Selenocystine Induces S-Phase Arrest and Apoptosis in Human Breast Adenocarcinoma MCF-7 Cells by Modulating ERK and Akt Phosphorylation

TIANFENG CHEN[†] AND YUM-SHING WONG*,[†,§]

State Key Laboratory (China) for Agrobiotechnology and Food and Nutritional Sciences Programme, Department of Biology, The Chinese University of Hong Kong, Hong Kong, China

Selenocystine (SeC) is a nutritionally available selenoamino acid with selective anticancer effects on a number of human cancer cell lines. The present study shows that SeC inhibited the proliferation of human breast adenocarcinoma MCF-7 cells in a time- and dose-dependent manner, through the induction of cell cycle arrest and apoptotic cell death. SeC-induced S-phase arrest was associated with a marked decrease in the protein expression of cyclins A, D1, and D3 and cyclin-dependent kinases (CDKs) 4 and 6, with concomitant induction of p21waf1/Cip1, p27Kip1, and p53. Exposure of MCF-7 cells to SeC resulted in apoptosis as evidenced by caspase activation, PARP cleavage, and DNA fragmentation. SeC treatment also triggered the activation of JNK, p38 MAPK, ERK, and Akt. Inhibitors of ERK (U0126) and Akt (LY294002), but not JNK (SP600125) and p38 MAPK (SB203580), suppressed SeC-induced S-phase arrest and apoptosis in MCF-7 cells. The findings establish a mechanistic link between the PI3K/Akt pathway, MAPK pathway, and SeC-induced cell cycle arrest and apoptosis in MCF-7 cells.

KEYWORDS: Selenium; selenocystine; MCF-7 cells; cell cycle arrest; apoptosis; ERK; Akt

INTRODUCTION

Breast cancer is a leading cause of morbidity and mortality in women, in developed and, increasingly, in developing countries (*1*). Recent progress in diagnosis and therapy has increased the survival of women in estrogen-dependent breast cancer. However, the treatment options available for breast cancer are far from satisfactory, which results in poor prognosis (*2*). Administration of naturally occurring and/or synthetic agents could prevent initiation, promotion, and progression events associated with carcinogenesis and may be a direct way to reduce cancer mortality and morbidity (*3*). In breast cancer, the balance between cell proliferation and cell death is lost, which contributes to the increase in cellular mass and tumor progression (*4*). In this regard, there is considerable emphasis in searching for novel agents that selectively activate cell death machinery of breast cancer cells without producing cytotoxic effects on normal cells (*5*).

The trace element selenium is important for the health of humans and animals (*6*). Converging data from epidemiological, preclinical, and clinical studies have implicated Se as an effective chemopreventive and chemotherapeutic agent, particularly for prostate cancer (*7, 8*). Important for breast cancer chemoprevention is the fact that in recent years, some orga-

noselenium compounds have been shown as promising preventive agents for breast cancer (*9–14*). For instance, our previous study showed selenazolo derivatives as potent cytotoxic agents against human breast cancer cells through the induction of mitochondria-mediated apoptosis (*12*). Recent studies have brought a renewed interest in the therapeutic potential of methylseleninic acid, as an enhancer of existing anticancer drugs against breast and prostate cancers (*9, 15*). Several mechanisms have been postulated to elucidate the anticancer activity of Se, such as the induction of cell apoptosis, arrest of cell proliferation, modulation of redox state, detoxification of carcinogen, and inhibition of angiogenesis (*7, 16, 17*). Among these proposed mechanisms, induction of apoptosis and cell cycle arrest have received most of the attention and have been postulated to be critical for anticancer action of Se (*7*). Selenocystine (SeC), a nutritionally available selenoamino acid, has been shown to reduce tobacco-derived nitrosamine-induced lung tumor and enhance hepatic chemoprotective enzyme activities in animal models (*18*). In our previous works, we contrasted the in vitro anticancer activities of SeC with some other selenocompounds and reported that SeC exhibited stronger antiproliferative effect against breast cancer cells through the induction of apoptosis with the involvement of oxidative stress (*13*). However, the molecular mechanisms of the apoptosis-inducing action of SeC remain to be elucidated.

Growing evidence suggests that reactive oxygen species (ROS) play an important role in the anticancer action of Se compounds (*16, 19*). Besides the induction of oxidative damage,

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overproduction of ROS is also able to activate a variety of stress responses, such as stress adaptation, survival, and cell death, through different signaling pathways (*20*). Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways are the major oxidative stress-sensitive signal transduction pathways in most cell types (*21, 22*). MAPKs are important intermediates that convert extracellular signals into intracellular responses (*23*). The PI3K/Akt pathway has been shown to regulate apoptosis in most cell types (*24*). For instance, the PI3K/Akt pathway is frequently activated in breast cancer cells and results in enhanced resistance to apoptosis through multiple mechanisms (*21*). Overproduction of ROS and the resulting cellular redox change have been found as part of the signal transduction pathways leading to apoptosis of MCF-7 cells treated with SeC (*13*). Thus, it is likely that SeC may induce growth inhibition by regulating the MAPK and PI3K/Akt pathways. The objectives of this study were to investigate the inhibitory activity of SeC on MCF-7 cells and to delineate the mechanisms and molecular targets by which SeC inhibited MCF-7 cell growth.

