Breast Cancer Research and Treatment (2005) 92: 239–250
DOI 10.1007/s10549-005-3203-5

Report

Attenuation of estrogen receptor α (ERα) signaling by selenium in breast cancer cells via downregulation of ERα gene expression

Yatrik M. Shah[1], Aparna Kaul[1], Yan Dong[2], Clement Ip[2], and Brian G. Rowan[1]
[1]*Department of Biochemistry & Cancer Biology, Medical College of Ohio, Toledo, OH;* [2]*Department of Cancer Chemoprevention, Roswell Park Cancer Institute, Buffalo, New York, USA*

Key words: breast cancer, estrogen receptor, MCF-7, selenium

Summary

Numerous studies have shown that selenium provides beneficial effects as a cancer chemoprevention agent. Although long-term intervention trials failed to confirm selenium protection against breast cancer in humans because of insufficient cases, the evidence of effective selenium chemoprevention in animal mammary tumor models or human breast cancer cells is substantial and convincing. The present study demonstrates that the selenium compound methylseleninic acid (MSA) inhibits estrogen receptor α (ERα) signaling in ER-positive MCF-7 breast cancer cells as evidenced by decreased estradiol-dependent cell growth and gene expression. MSA diminishes estradiol induction of endogenous ER-regulated pS2 and c-myc genes as well as the expression of an ER-regulated reporter gene. A major mode of MSA action on ER signaling is through a downregulation of ERα gene expression that precedes a decrease in ERα protein level. This study provides a mechanism driven rationale for using selenium as a chemopreventive agent for women at high risk for developing breast cancer or as a therapeutic strategy for ER-positive breast cancer.

Introduction

Breast cancer is the most common female neoplasia in the western world with one in eight women developing breast cancer in the United States [1]. Estrogens promote the growth of breast cancer and a strong correlation exists between prolonged estrogen exposure and breast cancer risk [2]. The major mechanism of estrogen action in breast tissues is through binding to a specific nuclear estrogen receptor (ER) [3,4]. Two isoforms of ER have been identified. The role of the major isoform, ERα, in breast cancer has been clearly defined although the role of the recently discovered ERβ remains unclear [5–7]. ER is a ligand-activated transcription factor that upon estrogen stimulation binds to promoter regions of ER-regulated genes to modulate the expression of genes important for estrogen-regulated cell proliferation [4].

The micronutrient selenium has beneficial effects in inhibiting cancer growth. In a clinical trial Clark et al. demonstrated the protective effects of selenium against prostate, lung and colon cancer [8,9]. In addition, selenium inhibits mammary tumorigenesis [10] and breast-derived cell growth [11]. A recent study demonstrated that selenium interferes with androgen receptor (AR) signaling to decrease prostate cancer cell growth [12]. Since the ER belongs to the same superfamily of ligand-activated nuclear receptors as does the AR, it was of interest to determine whether selenium compounds could impact on ER signaling in breast cancer cells.

The present study used methylseleninic acid (MSA), developed specifically for testing selenium effects in *in vitro* cell line experiments [13]. MSA is a monomethylated selenium compound that bypasses the need for metabolic enzymes required to convert other selenium species to the active metabolite, methylselenol. This allows MSA, as opposed to other selenium species to act rapidly in cell lines. Selenomethionine, currently used in the SELECT clinical trail of prostate cancer, cannot be used in cell culture studies because most epithelial cell lines lack enzymes needed for metabolism to the active methylselenol [14]. Our results in the estrogen-dependent MCF-7 breast cancer cell line demonstrate that MSA inhibits ERα-dependent gene transcription by decreasing ERα mRNA levels and resulting protein levels. Attenuation of ER signaling in breast cancer cells is likely a major mechanism contributing to the growth inhibitory effect of MSA.

Material and methods

MTT assay

MCF-7 cells were plated in 24 well plates (20,000 cells/well) and cultured in phenol red-free DMEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS). Cells were incubated with 1, 5, or 10 μM of MSA at 24 h after plating. An aliquot of 125 μl of MTT

reagent (5 mg/ml of 2,5-diphenyl tetrazolium bromide in PBS) was pipetted into each well after 24, 48, or 72 h of exposure to MSA. The media with the MTT reagent was removed after 15–30 min and 300 μl of DMSO (Fisher Biotech, Fairlawn, NJ) was added to each well. The plates were read at a wavelength of 570 nm.

BrdU labeling assay

Cells were seeded in T75 culture flasks at a density designed to reach 70–80% confluency at the time of assay. At 48 h after seeding, cells were exposed to 10 μM MSA for 16 or 24 h. During the last 30 min of MSA treatment, cells were labeled with 10 μM of bromodeoxyuridine (10 μl of 1 mM BrdU was added to each millilitre of culture media). BrdU-labeled cells were trypsinized, fixed, treated with DNase I, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody using the BrdU Flow Kit from BD Pharmigen (San Diego, CA). Stained cells were then quantified by flow cytometry, and the data were analyzed with the WinList software (Variety Software House, Topsham, ME).

Detection of apoptosis

Cells were seeded in triplicate in a 96-well microtiter plate at a density designed to reach $\sim 10^4$ cells per well at the time of assay. At 48 h after seeding, cells were exposed to either 5 or 10 μM MSA for 24 h. Detached cells were pulled with attached cells by centrifugation. Cytoplasmic histone-associated DNA fragments were quantified using the Cell Death Detection ELISA^{PLUS} Kit (Roche Applied Science, Indianapolis, IN) as per manufacturer's protocol. The absorbance measured at 405 nm (with reference wavelength 492 nm) was normalized by the protein concentration of the samples.

Quantitation of propidium-iodide-stained cells by flow cytometry

MCF-7 cells were seeded in T75 culture flasks at a density designed to reach 70–80% confluency at the time of assay. At 48 h after seeding, cells were exposed to either 5 or 10 μM MSA for 6, 16 or 24 h. Adherent cells harvested by mild trypsinization were pooled together with detached cells (if any). Cells were incubated with 1 μg/ml of propidium iodide (PI) for 5 min on ice. PI-stained cells were subsequently quantified by flow cytometry, and the data were analyzed with the WinList software (Variety Software House, Topsham, ME).

Luciferase assay

MCF-7 and HeLa cells were plated in 6-well plates (2×10^5 cell/well) and cultured in phenol red-free DMEM containing 2% FBS that had been charcoal stripped to remove endogenous steroids. At 24 h after plating, the MCF-7 cells were transfected with 500 ng of EREe1b-luciferase reporter, and the HeLa cells were cotransfected with 50 ng of ERα and 500 ng EREe1b-luciferase reporter using Fugene transfection reagent (Roche, Madison, WI). At 24 h posttransfection, the cells were incubated with either vehicle, estradiol (10^{-8} M) (Sigma, St. Louis, MO), MSA (1, 5 or 10 μM), or a combination of estradiol and MSA for 24 h. Luciferase expression was measured and normalized as previously described [15].

Western blot analysis

MCF-7 and HeLa cells were plated in 100 mm dishes (3×10^6 cell/plate), and cultured in 2% charcoal-stripped FBS in DMEM. MCF-7 cells were maintained in the stripped media for 3 days until 90% confluency. HeLa cells were transfected at 24 h post plating with the expression vector for hERα (0.5 μg /plate) or the empty vector (0.5 μg/plate) and maintained in 2% stripped FBS in DMEM for an additional 48 h. Both MCF-7 and HeLa cells were incubated with either vehicle, estradiol, MSA or a combination of estradiol and MSA at varying concentrations and times as indicated in the figure legends. The cells were lysed and prepared for Western blotting as previously described [15]. The membranes were incubated with an antibody against ERα (Novacastra, Newcastle on Tyne, UK), and normalized to β-actin (Santa Cruz Biotechnology INC, Santa Cruz, CA).

