Methylseleninic acid (MSA) inhibits 17 β -estradiol-induced cell growth in breast cancer T47D cells via enhancement of the antioxidative thioredoxin/thioredoxin reductase system

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ABSTRACT

The purpose of this study was to clarify the cell growth inhibitory mechanism of human breast cancer cells caused by selenium (Se) compounds. In the presence of 17 β -estradiol (E₂) at physiological concentrations, growth of estrogen receptor α (ER α)-positive T47D cells was markedly inhibited by 1×10^{-6} mol/L methylseleninic acid (MSA) with no Se related toxicity. Under conditions where cell growth was inhibited, MSA decreased ER α mRNA levels and subsequent protein levels; further decreasing expression of estrogen-responsive finger protein (Efp) which is a target gene product of ER α and promotes G2/M progression of the cell cycle. Therefore, the decline in Efp expression is presumed to be involved in G2 arrest. Coincidentally, the antioxidative thioredoxin/thioredoxin reductase (Trx/TrxR) system in cells was enhanced by the synergistic action of E₂ and MSA. It has been reported that ROS-induced oxidative stress enhanced ER α expression. E₂ increased production of intracellular ROS in T47D cells. Meanwhile, MSA significantly decreased E₂-induced ROS accumulation. From these results, activation of the Trx/TrxR system induced by the coexistence of MSA and E₂ suppresses oxidative stress and decreases expression of ER α , and finally induces the growth arrest of T47D cells through disruption of ER α signaling.

Breast cancer, the most common cancer in women, is classically an hormone-dependent cancer. Prolonged exposure to estrogen is a major risk for breast cancer (15). In Japan, the incidence of breast cancer among women has increased over the past three decades (10, 34). The Monitoring of Cancer Incidence in Japan (MCIJ) estimated that more than 49,000 Japanese women were diagnosed with breast cancer in 2006 (20).

Estrogen such as 17 β -estradiol (E₂) is known to

promote cell growth of breast cancer through binding to its cognate receptor, estrogen receptors (ERs) (18, 28, 29, 36, 40). At present, there are at least two different forms of ERs, usually referred to as ER α and ER β (39). Approximately two-thirds of all breast cancers express the ER α protein at the time of diagnosis (4). ER α is a ligand-transcriptional factor that belongs to the nuclear steroid/thyroid hormone receptor family (6). The classic mechanism of action of ER α has been proposed as follows: Upon binding of E₂, ER α undergoes conformational changes. Following dimerization, ER α binds to estrogen response elements (ERE) located in the promoter regions of estrogen-regulated genes to modulate expression of genes related to estrogen-responsive cell proliferation (39). In addition, ER α can mediate transcription of other target genes,

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through protein-protein interactions with other transcriptional factors such as activator protein-1 (AP-1) (13, 22). ER α signaling is closely related to cell growth of ER α -positive breast cancer cells.

Oxidative stress induced by reactive oxygen species (ROS) has been reported to modulate ER α expression in various cell lines. In human breast cancer MCF-7 cells, H₂O₂ increased ER α protein levels (37). Endogenous E₂ undergoes oxidative metabolism and generates ROS that include a number of chemically reactive oxygen derivatives such as the superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and the hydroxyl radical (OH[•]) (22).

The essential micronutrient selenium (Se) has beneficial effects in inhibiting cancer growth. Several clinical trials using Se-enriched yeast showed the protective effects of Se against prostate, lung, and colon cancers (3, 5). These trials failed to detect a significant change in breast cancer risk. One reason might be attributable to the fact that there were too few breast cancer cases in the cohorts to provide sufficient statistical power for a conclusive analysis of the data. Previous reports, however, showed the efficacy of Se chemoprevention in animal mammary tumor models (21) and human breast cancer cell lines (31). In ER α -positive MCF-7 breast cancer cells, methylseleninic acid (CH₃SeO₂H, abbreviated as MSA) inhibited cell growth and ER α -mediated gene transcription by decreasing ER α mRNA levels and subsequent protein levels (33). The disruption of ER α signaling induced by MSA is likely a major mechanism of MCF-7 cell growth inhibition. However, the mechanism that MSA uses to suppress ER α expression is still unknown. It is also unclear how Se compounds affect the growth of other breast cancer cells with varying ER α expression in the presence of E₂.