MATERIALS AND METHODS

Materials. Seleno-L-cystine, propidium iodide (PI), SP600125, and bicinchoninic acid kit for protein determination were purchased from Sigma. Caspase-3/7 substrate (Ac-DEVD-AMC) was purchased from Biomol (Germany). SB203580, U0126, and LY294002 were obtained from Calbiochem (San Diego, CA). The BrdU incorporation ELISA kit was purchased from Roche Applied Science (Basel, Switzerland). RPMI 1640 medium was purchased from Invitrogen Corp. (Carlsbad, CA), and fetal bovine serum (FBS) was purchased from Gibco BRL (Gaithersburg, MD). All of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). The ultrapure water used for all experiments was supplied by a Milli-Q water purification system from Millipore.

Cell Culture. The human breast adenocarcinoma MCF-7 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (50 units/mL) at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

Drug Treatment. MCF-7 cells were seeded in 6-well tissue culture plates at 1.2×10^5 cells/well for 24 h. The cells were then incubated with different concentrations of SeC for different periods of time. In some experiments, the cells were pretreated with 20 μM SP600125, SB203580, U0126, and LY294002, alone or in combination, for 1 h prior to the addition of SeC.

MTT Assay. The cell viability was determined by measuring the ability of cells to transform MTT to a purple formazan dye as described previously (*13, 14*). The cells were seeded in 96-well tissue culture plates at 2.5×10^3 cells/well for 24 h. The cells were then incubated with SeC at different concentrations for different periods of time. After incubation, 20 μL/well of MTT solution (5 mg/mL phosphate-buffered saline) was added and incubated for 5 h. The medium was aspirated and replaced with 150 μL/well of DMSO to dissolve the formazan salt formed. The color intensity of the formazan solution, which reflects the cell growth condition, was measured at 570 nm using a microplate spectrophotometer (SpectroAmax 250). The cell viability of treatment groups was expressed as percentage of the control.

Cell Proliferation Assay. The BrdU chemiluminescence assay was employed to determine the incorporation of BrdU into the cellullar DNA, which allows a direct evaluation of cell proliferation at the DNA level. Briefly, MCF-7 cells (2.5×10^3 cells/well) were seeded in 96-well plates and cultured for 24 h. The medium was then replaced by fresh medium (200 μL/well) containing different concentrations of SeC. After incubation in the presence of SeC for 24 h, BrdU (100 μM final concentration) was added to the cells and the incubation continued for an additional 2 h. After labeling solution was removed, the cells were fixed with FixDenat solution for 30 min, followed by incubation with anti-BrdU-POD solution for 90 min at room temperature. The chemiluminescence intensity was assessed using ELISA-BrdU assay kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's protocols. The cell proliferation was expressed as the percentage relative to the control.

Flow Cytometric Analysis. The cell cycle distribution was analyzed by flow cytometry as described previously (*12*). After treatment, the cells were trypsinized, washed with PBS, and fixed with 75% ethanol overnight at −20 °C. The fixed cells were washed with PBS and stained with PI working solution (1.21 mg/mL Tris, 700 U/mL RNase, 50.1 μg/mL PI, pH 8.0) for 4 h in darkness. The DNA contents were analyzed with a Beckman Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). The proportions of cells in G0/G1, S, and G2/M phases were represented as DNA histograms. Apoptotic cells with hypodiploid DNA contents were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10000 events per sample were recorded.

Caspase Activity Assay. The harvested cell pellets were suspended in the cell lysis buffer and incubated on ice for 1 h. After centrifugation at 11000g for 30 min, the supernatants were collected and immediately measured for protein concentration and caspase activity. Briefly, cell lysates were placed in 96-well plates, and then specific caspase-3/7 substrates (Ac-DEVD-AMC) were added. Plates were incubated at 37 °C for 1 h, and caspase activity was determined by measuring the fluorescence intensity with the excitation and emission wavelengths set at 380 and 440 nm, respectively.

Western Blot Analysis. The total cellular proteins were extracted by incubating the cells in the lysis buffer obtained from Cell Signaling Technology. The protein concentrations in the cell lysates were determined by bicinchoninic acid assay (Sigma) according to the manufacturer's protocols. SDS-PAGE was performed in 10% tricine gels with equal amounts of protein loaded per lane. After electrophoresis, the proteins were transferred from the gel to a nitrocellulose membrane, and the membrane was blocked with 5% nonfat milk in TBST buffer for 1 h. The membranes were then incubated with primary antibodies at 1:1000 dilution in 5% nonfat milk overnight at 4 °C, followed by secondary antibodies conjugated with horseradish peroxidase at 1:2000 dilution for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Kodak). The expression levels of proteins were relatively quantified using the Glyko BandScan software, and the expression ratio was calculated according to the reference band of β-actin.

Statistical Analysis. Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± standard deviations (SDs). Statistical analysis was performed using SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL). The difference between two groups was analyzed by two-tailed Student's *t* test, and that between three or more groups was analyzed by one-way analysis of variance multiple comparisons. Differences with $P < 0.05$ (∗) or $P < 0.01$ (∗∗) were considered to be statistically significant.