Real-Time RT-PCR

The culture and/or transfection conditions for MCF-7 and HeLa cells were identical to that described above for Western blot analysis. Total mRNA was extracted from the cell pellet, reverse transcribed and gene expression was measured by real-time RT-PCR as described previously [15].

C-myc:
FWD-5′-CGTCTCCACACATCAGCACAA-3′
REV-5′-TGTTGGCAGCAGGATAGTCCTT-3′
Probe-5′-56FAM/ACGCAGCGCCTCCCTCCACTC/3BHQ-1/-3′

pS2:
FWD-5′-CGTGAAAGACAGAATTGTGGTTTT-3′
REV-5′-CGTCGAAACAGCAGCCCTTA-3′
Probe-5′-/56FAM/TGTCACGCCCTCCCAGTGTGCA/3BHQ-1/-3′

ERα
FWD-5′-AGACGGACCAAAGCCACTTG-3′
REV-5′-CCCCGTGATGTAATACTTTTGCA-3′
Probe-5′-/56FAM/TGCGGGCTCTACTTCATCGCATTCC/3BHQ-1/-3′

ERβ
FWD-5′-CCCAGTGCGCCCTTCAC-3′
REV-5′-CAACTCCTTGTCGGCCAACT-3′
Probe-5′-/56FAM/AGGCCTCCATGATGTCCCTGA/3BHQ-1/-3′

Cofilin
FWD-5′-TGTGCCGGCTGGTTCCT-3′
REV-5′-CTTACTGGTCCTGCTTCCATGAG-3′
Probe-5′-/56FAM/CTTTTCCCCTGGTCACGGCT/3BHQ-1/-3′
CRABPII
FWD-5′-TTCTCTGGCAACTGGAAAATCA-3′
REV-5′-CATTCACCCCCAGCACTTTG-3′
Probe-5′-/56FAM/CCGATCGGAAAACTTCGAGGAATTGC/3BHQ-1/-3′
Smooth muscle actin
FWD-5′-TCCTCCCTTGAGAAGAGTTACGA-3′
REV-5′-GGCAGCGGAAACGTTCATT-3′
Probe-5′-/56FAM/TGCCTGATGGGCAAGTGATCA/3BHQ-1/-3′

Data analysis

Results are expressed as mean ± SD. *P*-values were calculated using Anova Dunnett's *T*-Test and Independent *t*-test. $p < 0.05$ was considered significant.

Results

MSA inhibits MCF-7 cell growth via inhibition of proliferation and induction of apopotosis

MCF-7 is an ER positive breast cancer cell line that is sensitive to estradiol-induced cell growth and ER-regulated gene expression. MCF-7 cells were incubated with MSA and cell growth was measured by the MTT assay. MSA at 5 or 10 μM inhibited cell growth by approximately 50% at 24, 48, and 72 h (Figure 1(a)). To assess the contribution of apoptosis and anti-proliferation to MSA growth inhibition in MCF-7 cells, *BrdU* incorporation and DNA fragmentation were assessed. MSA (10 μM) decreased *BrdU* incorporation by 50% and 70% at 16 and 24 h, respectively (Figure 1(b)). In the same time course, MSA increased apoptosis at 24 h by 8- and 15-fold at 5 and 10 μM MSA, respectively (Figure 1(c)). MSA had no effect on cell toxicity up to 24 h as assessed by trypan blue staining and only very modest effects at 24 h as assessed by propidium iodine (PI) staining (Figure 1 (d) and (e)).

MSA inhibits estradiol-dependent induction of estrogen response element (ERE)-luciferase reporter gene (ERE_2e1b-luciferase) and endogenous ERα-regulated genes

To assess whether MSA could alter ER signaling, MCF-7 cells were transfected with the ERE_2e1b-luciferase reporter gene and incubated with estradiol or MSA alone, or co-incubated with estradiol and MSA. MSA alone decreased basal luciferase expression and inhibited estradiol-dependent stimulation of the ERE_2e1b-luciferase reporter presence of 5 and 10 μM MSA (Figure 2(a)). Since MSA inhibited estradiol-dependent induction of the ERE_2e1b-luciferase reporter, it was of interest to determine whether MSA could inhibit two well-characterized estrogen-regulated genes, c-myc, and pS2. Real-time RT-PCR was used to measure c-myc and pS2 expression after incubation with estradiol alone or in combination with MSA for 6 h. As expected, estradiol induced pS2 and c-myc expression and this induction was inhibited in the presence of 1, 2.5, 5, or 10 μM MSA in a dose-dependent manner (Figure 2(b) and (c)). Additional time course experiments revealed that estradiol induction of pS2 and c-myc occurred at 2 h and 1 h, respectively (Figure 2(d) and (e)). A period of 4 h co-incubation with MSA was required to block the response to estradiol induction (Figure 2(d) and (e)).

MSA specifically inhibits estradiol-dependent signaling

In order to rule out the nonspecific effects of MSA on general transcription, MCF-7 cells were transfected with constitutively active RSV-luciferase reporter and incubated with 1, 5, or 10 μM MSA. No effect on the RSV-luciferase reporter was observed at any MSA concentration (Figure 3(a)). In addition to assess the specificity of MSA towards ER signaling, MSA effects on several genes that are expressed in MCF-7 cells but unrelated to estrogen signaling were examined. Cellular retinoic acid binding protein II (CRABPII) is expressed in MCF-7 cells but is not modulated by estradiol [16]. Cofilin [17] and smooth muscle actin [18] are expressed in breast cancer cell lines but not regulated by estradiol (data not shown). MSA had no effect on CRABPII, cofilin, or smooth muscle actin mRNA expression (Figure 3(b)). These data demonstrate a specificity of MSA towards estradiol-dependent signaling.

MSA reduces ERα protein expression in MCF-7 cells

Since MSA inhibited ER signaling, we proceeded to determine if MSA altered ERα protein levels. MCF-7 cells were incubated with estradiol, MSA, or estradiol + MSA for 6 h, and ERα levels were assessed by Western blot analysis. MSA alone at 5 or 10 μM reduced ERα level significantly compared to vehicle-incubated samples. Estradiol treatment alone reduced ERα protein level (Figure 4(a), lane 6), most likely due to ubiquitin-mediated downregulation as reported previously [15]. MSA at 5 or 10 μM further reduced ERα levels when co-incubated with estradiol (Figure 4(a), lanes 9 and 10). To measure time-dependent decreases of ER by MSA, MCF-7 cells were incubated with 10 μM of MSA for 2, 4, 6, or 12 h. A significant reduction in ERα protein level was detected after 4 h of incubation with MSA (Figure 4(b)); this time frame coincided with the time it took MSA to inhibit estradiol-dependent induction of pS2 and c-myc (Figure 2(d) and (e)). The effect of MSA on ERβ protein level was also assessed. However, Western blotting of ERβ in MCF-7 cells generated very weak signals, so the result was inconclusive (data