In the present study, we first investigated the growth inhibitory effect of various Se compounds against ER α -positive T47D cells derived from human mammary gland ducts. We found that MSA at 1×10^{-6} mol/L strongly inhibited E₂-induced cell growth in comparison with other selenium compounds. MSA was accompanied by decreases in ER α mRNA levels and resulting protein levels, and finally caused cell cycle arrest as a result of disruptions in ER α signaling. We further investigated the mechanism by which MSA decreases expression levels of the ER α protein. Our results suggested that MSA decreased E₂-induced intracellular ROS levels through activation of the thioredoxin/thioredoxin reductase (Trx/TrxR) system, but not the glutathione/glutathione peroxidase 1 (GSH/GPx1) system, and

inhibited ER α expression.

MATERIALS AND METHODS

Chemicals. Sodium selenite (Na₂SeO₃), sodium selenide (Na₂Se), selenocysteine, selenocystamine, and MSA were obtained from Sigma-Aldrich Co. LLC. (St Louis, MO). Selenomethionine and Se-methylselenocysteine were purchased from Acros Organics (Geel, Belgium). Sodium selenite (Na₂SeO₄) and sodium dioxide (SeO₂) were from Kanto Chemical Co. Inc. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Antibodies. Antibodies against ER α were purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan), and TrxR-1 and Trx were from LabFrontier (Seoul, Korea). Efp and β -actin were obtained from BD Biosciences (Franklin Lakes, NJ) and Novus Biologicals, LLC (Littleton, CO), respectively. HRP-conjugated secondary antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA).

Removal of sex steroids in serum. We removed sex steroids from fetal bovine serum (FBS) by charcoal-dextran stripping (23). Briefly, a suspension of 5% charcoal with 0.5% dextran was prepared. Aliquots of charcoal-dextran suspension of a volume similar to the serum aliquot to be processed were centrifuged at $1,000 \times g$ for 10 min. Supernatants were aspirated, and serum aliquots were mixed with charcoal pellets. This charcoal-serum mixture was maintained in suspension by rolling at 6 cycles/min at 37°C for 1 h. The suspension was centrifuged at $1,000 \times g$ for 20 min, and the supernatant was then filtered through a 0.20 μ m filter. Charcoal dextran-treated serum (CDFBS) was stored at -80°C until needed.

Cell lines and culture conditions. Human breast cancer T47D cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% FBS and maintained in an atmosphere of 5% CO₂ in a 37°C humidified incubator.

DNA determination by ethidium bromide fluorescence method. Cells were plated in 96-well plates (Nunc A/S, Roskilde, Denmark) at initial concentrations of 2.5×10^4 cells/well in 5% CDFBS-supplemented phenol red-free DMEM. Cells were allowed to attach for 24 h, and the different concentrations of E₂ and Se compounds were added. After 0–4

days culture, total DNA amounts in growth cells were determined by the ethidium bromide fluorescence method (32). Plates were centrifuged at 4°C, $320 \times g$ for 15 min and supernatants were removed. Cells were washed once with 200 μ L of PBS and 200 μ L of 0.05% SDS was added and left for 30 min to lyse the cells. Then, 100 μ L of 50 mg/mL ethidium bromide was added to each well, left for 30 min, and DNA-ethidium bromide complex was determined fluorometrically at Ex. 515 nm and Em. 620 nm as an indication of cell numbers.