RESULTS

Inhibition of MCF-7 Cell Proliferation by SeC. The inhibitory effect of SeC on the growth of MCF-7 cells was determined employing a MTT assay. As shown in **Figure 1A**, SeC treatment resulted in a dose- and time-dependent inhibition of cell growth, accounting for 4−35, 9−70, and 15−85% inhibition after 24, 48, and 72 h of treatment with various concentrations of SeC (5−40 μM), respectively. To further investigate the effects of SeC on cell proliferation, we treated the cells with different doses of SeC and analyzed DNA synthesis by the BrdU incorporation assay. As shown in **Figure 1B**, cell proliferation was noticeably inhibited in a dose-dependent manner after 24 h of treatment with SeC. The inhibitory effect of SeC on normal human fibroblast Hs68 cells was also assessed. After 24, 48, and 72 h treatments with SeC

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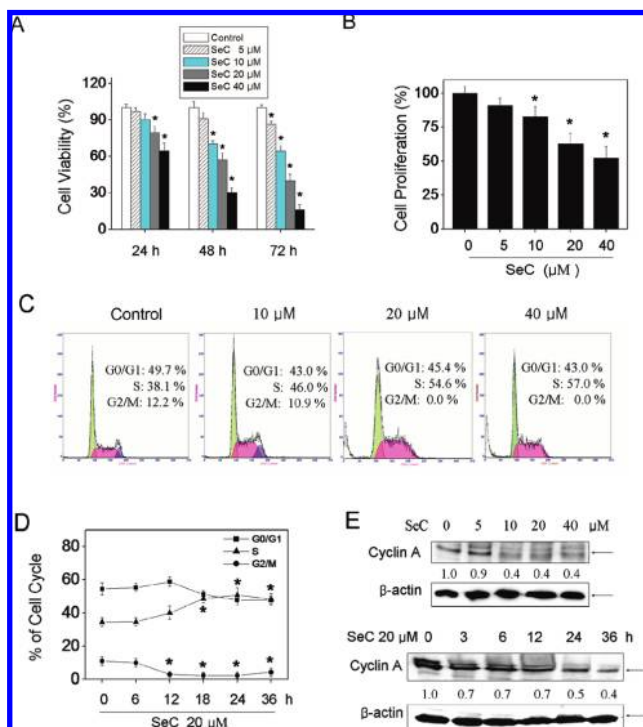
Figure 1. SeC induced S-phase arrest in MCF-7 cells. (**A**) Growth inhibitory effects of SeC. Cells were treated with specified concentrations of SeC for 24, 48, and 72 h, and cell viability was determined by MTT assay as described under Materials and Methods. ∗, $P < 0.05$ versus the control. (**B**) SeC inhibits proliferation of MCF-7 cells. Cells were treated with SeC for 24 h, and cell proliferation was determined by ELISA-BrdU chemiluminescence assay. ∗, $P < 0.05$ versus the control. (**C**) SeC induces S-phase arrest in MCF-7 cells. After treatment of SeC for 24 h, the cells were labeled with PI staining solution and analyzed by flow cytometry. (**D**) Time course analysis of SeC-induced S-phase arrest. MCF-7 cells were treated with 20 μM SeC for various periods of time. The value represents the mean $\pm$ SD of three independent experiments. ∗, $P < 0.05$ versus the control within the same phase. (**E**) Effects of SeC on the expression level of cyclin A in MCF-7 cells. Cells were treated with indicated concentrations of SeC for 24 h or 20 μM SeC for various times. Cell extracts were separated by 10% SDS-PAGE and immunoblotted with specific primary antibodies. The immunoblots were representatives of three independent experiments with similar results.

(5−100 μM), much lower growth inhibitory effects were observed in Hs68 cells (*13*). These results suggested the different susceptibilities of cancer and normal cells to SeC cytotoxicity.

Induction of S-Phase Arrest in MCF-7 Cells by SeC. To elucidate the mechanism of SeC-induced proliferation inhibition, we examined the effect of SeC on cell cycle distribution by flow cytometry. As shown in **Figure 1C**, concomitant with the growth inhibitory effect, SeC treatment induced a strong S-phase arrest in a dose-dependent manner. For instance, cell populations in the G0/G1, S, and G2/M phases were 49.7, 38.1, and 12.2% in control MCF-7 cells. However, after 24 h of incubation with 10, 20, and 40 μM SeC, the S population was noticeably enhanced by 8, 17, and 19%, respectively. This increase in the S-phase cell population was accompanied by a concomitant decrease in the G0/G1 and G2/M phase cell populations. Interestingly, the G2/M population of MCF-7 was markedly reduced after treatment with 20 and 40 μM SeC, indicating the complete blockage of the S-G2 transition in MCF-7 cells. Meanwhile, the sub-G1 population was slightly increased in cells exposed to 40 μM SeC. To study the time course for this S-phase

arrest, we treated MCF-7 cells with 20 μM SeC. Cells were harvested at various time points after the treatment, and the cell cycle profiles were determined by flow cytometry. The results showed that the S population increased as early as 12 h after SeC treatment, peaked at approximately 24 h, and decreased afterward (**Figure 1D**).

As another index for S-phase arrest, we measured the expression level of cyclin A, because the cyclin A−CDK2 complex is the primary regulator of the S-phase progression. As shown in **Figure 1E**, MCF-7 cells exposed to SeC for 24 h exhibited down-regulation of cyclin A expression, which coincided with the SeC-induced cell cycle arrest at the S-phase. A time course study showed that treatment with 20 μM SeC led to an apparent down-regulation of cyclin A expression that started as early as 3 h after treatment (**Figure 1E**).

