER-regulated genes. In only one case (IGFBP10 mRNA levels) was the effect of MSA statistically significant. It is uncertain if a 23% higher mRNA level for this gene would be biologically significant. The minimal, statistically insignificant changes seen for all other genes in mRNA levels, and for transcription rates of all genes tested, strongly support the conclusion that there was no significant biological effect of MSA treatment on any of these parameters. While it is possible that other chemical forms of Se, tested in other cell lines or animal models, could produce different results, our experiments suggest that the chemopreventive effects of MSA against prostate cancer are likely accounted for by mechanisms other than an inhibitory effect on ER-β activation and ER-regulated gene expression.

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