Semi quantitative reverse transcription-polymerase chain reaction (RT-PCR). Cells were seeded in a 90 mm tissue culture dish (BD Falcon, Durham, NC) at an initial concentration of 4×10^6 cells/dish in 5% CDFBS-supplemented phenol red-free DMEM. At 24 h after seeding, cells were exposed to 1×10^{-9} mol/L E_2 and 1×10^{-6} mol/L MSA for 4 days. Total RNA from cells was isolated using TRIzol Reagent (Life Technologies Co., Gaithersburg, MD) according to the manufacturer's protocols and quantified spectrophotometrically. Total RNA (1 μ g) of each sample was reverse-transcribed using the SuperScript First Standard System (Life Technologies Co.) according to the manufacturer's instructions. The primers for amplification during PCR were designed according to a previously published report (19). The following primer pairs were used to amplify ER α and β -actin respectively: (sense) 5'-TACTGCATCAGATCCAAGGG-3'/(antisense) 5'-ATCAATGGTGCACCTGGTTGG-3'; (sense) 5'-GTGGGGCGCCCCAGGCACCA-3'/(antisense) 5'-CTTCCTTAATGTCACGCACGATTTC-3'. For RCRs, 1 μ L of cDNA, 8 μ L of 25 mmol/L $MgCl_2$, 8 μ L of 2.5 mmol/L dNTP mix, 10 μ L of $10 \times$ Ex Taq buffer solution (Takara Shuzo Co. Ltd., Shiga, Japan), 0.5 μ L of 5 U/ μ L Ex Taq DNA polymerase (Takara Shuzo Co. Ltd.), 1 μ L of 50 μ mol/L gene specific primers as described above, and were mixed with 70.5 μ L of DEPC- H_2O in a 200 μ L PCR tube. PCR was performed with the PCR thermal cycler MP (Takara Shuzo Co. Ltd.). Cycling conditions for ER α were carried out with the first denaturation step at 95°C for 6 min and 94°C for 1 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and then a final extension step at 72°C for 10 min. Cycling conditions for β -actin were carried out with the first denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 45 s, 60°C for 45 s, 72°C for 45 s, and then a final extension step at 72°C for 10 min. PCR products were electrophoresed on a 2% agarose gel,

visualized with ethidium bromide, and the intensity of each band was quantitated with Light-Capture and a CS Analyzer (Atto Co., Tokyo). The RT-PCR signal obtained for each gene was normalized to that for β -actin.

Western blot. Cells were seeded in a 90 mm dish at an initial concentration of 4×10^6 cells/dish in 5% CDFBS-supplemented phenol red-free DMEM. Cells were allowed to attach for 24 h, and then both 1×10^{-9} mol/L E_2 and 1×10^{-6} mol/L MSA were exposed for 4 days. For extraction of total cell proteins, cells were washed with precooled PBS and subsequently lysed in precooled lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride, 0.11 unit/mL trypsin inhibitor). Cell lysis was performed on ice for 30 min and was centrifuged at 4°C, $18,500 \times g$ for 30 min, and supernatants were obtained. Protein concentrations of supernatants were measured using a BCA protein assay reagent kit (Thermo Fisher Scientific K.K., Yokohama, Japan). Aliquots of protein extract and $2 \times$ Leammli sample buffer were mixed and boiled for 5 min. Aliquots of protein solution were analyzed on 2% polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes. For immunoblotting, PVDF membranes were incubated with specific antibodies recognizing target proteins for 2 h at room temperature. Membranes were then incubated with HRP-conjugated secondary antibody for 1 h at room temperature and subsequently analyzed by enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and visualized by Light-Capture. The intensity of each band was quantitated with a CS Analyzer. β -Actin was used as a loading control.

Enzyme activity. Cells were seeded in a 90 mm dish at an initial concentration of 4×10^6 cells/dish in 5% CDFBS-supplemented phenol red-free DMEM. At 24 h after seeding, cells were exposed to 1×10^{-9} mol/L E_2 and 1×10^{-6} mol/L MSA for 4 days. Cultured cells were washed once with PBS after centrifugation at $900 \times g$ for 10 min at 4°C. Cells were suspended in 0.2% Triton-X 50 mmol/L phosphate buffer (pH 7.0), followed by one cycle of freezing and thawing. After centrifugation at $13,000 \times g$ for 15 min at 4°C, the supernatant was dialyzed with 8,000 M.W.-cut-off membrane against 50 mmol/L phosphate buffer (pH 7.0) to remove endogenous thiol substances.

GPx1 activity was determined according to the

coupled enzymatic method with a slight modification (42). The assay mixture contained 0.2 mmol/L NADPH, 0.5 mmol/L EDTA, 0.2 mmol/L NaN_3 , 2 mmol/L GSH, 1 unit/mL of GSH reductase, 10 mmol/L phosphate buffer (pH 7.0), and 0.015 mmol/L hydrogen peroxide as the substrate in a total volume of 1 mL including a dialyzed sample. GPx1 activity was calculated by measuring absorbance at 340 nm. Protein was determined using a BSA protein assay reagent kit.