Effect of SeC on the Expression of Cell Cycle Regulators Involved in S-Phase Arrest. Different regulators working in multiple pathways tightly regulate cell cycle control, such as cyclins, cyclin-dependent kinase (CDKs), CDK inhibitors (CKDIs), and growth suppressor genes. On the basis of an observed S-phase arrest in MCF-7 cells induced by SeC, we assessed the levels of cell cycle regulators associated with this effect. As shown by immunoblot analysis in **Figure 2A**, SeC treatment caused a dose-dependent decrease in the expression levels of cyclin D1, cyclin D3, and cyclin-dependent kinases CDK 4 and CDK 6. SeC treatment also up-regulated the expression level of cyclin E and the phosphorylation of Rb protein, which may contribute to the cell cycle progression from G0/G1 to S-phase. Binding of cyclins to CDKs would form active kinase complexes, which are regulated and inhibited by various CKDIs and growth suppressor genes. We next assessed the effect of SeC treatment on the expression levels of p21waf1/Cip1, p27Kip1, and tumor suppressor gene p53 in MCF-7 cells. As shown by immunoblot analysis in **Figure 2B**, the expression levels of p21waf1/Cip1, p27Kip1, and p53 were up-regulated in a dose-dependent manner by SeC treatment.

Induction of Apoptosis in MCF-7 Cells by SeC. During the cell proliferation assay, we observed that SeC caused a significant decrease in MCF-7 cell growth and proliferation, where higher doses and longer treatment time were more effective. As shown in **Figure 1A**, SeC induced MCF-7 cell death in a dose-dependent manner. Moreover, we confirmed that apoptosis was the major mode of cell death induced by SeC in MCF-7 cells, by measuring three different apoptotic markers, including caspase-3/7 activation, PARP cleavage, and DNA fragmentation (**Figure 3**). To examine whether SeC-induced apoptosis is preceded by S-phase arrest, we compared the time courses of S-phase arrest and apoptosis in MCF-7 cells exposed to SeC. **Figure 1** shows that the S population increased as early as 12 h after SeC treatment. However, an increase in enzymatic activity of caspase-3/7 was evident 36 h after SeC treatment (**Figure 3A**). Consistent with the effect on caspase-3/7 activation, SeC also caused a strong cleavage of PARP, yielding an 89 kDa fragment in cells exposed to SeC for 36 h (**Figure 3B**). As another index of apoptotic cell death, we measured the sub-G1 cell population by flow cytometry. **Figure 3C** shows that SeC induced a time-dependent increase in the sub-G1 cell population, which reached 16.5 and 64.7% after treatment with 20 μM SeC for 24 and 36 h, respectively. These results suggested that SeC triggered S-phase arrest prior to the induction of apoptosis in MCF-7 cells.

Effects of SeC on MAPK Signaling Pathway. Studies have shown that the MAPK signaling pathway plays an important role in the action of chemotherapeutic drugs (*25*). Therefore,

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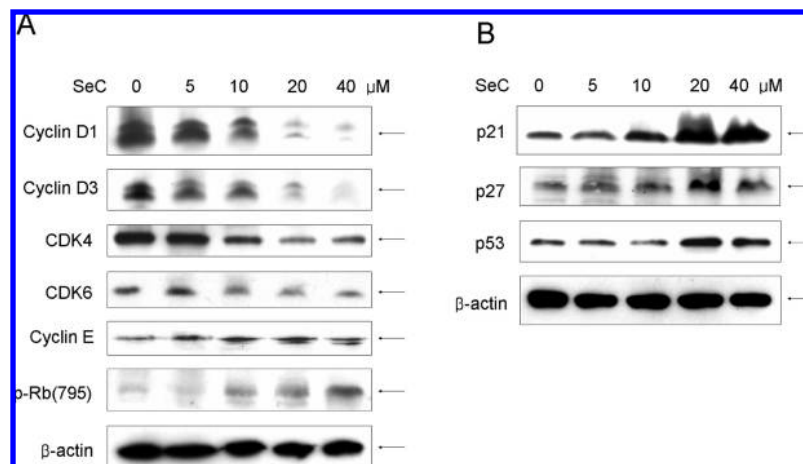
Figure 2. Effects of SeC on the expression levels of (**A**) cyclin D1, cyclin D3, CDK 4, CDK 6, cyclin E, and phosphorylated Rb and (**B**) p21, p27, and p53. Cells were treated with indicated concentrations of SeC for 24 h. Cell proteins (60 μg/lane) were separated by SDS-PAGE and immunoblotted with specific primary antibodies. Equal loading was confirmed by stripping immunoblots and reprobing for β-actin. The immunoblots were representatives of three independent experiments with similar results.
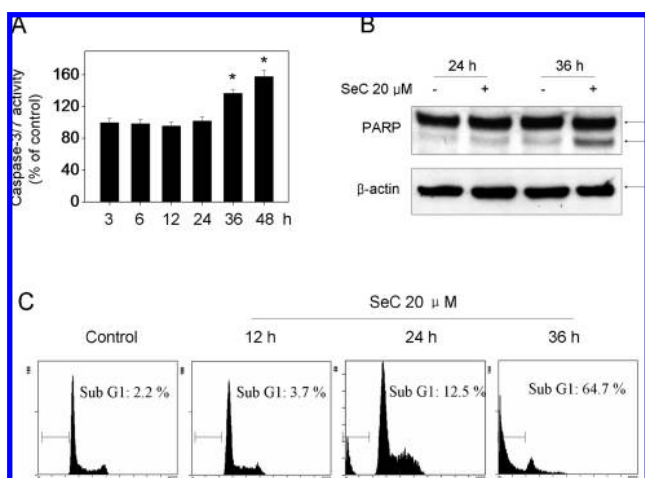