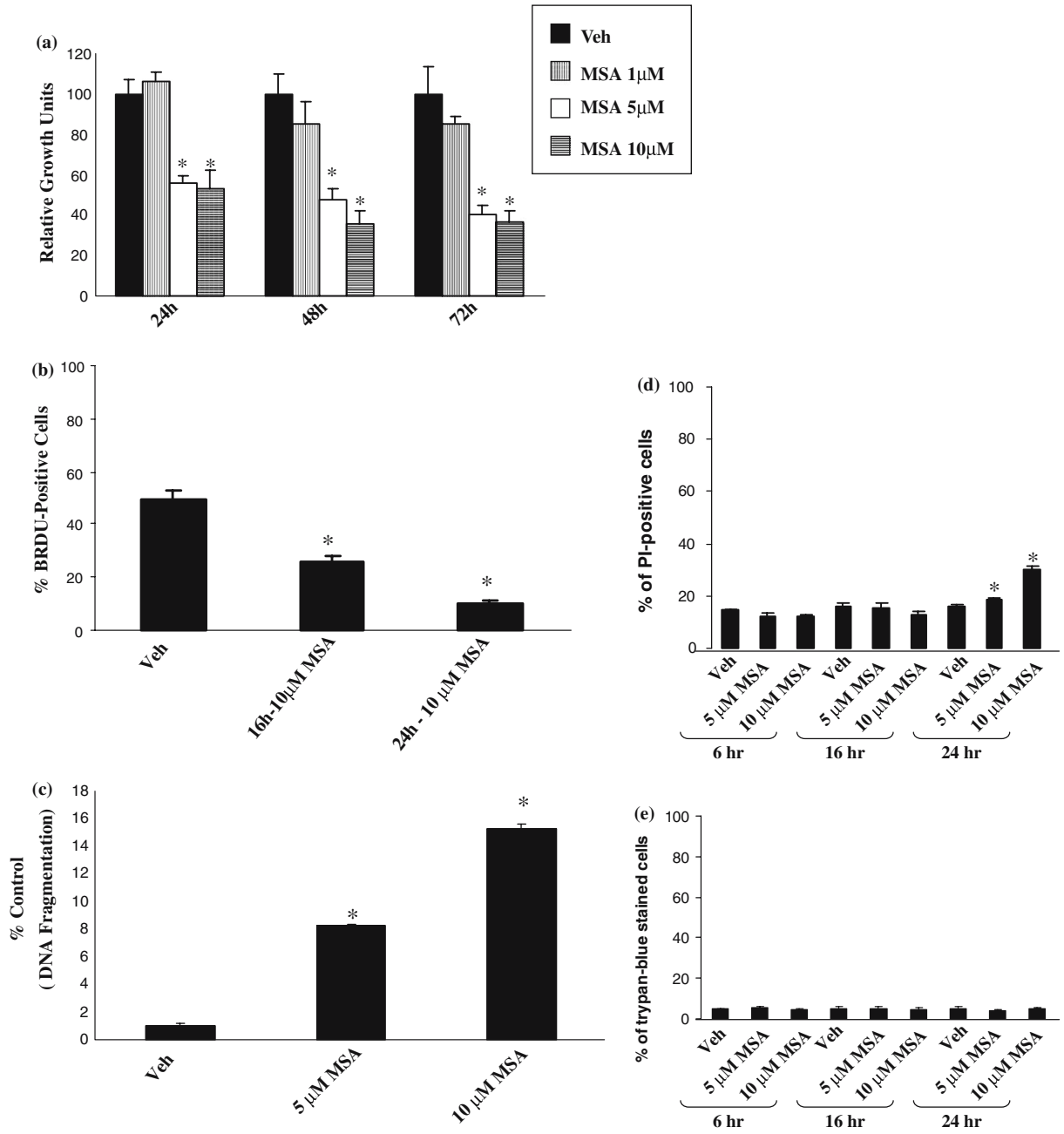

Figure 1. MSA inhibits MCF-7 cell growth through altering proliferation and apoptosis. (a) Cell growth analysis by MTT assay of MCF-7 cells incubated with vehicle (Veh) or 1, 5, and 10 μM of MSA for 24, 48, and 72 h. Each bar represents the mean value $\pm$ SD. Vehicle treated samples were set at 100%. *$p < 0.05$ compared to vehicle. (b) MCF-7 cell proliferation was measured by BrdU incorporation. MCF-7 cells were incubated with 10 μM MSA for 16 and 24 h. BrdU incorporation was measured as described in Material and methods, each bar represents the mean value $\pm$ SD. *$p < 0.05$ compared to vehicle (Veh) incubated samples. (c) MCF-7 cells were incubated with 5 and 10 μM MSA for 24 h. Following incubation with MSA DNA fragmentation was assessed as described in Material and methods, each bar represents the mean value $\pm$ SD. *$p < 0.05$ compared to vehicle (Veh) incubated samples. (d) MCF-7 cells were incubated with 5 and 10 μM MSA for 6, 16, and 24 h. Cell toxicity was measured by PI staining (top panel) and by trypan blue staining (bottom panel) as described in Material and methods, each bar represents the mean value $\pm$ SD. *$p < 0.05$ compared to vehicle (Veh) incubated samples.

not shown). To determine whether ERα protein downregulation was a major mechanism by which MSA inhibits ER signaling, we constitutively expressed ERα protein in the well-characterized, ER-negative HeLa cell line by transfection with a constitutively expressed ERα expression plasmid. HeLa cells are cervical carcinoma cells in which reexpression of ERα results in robust estradiol-dependent reporter activation. Incubation of transfected cells with 10 μM MSA did not affect ERα protein level (Figure 4(c) inset). Under these conditions in which ERα protein levels remained constant, MSA did not inhibit estradiol induction of the ERE_2e1b-luciferase reporter (Figure 3(c)), nor endogenous pS2 (Figure 4(d)) or c-myc (Figure 4(e)). These results suggest that a major mechanism contributing to MSA inhibition of estrogen signaling is through reduction in ERα protein. One caveat to this finding is that HeLa is a cervical carcinoma and not a breast cancer cell line.