To measure TrxR activity, the dialyzed sample was heated at 55°C for 10 min to inactivate heat-labile thiol-reduction enzymes. The sample was cooled and centrifuged at $13,000 \times g$ for 30 min at 4°C. TrxR activity was determined by a spectrophotometric insulin-reduction assay (9, 11). The assay mixture contained 0.8 mmol/L NADPH, 0.34 mmol/L insulin, 4.5 mmol/L EDTA-2Na, 90 mmol/L HEPES buffer (pH 7.0), and with or without 4.5 $\mu\text{mol/L}$ *E. coli* Trx in 200 μL . The mixture was started by addition of 100 μL of the supernatant sample at 37°C, incubated for 60 min, and stopped by the addition of 750 μL of 0.3 mg/mL DTNB-6 mol/L guanidine hydrochloride-0.2 mol/L Tris-HCl (pH 8.0). TrxR activity was calculated by measuring absorbance at 412 nm. Protein was determined using a BSA protein assay reagent kit.

Determination of GSH. GSH content was determined by HPLC using SBD-F as a fluorescent reagent according to the method described in a previous report (38).

Determination of ROS by flow cytometry. Cells were seeded in a 90 mm dish at an initial concentration of 4×10^6 cells/dish in 5% CDFBS-supplemented phenol red-free DMEM. At 24 h after seeding, 1×10^{-9} mol/L E_2 and 1×10^{-6} mol/L MSA were exposed for 0, 6, 12, and 24 h. Intracellular ROS levels were detected by flow cytometry using carboxy dichlorofluorescein diacetate (carboxy DCFH-DA) as described previously (2). Cells (10,000 cells measured) were analyzed in a flow cytometer (JASCO Co., Tokyo).

Statistical analysis. Statistical analyses were carried out using Origin 7.5 software (OriginLab, USA). A student's *t*-test was used to determine the significance between treatments and untreated controls, and $P < 0.05$ was considered significant.

RESULTS

Effect of Se compounds on T47D cell growth in the presence of E_2

The inhibitory effects of Se compounds on the growth of ER α -positive T47D cells were investigated. E_2 promoted the growth of T47D cells in a concentration-dependent manner. At 4 days, a physiological concentration of 1×10^{-9} mol/L E_2 increased the cell number to about 3-fold of untreated controls. Meanwhile, Na_2SeO_3 , selenomethionine or MSA at each concentration of 1×10^{-6} mol/L significantly inhibited E_2 -induced cell number accumulation in the presence of E_2 (Table 1). Because MSA strongly inhibited E_2 -induced cell growth, we chose MSA to further elucidate the inhibitory mechanism of cell growth. As shown in Fig. 1, MSA inhibited

Table 1 Effect of various Se compounds on the growth of E_2 -induced T47D cells

Se compounds (1×10^{-6} mol/L)	Cell growth rate (%)
Control	100.0 $\pm$ 1.0
Na_2SeO_3	82.7 $\pm$ 1.1*
Na_2SeO_4	90.3 $\pm$ 11.2
Na_2Se	99.2 $\pm$ 7.5
SeO_2	100.8 $\pm$ 11.3
Selenomethionine	79.9 $\pm$ 4.5*
Se-Methylselenocysteine	99.2 $\pm$ 6.8
Selenocystine	98.2 $\pm$ 8.8
Selenocystamine	90.1 $\pm$ 8.1
MSA	61.2 $\pm$ 8.1*

E_2 was exposed at the concentration of 1×10^{-9} mol/L.

Values are mean $\pm$ SD ($n = 6$).

*Significantly different from the control ($P < 0.01$).

Fig. 1 Dose-response effect of MSA on E_2 -induced cell growth of T47D cells. Values are mean $\pm$ SD ($n = 4-5$). E_2 was exposed at 1×10^{-9} mol/L. * Significantly different from the E_2 -induced control group ($P < 0.01$). † Significantly different from the untreated control group ($P < 0.01$).