Figure 3. SeC induced apoptosis in MCF-7 cells. (**A**) Activation of caspases by SeC. Cells were treated with 20 μM SeC for various periods of time. Cells were then lysed, and caspase-3/7 activity was determined with a fluorescence assay kit using Ac-DEVD-AMC as the substrate. The value represents the mean ± SD of three independent experiments. ∗, $P < 0.05$ versus the control. (**B**) SeC induced PARP cleavage. MCF-7 cells were treated with 20 μM SeC for 24 and 36 h. Cell lysates were electrophoresed on 8% SDS-PAGE, and full length PARP (115 kDa) and its cleaved form (89 kDa) were detected using the anti-PARP antibody. β-Actin level was measured as the loading control. (**C**) Quantitative analysis of apoptotic cell death induced by SeC by measuring the sub-G1 cell population. Cells treated with SeC for various periods of time were fixed with 70% ethanol, stained with PI, and analyzed by flow cytometry.

we next determined whether the MAPKs were activated in SeC-treated MCF-7 cells by Western blot analysis using specific antibodies against the phosphorylated (activated) forms of the kinases. It was found that SeC treatment induced differential phosphorylation of JNK, ERK, and p38 MAPK in cells exposed to SeC (**Figure 4A**). A time course study (**Figure 4B**) showed that JNK activation displayed a rapid onset after 1 h of treatment, followed by a progressive decline, returning to the basal level after 4 h. Phosphorylation of ERK was detected as a sustained activation from 1 to 12 h, which decreased thereafter and reached the control level at 24 h. Activation of p38 by SeC was also observed as early as 1 h after SeC treatment, which peaked at approximately 12 h and declined from 12 to 24 h. Phosphorylated c-Jun, a target of JNK, was also detected in

SeC-treated cells, confirming the activation of JNK. In contrast, the total protein levels of JNK, ERK, and p38 MAPKs remained constant over the time course (data not shown).

To study the role of MAPK activation in SeC-induced growth inhibition, we examined the effects of specific MAPK inhibitors on overall cell death. The results of the MTT assay showed that pretreatment with SP600125 (a JNK inhibitor) or SB203580 (a p38 inhibitor) had no effect on SeC-induced cell death (**Figure 4C**), although these inhibitors reduced the phosphorylation of their target kinases. The results suggested that JNK and p38 did not play important roles in regulating cell death in MCF-7 cells induced by SeC. However, pretreatment with U0126 (an ERK inhibitor) significantly decreased the extent of cell death induced by SeC (**Figure 4C**). Consistent with these results, microscopic examinations revealed that U0126 effectively inhibited SeC-induced morphological changes indicative of cell death, such as cell shrinkage, cell rounding, and the appearance of apoptotic bodies (**Figure 4D**). These results suggested that activation of the ERK pathway was involved in the apoptotic cell death of MCF-7 cells induced by SeC.

Akt Pathway and SeC-Induced Growth Inhibition. To investigate the possible role of the Akt pathway in SeC-induced cell growth inhibition, we performed time- and dose-dependent experiments to determine the expression and phosphorylation of Akt. As shown in **Figure 5A**, SeC treatment resulted in a decrease of phosphorylated AKT in MCF-7 cells exposed to SeC at concentration >20 μM. A time course study showed that Akt phosphorylation increased rapidly after 1 h of treatment, peaked at approximately 4 h, progressively declined from 8 to 12 h, and almost vanished after 24 h (**Figure 5B**). Total Akt protein level remained constant throughout the course of the experiment. We next investigated whether activation of the Akt pathway was necessary in the cell death induced by SeC. As shown in **Figure 5C**, treatment with a PI3K inhibitor LY294002 (Akt-upstream inhibitor) alone for 24 h showed no significant inhibitory effect on the growth of MCF-7 cells. Pretreatment with LY294002 resulted in a marked increase in cell viability from 60.5% (SeC alone) to 92.0%. Microscopic examinations also revealed the prevention of cell shrinkage, cell rounding, and the appearance of apoptotic bodies in SeC-treated MCF-7 cells by LY294002 (**Figure 5D**). These results indicated that regulation of Akt phoshorylation was associated with SeC-induced cell death.

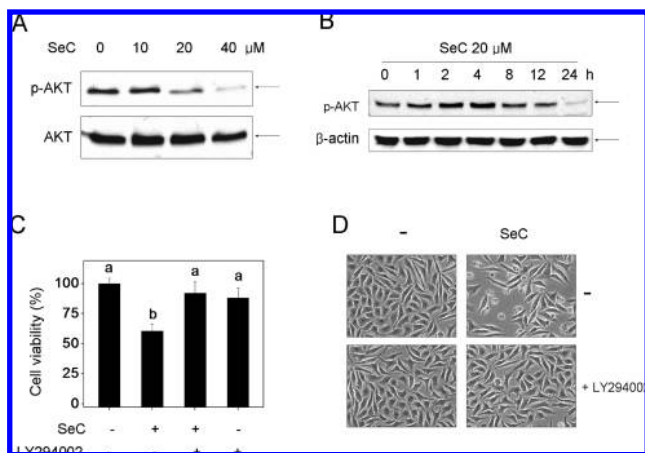**10578** *J. Agric. Food Chem.,* Vol. 56, No. 22, 2008