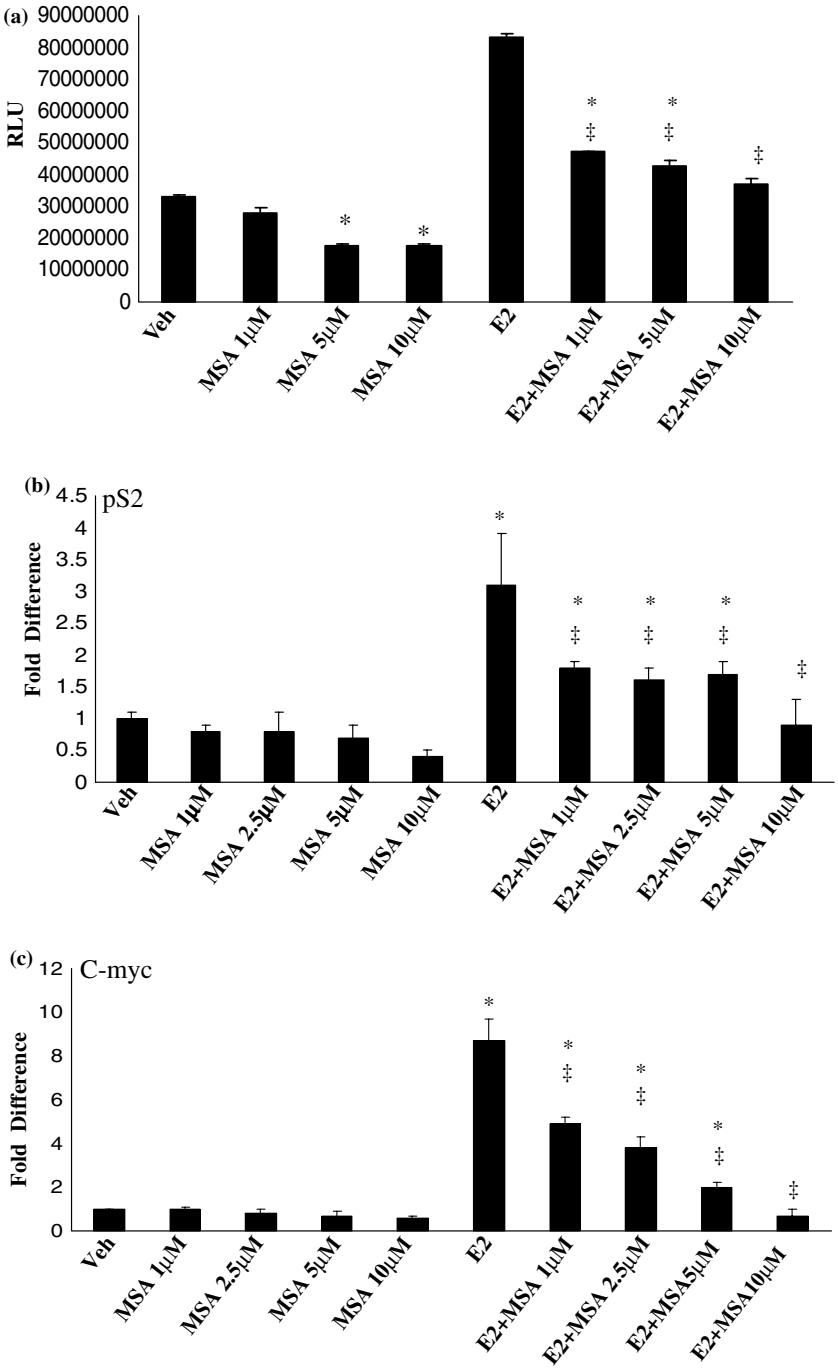

Figure 2. MSA inhibits estradiol-dependent activation of an EREe1b-luciferase reporter, pS2, and c-myc gene expression. (a) MCF-7 cells were transfected with ERE_2e1b-luciferase reporter (0.5 μg/well). Twenty-four hours posttransfection cells were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or 1, 5, or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol and 1, 5, or 10 μM MSA. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and methods. Each bar represents the mean value ± SD. *$p < 0.05$ compared to vehicle incubated samples ‡$p < 0.05$ compared to estradiol (E2) incubated samples. Dose response of MSA on (b) pS2 or (c) c-myc gene expression. MCF-7 cells (2×10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or 1, 2.5, 5, or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol and 1, 2.5, 5, or 10 μM MSA for 6 h. Time titration of MSA on (d) pS2 or (e) c-myc gene expression. MCF-7 cells (2×10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol (E2) and 10 μM MSA. Expression of (b and d) pS2 and, (c and e) c-myc genes was measured by real time RT-PCR as described in the Materials and methods. Expression was normalized to GAPDH and each bar represents the mean value ± SD. *$p < 0.05$ compared to vehicle incubated samples ‡$p < 0.05$ compared to estradiol (E2) incubated samples.

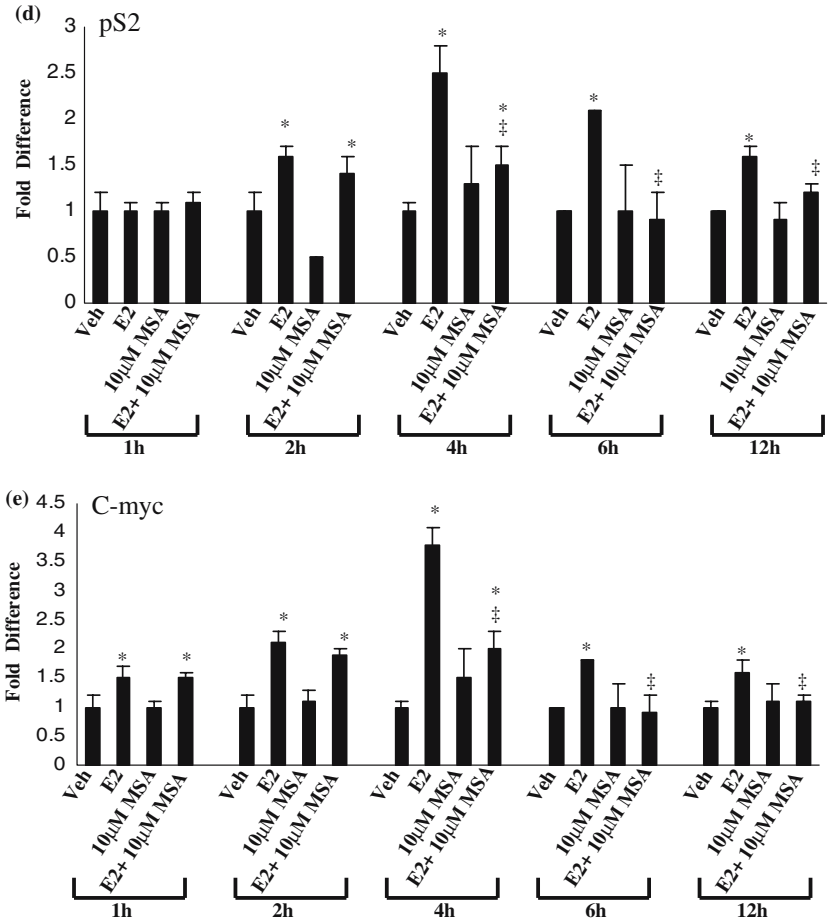

Figure 2. Continued.

Although similar experiments were attempted in two ER-negative breast cancer cell lines (MDA-MB-231, MDA-MB-468) transfected with ERα, results were inconclusive since neither cell line displayed estradiol activation of cotransfected ERE_2e1b-luciferase (data not shown).

MSA reduces ERα mRNA

To determine whether the effect of MSA on ERα was at the transcriptional level, ERα mRNA in MCF-7 cells was measured by real time RT-PCR following incubation of cells with estradiol, MSA, or estradiol + MSA. MSA at 5 or 10 μM inhibited ERα gene expression (Figure 5(a)) but had no effect on ERβ gene expression (Figure 5(b)). Time course studies revealed that ERα mRNA was inhibited by 10 μM MSA within 2 h (Figure 5(c)), thereby preceding the decrease in protein level that was not detected until after 4 h, (see Figure 5(b)), suggesting that the mechanism by which MSA reduces ERα protein level is through decreased ERα mRNA transcription.

Discussion

ER is a major therapeutic target for hormone-dependent, ER-positive breast cancers. Antiestrogen therapies designed to compete with estrogen for binding to ER have proven effective in reducing estrogen-dependent tumor growth. In addition to targeting ER function, strategies designed to lower ER protein level are also useful in the management of hormone-dependent, ER-positive tumors. The present study is the first to describe the disruption of ER signaling in breast cancer cells by a selenium compound. We found that MSA blocked estradiol-dependent activation of a reporter gene (ERE_2e1b-luciferase) as well as the transcription of endogenous ER-regulated genes. A major underlying mechanism by which MSA interferes with ER signaling was through a decrease in ERα gene transcription and the subsequent reduction of ERα protein.