E_2 -induced cell growth in a concentration-dependent manner. No effect on cell growth was seen up to a concentration of 1×10^{-7} mol/L. Beyond this concentration, MSA significantly inhibited cell growth in the presence of E_2 . After treatment with greater than 1×10^{-5} mol/L MSA, a strong inhibitory effect against T47D cells was observed regardless of the existence of E_2 . This effect was presumed to be mediated by Se related toxicity. Interestingly, MSA at a concentration of 1×10^{-6} mol/L specifically inhibited cell growth in the presence of E_2 , and exhibited no Se-induced toxicity. From these results, inhibition caused by MSA at a concentration of 1×10^{-6} mol/L may be closely related to the mechanism of E_2 -dependent cell growth.

Expression of ER α mRNA and protein by treatment with E_2 plus MSA

To determine whether ER α is related to the inhibition of cell growth caused by MSA, expression of ER α in T47D cells was measured by RT-PCR and Western blotting. As shown in Fig. 2, 4 days after treatment with 1×10^{-9} mol/L E_2 , expression levels of ER α mRNA and ER α protein increased to 160% and 165% of untreated controls, respectively. These results suggest that the increase in ER α expression plays key roles in E_2 -dependent cell growth. On the

other hand, the combination of MSA, at a concentration of 1×10^{-6} mol/L, with E_2 did not increase expression levels of ER α mRNA or ER α protein over that of untreated controls (Fig. 2). Furthermore, it was recognized that MSA at concentrations between 1×10^{-7} and 1×10^{-6} mol/L decreased expression of ER α protein in a dose-dependent manner (data not shown). It was also found that MSA-induced inhibition of cell growth recovered by about 20% in T47D cells knocked down for ER α by siRNA in the presence of E_2 . Expression levels of estrogen-responsive finger protein (Efp), a cell cycle-related gene modulated by ER α , that increased to approximately 1.6-fold in cells exposed to E_2 was suppressed by MSA treatment. These results indicated that MSA may cause the disruption in ER α signaling through decreases in ER α mRNA levels and subsequent protein levels. It is also suggested that cell growth inhibition induced by MSA is related to cell cycle arrest.

Induction of oxidative stress defense system by treatment with E_2 plus MSA

To clarify whether resistance to oxidative stress induced by MSA was related to inhibition of ER α expression, we investigated the activation of antioxidative enzymes such as GPx1 and TrxR by treat-

Fig. 2 Effect of E_2 and MSA on ER α mRNA levels (A) and ER α protein levels (B) in T47D cells. E_2 and MSA were exposed at concentrations of 1×10^{-9} mol/L and 1×10^{-6} mol/L, respectively. Relative expression of ER α mRNA and ER α protein of untreated controls was taken as 100%, respectively. Values are mean $\pm$ SD ($n = 3-5$).

ment with E_2 and MSA. In T47D cells, GPx1 protein expression and GPx1 activity were hardly detected regardless of treatment with E_2 or MSA. Additionally, there was little content of GSH as a coupling factor of GPx1 (26 ± 2 nmol/mg protein). On the other hand, as shown in Fig. 3, MSA significantly increased TrxR protein levels. MSA also increased enzyme activity of TrxR (Fig. 4). In cells exposed to E_2 , as shown in Fig. 5, Trx protein levels increased to about 1.5-fold of untreated controls. These results suggest that the Trx/TrxR system plays an important role in the resistance to oxidative stress in T47D cells exposed to both E_2 and MSA.

Fig. 3 Effect of E_2 and MSA on TrxR protein levels in T47D cells. E_2 and MSA were exposed at concentrations of 1×10^{-9} mol/L and 1×10^{-6} mol/L, respectively. Relative expression of TrxR protein of untreated controls was taken as 100%. Values are mean $\pm$ SD ($n = 3-5$).

Fig. 4 Effect of E_2 and MSA on TrxR activity in T47D cells. E_2 and MSA were exposed at 1×10^{-9} mol/L and 1×10^{-6} mol/L, respectively. Values are mean $\pm$ SD ($n = 3-5$).