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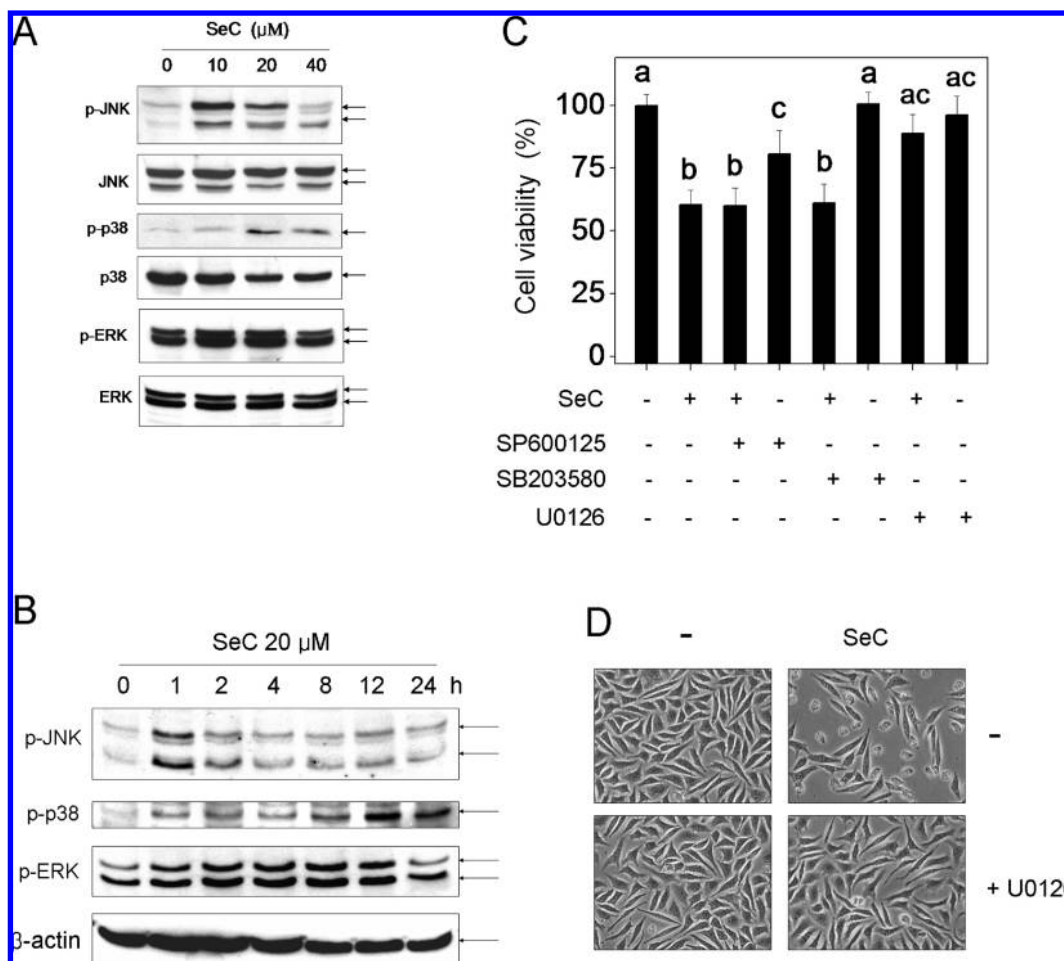
Figure 4. Roles of MAPKs in SeC-induced growth inhibition in MCF-7 cells. (**A**) Effects of SeC on the phosphorylation status and expression levels of MAPKs. Cells were treated with different concentrations of SeC for 24 h. Cell extracts were separated by SDS-PAGE and immunoblotted with specific primary antibodies. The immunoblots were representatives of three independent experiments with similar results. (**B**) Cells were treated with 20 μM SeC for various periods of time. (**C**) Effects of SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor), and U0126 (ERK inhibitor) on SeC-induced growth inhibition. Cells were pretreated with 20 μM concentrations of different inhibitors 1 h prior to the treatment with 20 μM SeC for 24 h (total inhibitor exposure time was 25 h). Cell viability was determined by the MTT assay as described under Materials and Methods. Bars with different characters are statistically different at the $P < 0.05$ level. (**D**) Morphology of MCF-7 cells under different treatments (magnification, 200×). U0126, pretreatment with 20 μM U0126 for 1 h; SeC, treatment with 20 μM SeC for 24 h.

Attenuation of SeC-Induced S-Phase Arrest and Apoptosis by Akt and ERK Inhibitors. We next examined the effects of PI3K inhibitor LY294002 and MEK inhibitor U0126, alone or in combination, on the overall cell death, S-phase arrest, and apoptosis induced by SeC. As shown in **Figure 6A**, pretreatment with LY294002 in combination with U0126 significantly suppressed SeC-induced cell growth inhibition in a dose-dependent manner. In addition, we found that LY294002 and U0126, alone or in combination, almost completely prevented the SeC-induced apoptosis as detected by flow cytometry (**Figure 6B**). Consistent with these results, pretreatment with LY294002 and U0126 effectively blocked the SeC-induced caspase-3/7 activation (data not shown). Moreover, it was also found that LY294002 and U0126 effectively blocked the SeC-induced S-phase arrest (**Figure 6C**). These results suggested the important roles of ERK and AKT pathways in SeC-induced cell cycle arrest and apoptosis.