Several mechanisms may contribute to the growth inhibitory effects of MSA including antioxidant properties and alteration of redox reactions that may subsequently induce apoptosis [14,19]. Several independent studies have demonstrated MSA-induced growth inhibition in *in vitro* cell lines via induction of apoptosis. In hyperplastic mammary epithelium cells TM12 and TM2H [13] and in premalignant human breast cells MCF10AT1 and MCF10AT3B [20], MSA inhibited cell proliferation and induced apoptosis. MSA also demonstrated similar effects in human lung cancer cell lines [21] and human prostate carcinoma cell lines [22, 23]. Moreover, MSA effects on cell growth were not mediated by selenium related toxicity [13] as is detected with

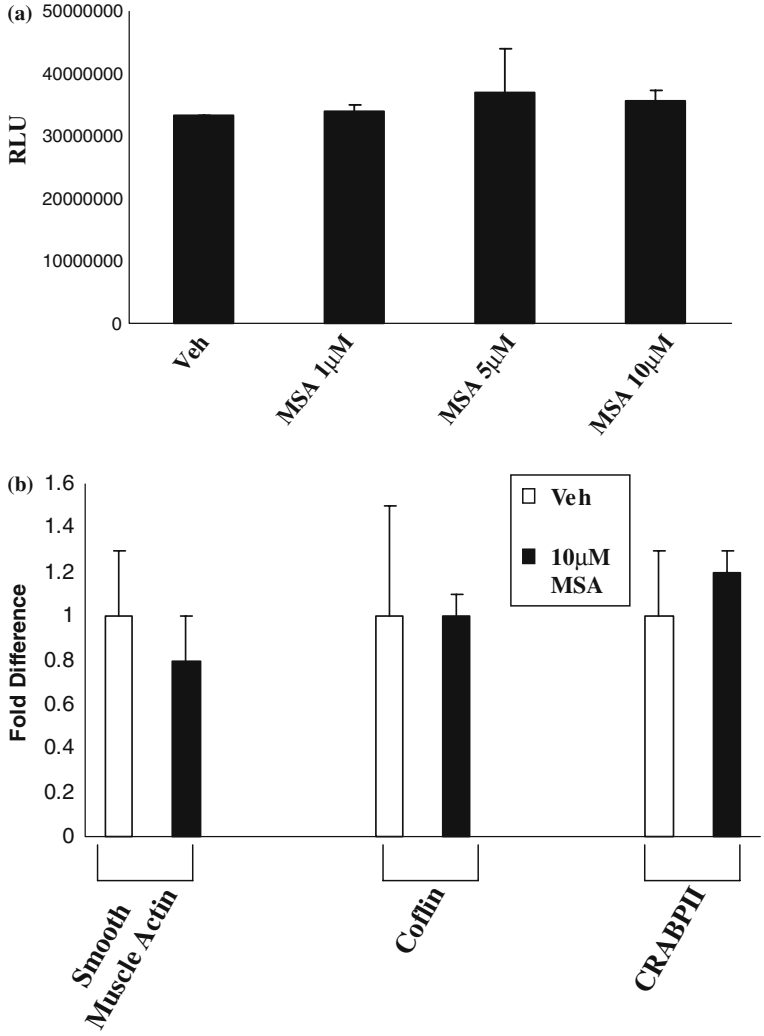

Figure 3. MSA specifically inhibits estradiol-dependent signaling. (a) MCF-7 cells were transfected with RSV-luciferase reporter (0.5 μg/well). Twenty-four hours posttransfection cells were incubated with vehicle 1, 5, or 10 μM MSA. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and methods. (b) MCF-7 cells (2×106 cells/plate) were incubated with vehicle (Veh) or 10 μM MSA for 2 h. Smooth muscle actin, cofilin and CRABPII genes were measured by real time RT-PCR as described in the Materials and methods and expression was normalized to GAPDH.

inorganic selenium compounds such as sodium selenite and sodium selenide [24]. In addition, based on our characterization of MSA action in MCF-7 cells, the growth inhibitory effects of MSA occur through both apoptosis and antiproliferation mechanisms (Figure 1(b) and (c)). Comprehensive analysis of these mechanisms in a variety or hormone-dependent and hormone-independent breast cancer cell lines is currently being assessed. What is demonstrated here for the first time in estrogen-dependent breast cancer cells is a novel component of the overall antiproliferative effect of MSA; the inhibition of ER signaling. Estrogens are important mitogenic signals for the growth of breast cancers, and have been shown to induce G_1/S transition in breast cancer cells [25] by control of several key cell-cycle regulators (for ref see [26]). In addition to inducing cellular proliferation, estrogens increase cell survival by upregulating the antiapoptotic factor bcl-2 [27] and downregulating several proapoptotic factors [28]. The overall growth inhibitory effects of MSA in estrogen dependent breast cancer cells are likely attributed to MSA disruption of ER signaling and non ER antiproliferative and apoptotic effects of MSA in hormone dependent breast cancer. Indeed, MSA also inhibited growth of an ERα-negative breast cancer cell line (MDA-MB-231, data not shown). Future work is being performed to assess the relative contribution of loss of ER-signaling to the overall growth inhibitory effect of MSA in ERα positive breast cancer cells.

Low concentrations of MSA (1 or 2.5 μM) had no significant effect on ERα protein levels although both concentrations were capable of inhibiting estradiol-dependent reporter gene activation (Figure 2(a)) and endogenous pS2 and c-myc genes (Figure 2(b) and (c)). This apparent discrepancy may be attributed to MSA effects on other proteins important for ERα action. Dong et al. [20] demonstrated that MSA regulates expression of several proteins shown to be key mediators of ER signaling. For example, MSA decreased expression of cyclin D1, a known coregulator for ER