Suppression of intracellular ROS generation by MSA

We next examined whether the amount of intracellular ROS generated by E_2 metabolism was scavenged by MSA treatment. As shown in Fig. 6, intracellular ROS content in T47D cells after 12 h and 24 h treatment of 1×10^{-9} mol/L E_2 was 1.2- and 1.5-fold higher than that of untreated controls, respectively. MSA markedly decreased intracellular ROS content induced by E_2 treatment (Fig. 6). These results suggest that MSA scavenges E_2 -induced ROS in T47D cells through an enhanced antioxidant Trx/TrxR system.

DISCUSSION

As a first step toward elucidation of the mechanisms important for the anticancer activity of Se compounds, we evaluated the growth inhibitory effects of 9 kinds of commercial Se compounds against ER α -positive T47D human breast cancer cells in the presence of E_2 . Previous studies have shown that organic Se compounds provide beneficial inhibitory effects on breast cancer cell growth *in vitro*. Redman *et al.* (31) reported that selenomethionine inhibited growth of ER α -positive MCF-7 cells at concentrations greater than 1×10^{-5} mol/L. MSA was able to suppress growth of MCF-7 cells at concentrations of 5×10^{-6} and 1×10^{-5} mol/L, whereas Se-methylselenocysteine had no effect (33). MSA

Fig. 5 Effect of E_2 and MSA on Trx protein levels in T47D cells. E_2 and MSA were exposed at 1×10^{-9} mol/L and 1×10^{-6} mol/L, respectively. Relative expression of Trx protein of untreated controls was taken as 100%. Values are mean $\pm$ SD ($n = 3-5$).

Fig. 6 Effect of E_2 and MSA on intracellular ROS production in T47D cells. E_2 and MSA were exposed at 1×10^{-9} mol/L and 1×10^{-6} mol/L, respectively. Relative ROS content of untreated controls was taken as 1.0. Values are mean $\pm$ SD ($n = 3-4$).

also inhibited growth of noncancerous cells, MC-F10AT1 and MCF10AT3B breast epithelial cells, at a concentration range between 2.5×10^{-6} and 1×10^{-5} mol/L. However, these doses are slightly higher than human blood levels noted in individuals receiving dietary Se supplements. Moreover, there is a possibility that the response to cell growth inhibition differs with Se forms and cell lines. Previous reports imply that the mechanism of growth inhibition by MSA between T47D cells and other ER α -positive breast cancer cells may be different. Until now, the inhibitory effect of various Se compounds against T47D cells is not clear. Our data show that Na_2SeO_3 , selenomethionine and MSA were sufficient to inhibit E_2 -dependent cell growth of T47D cells at a concentration of 1×10^{-6} mol/L (Table 1). In contrast, no growth inhibitory effects were observed after treatment with other Se compounds at 1×10^{-6} mol/L in the presence of E_2 . Interestingly, the inhibitory action of MSA was stronger than that of both Na_2SeO_3 and selenomethionine. We demonstrated, for the first time, that MSA at 1×10^{-6} mol/L specifically inhibited only E_2 -induced cell growth (Fig. 1). These results suggest that the inhibitory action of MSA at 1×10^{-6} mol/L may be closely related to E_2 -induced cellular proliferation.

E_2 is known to diffuse across the cell membrane and bind to ERs. ER α and ER β show different tissue distributions (26), affinity for coactivators (1), and responses to hormones (25). It is generally recognized that E_2 transcriptional activities implicated

in breast cancer cell proliferation are mediated through ER α (35, 41). A very small percentage of epithelial cells in the normal, adult mammary gland are proliferating and these proliferating cells express very little or no ER α . In contrast, epithelial breast cancer cells are ER α positive. The ER α signaling pathway plays an important role in promoting growth of ER α -positive breast cancer cells. ER α regulates the transcription of various genes as follows: The E_2 -ER α complex binds to a specific DNA sequence ERE present in target genes and rapidly induces transcription, which may, in turn, lead to activation of other downstream genes (16). Alternatively, the E_2 -ER complex can interact with other DNA-bound transcription factors that in turn bind DNA sequences (e.g. via AP-1, SP1 complex) (17, 30). Shah *et al.* (33) reported that MSA disrupted ER α signaling in MCF-7 cells. Efp, a target gene product of ER α , was expressed in human breast cancer cells and E_2 -induced expression of Efp was only found in MCF-7 cells until now (12). Orimo *et al.* (24) suggested that Efp is essential for estrogen induced cell growth. Efp is a RING-finger-dependent ubiquitin ligase (E3) that targets proteolysis of 14-3-3 σ , a negative cell cycle regulator that causes G2 arrest. (8). In our present study using T47D cells, MSA significantly blocked E_2 -dependent expression of ER α (Fig. 2), and then reduced expression of Efp. Therefore, we speculate that MSA causes G2 arrest through disruption of the ER α signaling pathway in T47D cells.