DISCUSSION

Because of unsatisfactory treatment options for breast cancer, there is a need to develop novel preventive approaches for this malignancy. One such strategy is through chemoprevention by

the use of nontoxic naturally occurring and/or synthetic agents. Organoselenium compounds have been implicated as a promising chemopreventive agent for breast cancer (*9−13*). SeC, a nutritionally available selenocompound, has been shown as a novel chemopreventive agent against human breast cancer cells through induction of apoptosis (*13*). ROS production and the resulting cellular redox change induced by SeC have been found as part of the signal transduction pathways leading to cell apoptosis (*13*). Different susceptibilities of cancer and normal cells to the cytotoxic action of SeC could be ascribed to their different sensitivities to the formation and action of ROS (*13*). MAPK and PI3K/Akt pathways are the major oxidative stress-sensitive signal transduction pathways in most cell types (*21, 22*). Thus, the present study characterized the effects of SeC on cell cycle arrest and apoptosis signaling with respect to PI3K/AKT and MAPK pathways in MCF-7 cells. It showed that SeC caused S-phase arrest via the inhibition of cyclins and CDKs together with the induction of p21waf1/Cip1, p27Kip1, and p53. Furthermore, SeC caused the onset of apoptosis in MCF-7 cells with the involvement of caspase activation. Our results also provide evidence supporting Akt and ERK as key protein kinases for regulating the apoptosis sensitivity of MCF-7 cells to SeC.

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Figure 5. Role of Akt in SeC-induced growth inhibition in MCF-7 cells. (**A**) Effects of SeC on the phosphorylation status and the expression level of Akt. Cells were treated with different concentrations of SeC for 24 h. Cell extracts were separated by SDS-PAGE and immunoblotted with specific primary antibodies. The immunoblots shown were representatives of three independent experiments with similar results. (**B**) Cells were treated with 20 μM SeC for various periods of time. (**C**) Effect of LY294002 (PI3K inhibitor) on SeC-induced growth inhibition. Cells were pretreated with 20 μM LY294002 1 h prior to the treatment with 20 μM SeC for 24 h (total inhibitor exposure time was 25 h). Cell viability was determined by the MTT assay as described under Materials and Methods. Bars with different characters are statistically different at the $P < 0.05$ level. (**D**) Morphology of MCF-7 cells under different treatments (magnification, 200×). LY294002, pretreatment with 20 μM LY294002 for 1 h; SeC, treatment with 20 μM SeC for 24 h.

Inhibition of deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth (*26*). Cyclins, CDKs, and CDKIs play essential roles in the regulation of cell cycle progression. CDKIs, such as p21waf1/Cip1 and p27Kip1, are tumor suppressor proteins that down-regulate the cell cycle progression by binding with active cyclin−CDK complexes and thereby inhibiting their activities (*5*). Chemopreventive agents usually cause apoptosis or cell cycle arrest at the G0/G1 or G2/M phases (*27*). Consequently, molecular mechanisms regulating the G1/S and G2/M transitions have been intensively investigated in mammalian cells. In contrast, relatively little is known about mechanisms that control progress within the S-phase. With regard to seleno-compounds, selenite, selenomethionine, and methylseleninic acid elicited cell-cycle arrest at G0/G1 and G2-M phases in human prostate and colon cancer cells and vascular cancer epithelial cells, through the induction of CDKIs and the inhibition of cyclins and CDKs (*28−32*). In mammary cancer cell lines, exposure to Se-methylselenocysteine arrested the cells in S-phase (*33*). Although these results offered much insight for the cell cycle arrest action of Se, the detailed molecular mechanisms remain to be clarified. Consistent with the previous paper (*33*), cell cycle analysis data showed that SeC treatment caused a strong S-phase arrest in cell cycle progression of MCF-7 cells. Furthermore, mechanistic investigation showed that SeC-induced S-phase arrest is mainly mediated via up-regulation of CDKIs and down-regulation of several cyclins and their corresponding CDKs. SeC treatment also resulted in elevation of the expression levels of cyclins E and phosphorylated Rb. Taken together, our results indicated that SeC modulates multiple regulatory molecules important in S-phase progression.
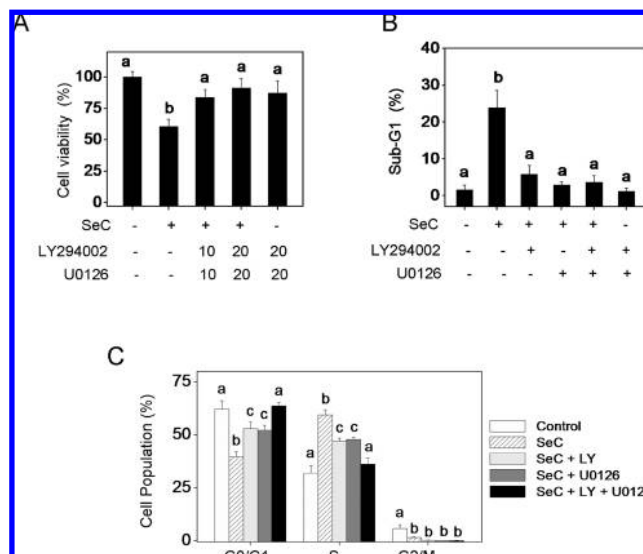
Figure 6. Roles of ERK and Akt in SeC-induced apoptosis and cell cycle arrest. Cells were pretreated with 20 μM LY294002 and U0126, alone or in combination, 1 h prior to the treatment of 20 μM SeC for 24 h (total inhibitor exposure time was 25 h). (**A**) Cell viability was determined by the MTT assay as described under Materials and Methods. Bars with different characters are statistically different at the $P < 0.05$ level. (**B**) Quantitative analysis of apoptotic cell death by measuring the sub-G1 cell population. Cells were fixed with 70% ethanol, stained with PI, and analyzed by flow cytometry. Bars with different characters are statistically different at the $P < 0.05$ level. (**C**) Effects of LY294002 and U0126 on SeC-induced S-phase arrest. Cell cycle distribution was analyzed by flow cytometry. The value is the mean ± SD of three different experiments. Within the same phase, the values with different letters are significantly different from each other ($P < 0.05$).