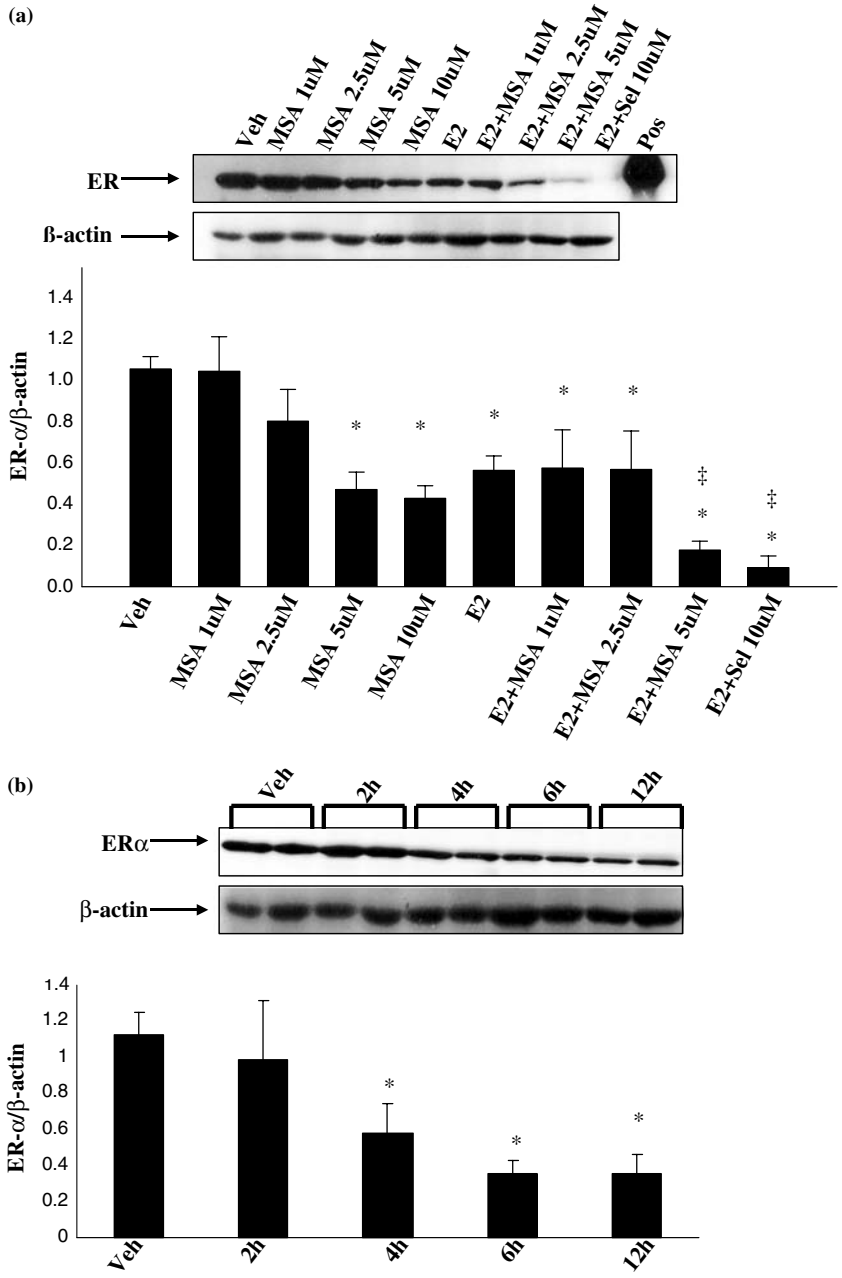

Figure 4. MSA inhibits ERα protein expression, which is required for its effect on ER-dependent gene expression. (a) Dose response of MSA on ERα protein expression. MCF-7 cells (2×10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or 1, 2.5, 5, or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol and 1, 2.5, 5 or 10 μM MSA for 6 h and Western blot analysis was performed. (b) Time titration of MSA on ERα protein expression. MCF-7 cells (2×10^6 cells/plate) were incubated with vehicle (Veh) or 10 μM MSA and Western blot analysis was performed. Quantitation of the Western blot signal for ERα was normalized to the Western blot signal for β-actin levels (Figure 3(a) and (b), bottom panel), and each bar represents the mean value ± SD. *$p < 0.05$ compared to vehicle incubated samples ‡$p < 0.05$ compared to estradiol (E2) incubated samples. (c) HeLa cell were transfected with 500 ng of constitutively expressed ER and incubated with vehicle or 10 μM MSA for 6 h and Western blot analysis was performed for ERα (Inset) or transfected with 50 ng of constitutively expressed ERα and 500 ng ERE_2e1b-luciferase reporter and incubated with (Veh), 10^{-8} M estradiol (E2) or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol (E2) and 10 μM MSA. Standard luciferase assays were performed on cell extracts in triplicate as described in the Materials and methods. *$p < 0.05$ compared to vehicle incubated samples. (d and e) HeLa cells were transfected with 500 ng of constitutively expressed ERα and incubated with (Veh), 10^{-8} M estradiol (E2) or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol (E2) and 10 μM MSA for 6 h. Expression of (d) pS2 and, (e) c-myc genes was measured by real time RT-PCR as described in the Materials and methods. Expression was normalized to GAPDH and each bar represents the mean value ± SD. *$p < 0.05$ compared to vehicle incubated samples.

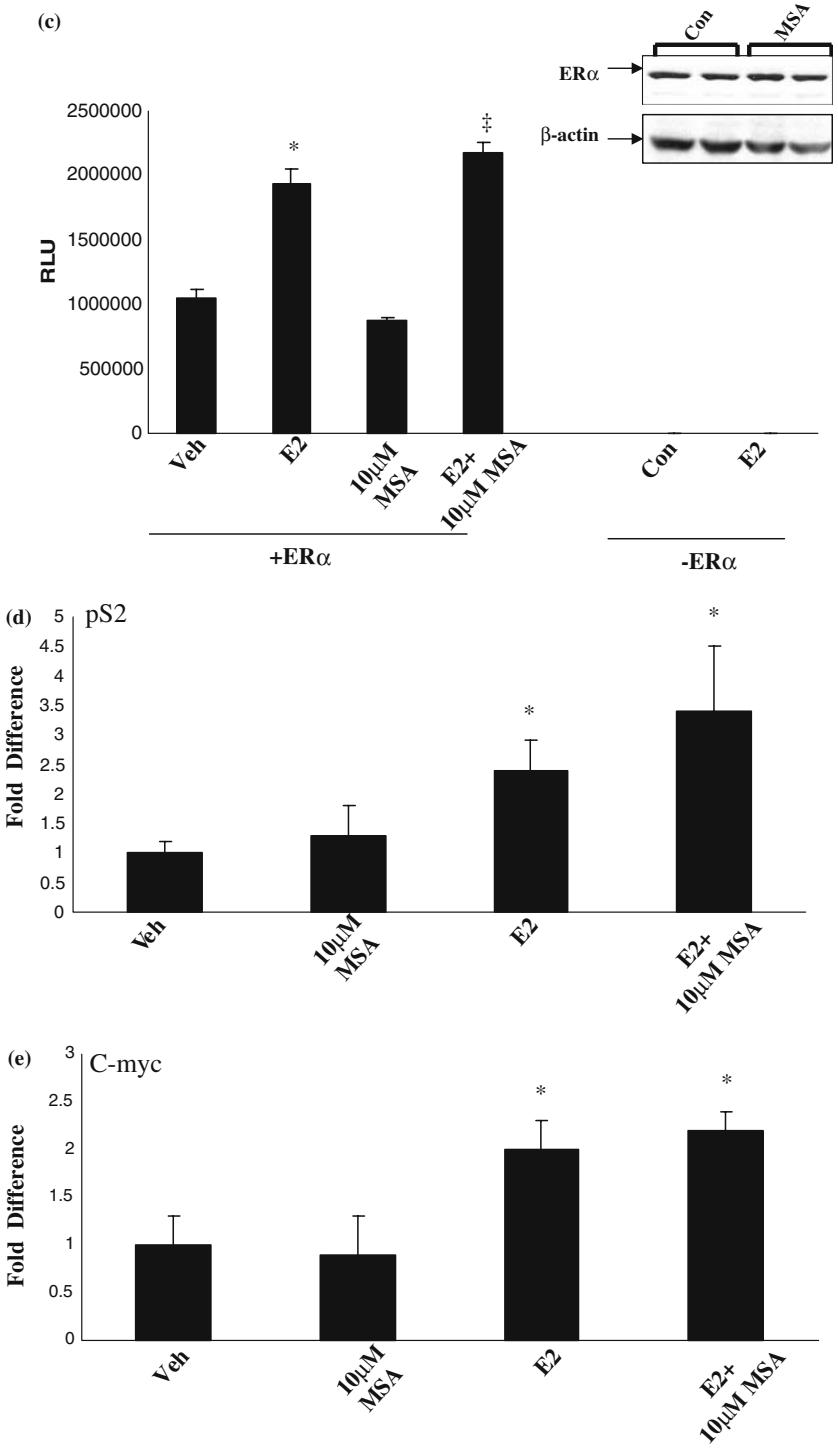

Figure 4. Continued.

action [29]. In addition, MSA decreased the expression of AKT2, a kinase known to phosphorylate ERα resulting in increased transcriptional activity [30] and stabilization of ERα binding to the pS2 and c-myc promoters (Shah and Rowan, Molecular Endocrinology, in press).

Although both ERα (present study) and AR [12] gene expression and protein levels were reduced by MSA, ERβ mRNA expression was unaltered. The lack of unanimity suggests a specificity of MSA for some, but not all, nuclear receptors. Future studies will examine the promoter regions of ERα and AR genes to assess whether homologous transcription factor binding sites are present, and to delineate the promoter regions required for MSA downregulation of ERα and AR transcription.