Fig. 7 Proposed suppression mechanism of E₂-induced T47D cell growth by MSA. E₂: 17β-estradiol, CE₂-Q: catechol E₂ quinone, CYP: cytochrome P-450, ERα: estrogen receptor α, Efp: estrogen receptor finger protein, ROS: reactive oxygen species, MSA: methylseleninic acid, Trx: thioredoxin, TrxR: thioredoxin reductase. $\Rightarrow$: Promotion, $\square$: Inhibition

In addition to inducing cellular proliferation, E₂ is also known to increase cell survival by upregulating the antiapoptotic factor bcl-2 (27) and downregulating several proapoptotic factors (7). Meanwhile, a previous report demonstrated MSA-induced growth inhibition in cell lines via induction of apoptosis (14). In the presence of E₂, induction of apoptosis was hardly observed in T47D cells exposed to MSA at 1×10^{-6} mol/L (data not shown). These results suggest that E₂-induced cell growth inhibited by MSA was due to arrest of the cell cycle rather than promotion of apoptotic cell death.

MSA markedly decreased ERα expression in T47D cells exposed to E₂ at a physiological concentration (Fig. 2), but the underlying mechanism has been unknown until now. Physiologically achievable concentrations of E₂ generate ROS such as O₂^{•-}, H₂O₂, and OH[•] via metabolism in cells (22). ROS have been reported to modulate ERα expression: low concentrations of H₂O₂ increased ERα protein levels in MCF-7 cells and T47D cells (37). This time, intracellular ROS content increased 12 h and 24 h after treatment with E₂ at a physiological concentration (Fig. 6). However, coexistence of MSA decreased E₂-induced intracellular ROS amounts to untreated control levels (Fig. 6). GSH, a tripeptide thiol of cysteine-glutamine-glycine, and Trx, a small protein that has two cysteines in its active site, are responsible for the antioxidant defense of cells. It has been reported that GSH content in MCF-7 cells was about 75 nmol/mg protein (43). In contrast, GSH content in T47D cells was 2.6 ± 0.2 nmol/mg protein. Expression and activity of GPx1 were not

detected in T47D cells regardless of E₂ and/or MSA exposure. These results suggest that the GSH/GPx1 system hardly functions as a scavenger for H₂O₂ in T47D cells. It is also known that TrxR is a key player in regulation of the redox state in cells. Oxidized Trx is inactive, but is converted to the active reduced form by TrxR. Trx binds ROS and protects cells against oxidative stress. In T47D cells, MSA at 1×10^{-6} mol/L significantly increased not only expression of the TrxR protein, but also enzyme activity of TrxR (Fig. 3 and 4). Moreover, E₂ at a physiological concentration caused increases in Trx protein levels (Fig. 5). These findings suggest that the Trx/TrxR system is effectively activated by the synergistic action of E₂ and MSA, and plays a central role in defense against oxidative stress in T47D cells.

In conclusion, we show that MSA exerted strong antiproliferation activity against ERα-positive T47D cells. It is possible that MSA-mediated growth arrest is mediated in part through disruption of estrogen signaling in ERα-positive T47D cells. We speculate that one of the inhibitory mechanisms of ERα expression is as follows (Fig. 7). First, in the presence of E₂, MSA effectively enhances the oxidative stress defense system, the Trx/TrxR system, which increases reduced Trx. The reduced Trx scavenges intracellular ROS generated by E₂ metabolism, resulting in suppression of ERα expression in ERα-positive cells. Thus, decreases in ERα protein levels cause the disruption of ERα signaling, and inhibits G2/M cell cycle progression through decreases in Efp expression.

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