Although our study demonstrates a strong MSA inhibition of estrogen signaling and cell growth in MCF-7 breast cancer cells *in vitro*, selenium compounds appear to have negligible effects on proliferation of normal rodent

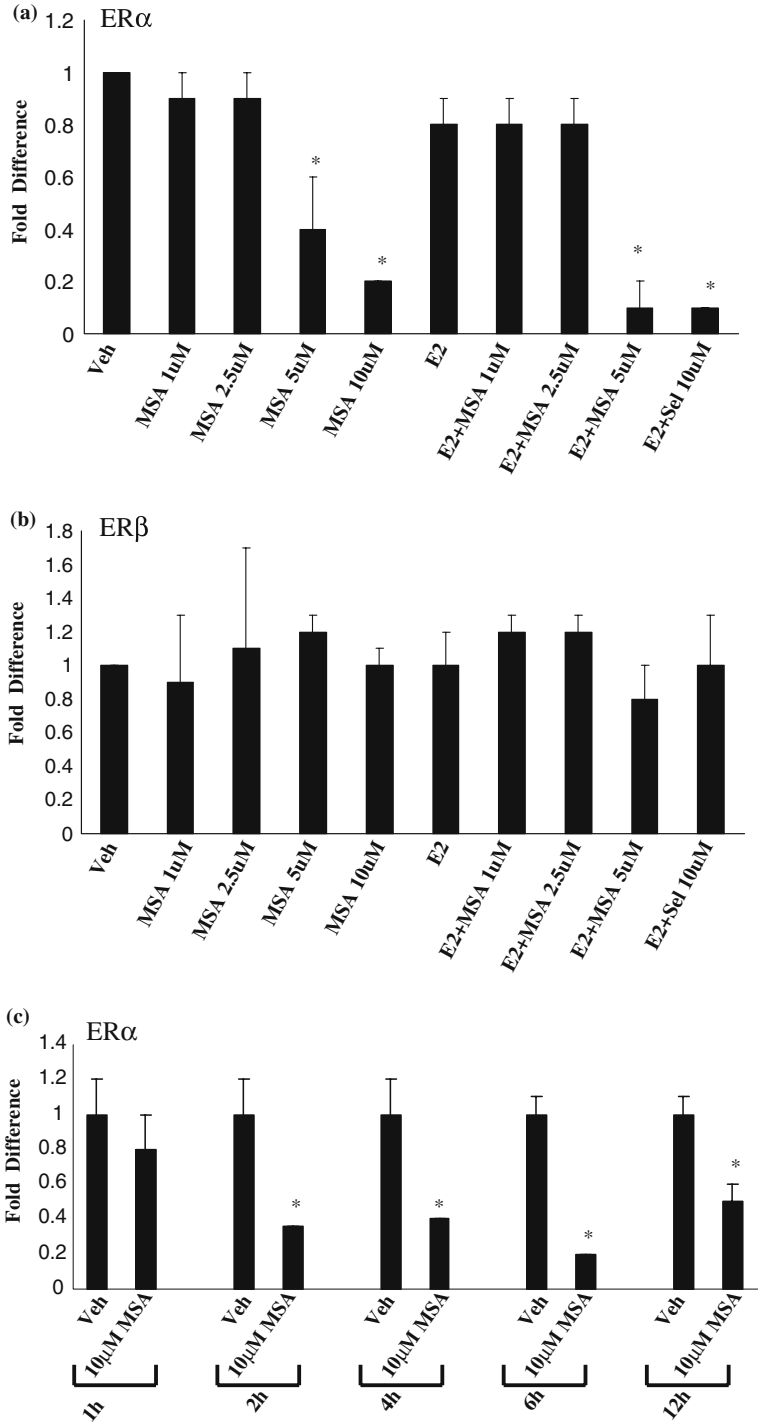

Figure 5. MSA inhibits ERα gene expression. Dose response of MSA on (a) ERα or (b) ERβ gene expression. MCF-7 cells (2×10^6 cells/plate) were incubated with vehicle (Veh), 10^{-8} M estradiol (E2) or and 1, 2.5, 5, or 10 μM MSA alone or co-incubated with 10^{-8} M estradiol and 1, 2.5, 5, or 10 μM MSA for 6 h. (c) Time titration of MSA on ERα gene expression. MCF-7 cells (2×10^6 cells/plate) were incubated with vehicle (Veh) or 10 μM MSA. ERα and ERβ genes were measured by real time RT-PCR as described in the Materials and methods. Expression was normalized to GAPDH and each bar represents the mean value ± SD. *$p < 0.05$ compared to vehicle incubated samples.

mammary gland [31–33]. This discrepancy may be due to differences in ERα expression in the normal versus malignant mammary gland. A very small percentage of epithelial cells in the normal, adult mammary gland are proliferating and these proliferating cells express very little or no ERα. In contrast, proliferating, epithelial breast cancer cells are ERα positive [34] and these cells are strong candidates for growth inhibition by antiestrogens [35–37]. If disruption of ER signaling is one of the major mechanisms for selenium-mediated growth inhibition, then the normal mammary epithelium may only be modestly affected due to absence of ERα in the proliferating epithelial cells. These possibilities remain under intense investigation by our laboratories.

Lastly, the present study presents the possibility that selenium compounds may not only be useful as a chemopreventative, but may also be efficacious as a therapy for existing tumors. Several studies have shown growth inhibition of established tumors by selenium compounds in *in vivo* models [33,38–41]. However these studies used inorganic selenium compounds that are genotoxic and no longer used for selenium chemoprevention and/or chemotherapy studies. In addition, Yan et al. demonstrated that supplementation with dietary selenium and magnesium had no effect on HTB123 human mammary cancer cells inoculated in athymic nude mice [42]. To our knowledge no study has examined the effects of MSA on established tumors in an *in vivo* model. However, Cao et al. have shown a synergistic interaction of organic selenium compounds with the anticancer drug irinotecan [43]. Mice bearing squamous cell carcinoma of the head/neck and colon carcinoma xenografts were given selenium in form of 5-methylselenocysteine and seleno-L-methionine orally 7 days prior to intravenous injection of irinotecan. Combination treatment of irinotecan + selenium decreased the toxicity of the chemotherapeutic agent and increased the cure rate of the tumor bearing mice inoculated with cancer cells sensitive and resistant to irinotecan [43]. Although it is unknown whether selenium monotherapy would be efficacious against MCF-7 xenografts, these tumors are tamoxifen sensitive [44] suggesting the possibility of combination therapy of tamoxifen with selenium compounds to improve efficacy.

Tamoxifen is currently the major antiestrogen used for breast cancer therapy, even though tamoxifen resistance and tamoxifen uterotropic effects represent significant drawbacks of this modality. ER signaling remains functional in the majority of breast cancers that exhibit tamoxifen resistance [45]. This suggests that any strategy designed to disrupt ER signaling or remove ERα protein in tamoxifen-resistant cells may provide some measure of efficacy. For example the antiestrogen fulvestrant results in ERα protein degradation and apoptotic cell death. Fulvestrant is recommended for postmenopausal women who exhibit breast cancer progression following tamoxifen therapy (for a review see ref. [46]). We are currently assessing whether MSA may be useful in a similar manner as Fulvestrant since MSA potentiates the growth inhibitory effects of tamoxifen in tamoxifen sensitive breast cancer cells and resensitize tamoxifen resistant breast cancer and endometrial cancer cells *in vitro* (submitted for publication).

An important goal in the endocrine management of hormone dependent breast cancer is to increase the proportion of cells undergoing apoptotic cell death relative to cell cycle arrest. Increasing the proportion of cells undergoing apoptosis would prevent cells from reentering the cell cycle once tamoxifen resistant mechanisms are acquired resulting in disease recurrence. MSA induces significant apoptosis in MCF-7 cells. Preclinical therapeutic regimens combining tamoxifen with selenium are being explored for efficacy in preventing or delaying tamoxifen resistance and/or reversing tamoxifen resistance.

Acknowledgements

Grant Support: This work was supported in part by National Institutes of Health Grant RO1 DK06832 (to B.G.R.) and Grant R01 CA91990 (to C.I). Y.K.S. was supported by a Predoctoral Dissertation Award (DISS0100539) from the Susan G. Komen Breast Cancer Foundation.

References

1. Kelsey JL, Berkowitz GS: Breast cancer epidemiology. Cancer Res 48: 5615–5623, 1988
2. Endogenous sex hormones and breast cancer in postmenopausal women (2002) reanalysis of nine prospective studies. J Natl. Cancer Inst. 94:606–616
3. Evans RM: The steroid and thyroid hormone receptor superfamily. Science 240: 889–895, 1988
4. Tsai MJ, O'Malley BW: Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63: 451–486, 1994
5. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA: Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93: 5925–5930, 1996
6. Mosselman S, Polman J, Dijkema R: ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 19(392), 49–53, 1996
7. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V: Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Mol Endocrinol 11: 353–365, 1997
8. Duffield-Lillico AJ, Reid ME, Turnbull BW, Combs GF Jr, Slate EH, Fischbach LA, Marshall JR, Clark LC: Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: a summary report of the Nutritional Prevention of Cancer Trial. Cancer Epidemiol Biomarkers Prev 11: 630–639, 2002
9. Clark LC, Combs GF Jr., Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL Jr, Park HK, Sanders BB Jr, Smith CL, Taylor JR: Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. JAMA 276: 1957–1963, 1996
10. el Bayoumy K, Chae YH, Upadhyaya P, Ip C: Chemoprevention of mammary cancer by diallyl selenide, a novel organoselenium compound. Anticancer Res 16: 2911–2915, 1996
11. Redman C, Scott JA, Baines AT, Basye JL, Clark LC, Calley C, Roe D, Payne CM, Nelson MA: Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines. Cancer Lett 125: 103–110, 1998
12. Dong Y, Lee SO, Zhang H, Marshall J, Gao AC, Ip C: Prostate specific antigen expression is down-regulated by selenium through disruption of androgen receptor signaling. Cancer Res 64: 19–22, 2004
13. Ip C, Thompson HJ, Zhu Z, Ganther HE: *In vitro* and *in vivo* studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. Cancer Res 60: 2882–2886, 2000

14. Ip C, Dong Y, Ganther HE: New concepts in selenium chemoprevention. Cancer Metastasis Rev 21: 281–289, 2002
15. Shah YM, Basrur V, Rowan BG: Selective estrogen receptor modulator regulated proteins in endometrial cancer cells. Mol Cell Endocrinol 219: 127–139, 2004
16. Delva L, Bastie JN, Rochette-Egly C, Kraiba R, Balitrand N, Despouy G, Chambon P, Chomienne C: Physical and functional interactions between cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear complex. Mol Cell Biol 19: 7158–7167, 1999
17. Bakin AV, Safina A, Rinehart C, Daroqui C, Darbary H, Helfman DM: A critical role of tropomyosins in TGF-beta regulation of the actin cytoskeleton and cell motility in epithelial cells. Mol Biol Cell 15: 4682–4694, 2004
18. Twal WO, Czirok A, Hegedus B, Knaak C, Chintalapudi MR, Okagawa H, Sugi Y, Argraves WS: Fibulin-1 suppression of fibronectin-regulated cell adhesion and motility. J Cell Sci 114: 4587–4598, 2001
19. Patrick L: Selenium biochemistry and cancer: a review of the literature. Altern.Med Rev 9: 239–258, 2004
20. Dong Y, Ganther HE, Stewart C, Ip C: Identification of molecular targets associated with selenium-induced growth inhibition in human breast cells using cDNA microarrays. Cancer Res 62: 708–714, 2002
21. Swede H, Dong Y, Reid M, Marshall J, Ip C: Cell cycle arrest biomarkers in human lung cancer cells after treatment with selenium in culture. Cancer Epidemiol Biomarkers Prev 12: 1248–1252, 2003
22. Dong Y, Zhang H, Hawthorn L, Ganther HE, Ip C: Delineation of the molecular basis for selenium-induced growth arrest in human prostate cancer cells by oligonucleotide array. Cancer Res 63: 52–59, 2003
23. Jiang C, Wang Z, Ganther H, Lu J: Caspases as key executors of methyl selenium-induced apoptosis (anoikis) of DU-145 prostate cancer cells. Cancer Res 61: 3062–3070, 2001
24. Lu J, Jiang C, Kaeck M, Ganther H, Vadhanavikit S, Ip C, Thompson H: Dissociation of the genotoxic and growth inhibitory effects of selenium. Biochem Pharmacol 50: 213–219, 1995
25. Leung BS, Potter AH: Mode of estrogen action on cell proliferative kinetics in CAMA-1 cells. I. Effect of serum and estrogen. Cancer Invest 5: 187–194, 1987
26. Foster JS, Henley DC, Ahamed S, Wimalasena J: Estrogens and cell-cycle regulation in breast cancer. Trends Endocrinol Metab 12: 320–327, 2001
27. Perillo B, Sasso A, Abbondanza C, Palumbo G: 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. Mol Cell Biol 20: 2890–2901, 2000
28. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS: Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144: 4562–4574, 2003
29. Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M, Ewen ME: Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol Cell Biol 17: 5338–5347, 1997
30. Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV, Cheng JQ: Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer Res 61: 5985–5991, 2001
31. Ip C, Thompson HJ, Ganther HE: Selenium modulation of cell proliferation and cell cycle biomarkers in normal and premalignant cells of the rat mammary gland. Cancer Epidemiol Biomarkers Prev 9: 49–54, 2000
32. Fico ME, Poirier KA, Watrach AM, Watrach MA, Milner JA: Differential effects of selenium on normal and neoplastic canine mammary cells. Cancer Res 46: 3384–3388, 1986
33. Watrach AM, Milner JA, Watrach MA, Poirier KA: Inhibition of human breast cancer cells by selenium. Cancer Lett 25: 41–47, 1984
34. Clarke RB, Howell A, Potten CS, Anderson E: Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res 57: 4987–4991, 1997
35. Clarke RB, Laidlaw IJ, Jones LJ, Howell A, Anderson E: Effect of tamoxifen on Ki67 labelling index in human breast tumours and its relationship to oestrogen and progesterone receptor status. Br J Cancer 67: 606–611, 1993
36. Jordan VC, Koerner S: Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 11: 205–206, 1975
37. Johnston SR, MacLennan KA, Sacks NP, Salter J, Smith IE, Dowsett M: Modulation of Bcl-2 and Ki-67 expression in oestrogen receptor-positive human breast cancer by tamoxifen. Eur J Cancer 30A: 1663–1669, 1994
38. Watrach AM, Milner JA, Watrach MA: Effect of selenium on growth rate of canne mammary carcinoma cells in athymic nude mice. Cancer Lett 15: 137–143, 1982
39. Poirier KA, Milner JA: Factors influencing the antitumorigenic properties of selenium in mice. J Nutr 113: 2147–2154, 1983
40. Greeder GA, Milner JA: Factors influencing the inhibitory effect of selenium on mice inoculated with Ehrlich ascites tumor cells. Science 209: 825–827, 1980
41. Medina D, Oborn CJ: Differential effects of selenium on the growth of mouse mammary cells in vitro. Cancer Lett 13: 333–344, 1981
42. Yan L, Boylan LM, Spallholz JE: Effect of dietary selenium and magnesium on human mammary tumor growth in athymic nude mice. Nutr Cancer 16: 239–248, 1991
43. Cao S, Durrani FA, Rustum YM: Selective modulation of the therapeutic efficacy of anticancer drugs by selenium containing compounds against human tumor xenografts. Clin Cancer Res 10: 2561–2569, 2004
44. Gottardis MM, Robinson SP, Satyaswaroop PG, Jordan VC: Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res 48: 812–815, 1988
45. Johnston SR, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M: Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Res 55: 3331–3338, 1995
46. McKeage K, Curran MP, Plosker GL: Fulvestrant: a review of its use in hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression following antiestrogen therapy. Drugs 64: 633–648, 2004

Address for offprints and correspondence: Brian G. Rowan, Department of Structural & Cellular Biology, Tulane University School of Medicine, Box SL49, 1430 Tulane Avenue, New Orleans, LA 70112; *Tel.:* 504-988-1365; *Fax:* 504-988-1687; *E-mail:* browan@tulane.edu