Apoptosis is a physiological process that functions as an essential mechanism of tissue homeostasis and is regarded as the preferred way to eliminate unwanted cells. Most of the present cytotoxic anticancer drugs mediate their effects via induction of apoptosis in cancer cells (*34*). Apoptosis has been evidenced as a critical mechanism for cancer chemoprevention and chemotherapy by selenocompounds (*7*). In the case of breast cancer, cancer cells become resistant to apoptosis and do not respond to chemotherapeutic agents, partially due to disruptions of apoptotic signaling pathways and changes in the expression of proteins and enzymes associated with tumor resistance (*4*). Thus, the agents that selectively induce apoptosis in breast cancer cells could be useful in controlling this cancer. We have previously shown that SeC induced apoptotic cell death in melanoma A375 cells, liver adenocarcinoma HepG2 cells, and breast adenocarcinoma MCF-7 cells as evaluated by flow cytometric analysis and annexin-V staining assay (*13*). In this study, it was found that SeC treatment led to a time-dependent increase in enzymatic activity of caspases-3/7, DNA fragmentation, and PARP cleavage in MCF-7 cells. These apoptotic events were evident after 36 h of SeC treatment, whereas S-phase arrest was detected in MCF-7 cells as early as 12 h after treatment. Our results suggested that SeC-induced apoptosis is preceded by S-phase arrest in MCF-7 cells.

Several protein kinase pathways have been known to regulate cell proliferation and survival. MAPKs, a family of serine−threonine protein kinases, have been implicated in apoptosis and cell cycle regulation signaling in diverse cell models (*21, 22, 25*). The MAPK family includes three kinase members, namely, c-Jun NH$_2$-terminal protein kinase/stress-activated protein kinases (JNK/SAPKs), p38 MAPK, and

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extracellular signal-regulated kinase (ERK). In general, JNK and p38 MAPK are activated by diverse stimuli such as oxidative stress, UV irradiation, and osmotic shock and required for the induction of apoptosis (*25*). In contrast, ERK plays vital roles in cell growth and division and is generally considered to be a survival mediator (*35*). However, the detailed functions of these MAPK members depend on kinds of stimuli and types of cell systems. In addition to the MAPKs, another protein kinase Akt is able to mediate cell growth via the phosphorylation of a variety of substrates including Bad, glycogen synthase kinase 3β (GSK3β), and FOXO transcription factors (*24*). With regard to the anticancer action of selenocompounds, many studies have implicated the regulation of the MAPK and PI3K/Akt pathways as likely mechanisms for induction of apoptosis and/or cell cycle arrest in human prostate cancer cells by either methylseleninic acid or selenite (*28–31, 36*). Studies also showed that inhibition of Akt phosphorylation by methylseleninic acid or Se-methylselenocysteine induced apoptosis or enhanced the apoptosis-inducing effects of chemotherapeutic drugs in mammary cancer cells (*9, 33*). However, limited information is available on the significance of MAPK and PI3K/Akt pathway as potential targets for anticancer action of SeC. In the present study, our findings established a mechanistic link between the MAPK pathway, Akt, and SeC-induced cell cycle arrest and apoptosis in MCF-7 cells. SeC treatment triggered the differential activation of JNK, p38 MAPK, ERK, and Akt pathways (**Figures 4A** and **5A**). Further examination using specific inhibitors suggested that JNK and p38 did not play important roles in regulating cell death induced by SeC in MCF-7 cells. To investigate the relationship between ERK and Akt phosphorylation and SeC-induced S-phase arrest and apoptosis, we contrasted the time course of their changes. The results in **Figures 3−5** provided a temporal sequence of these events during SeC treatment. Exposure of MCF-7 cells to SeC led to a rapid elevation of ERK and Akt phosphorylation after 1 h of treatment. However, the SeC-induced S-phase arrest was evident 12 h after SeC treatment (**Figure 1D**). Meanwhile, the onset of apoptosis as revealed by caspase-3/7 activation and PARP cleavage could be observed only 36 h after SeC treatment (**Figure 3**). Furthermore, inhibitors of ERK and Akt, alone or in combination, almost completely blocked the SeC-induced S-phase arrest and apoptosis in MCF-7 cells, indicating that ERK and Akt are critical in mediating SeC-induced growth inhibition. Collectively, our results suggest that modulation of ERK and Akt phosphorylation contribute to the anticancer effect of SeC.

In conclusion, this study provides mechanistic insights into how SeC regulates the components of cell cycle progression and apoptotic machinery to delay S to G2/M transition and induces apoptosis in MCF-7 cells. Our data imply the potential of SeC as a chemotherapeutic agent because many anticancer drugs are known to achieve their anticancer function by inducing apoptosis and/or cell cycle arrest in susceptible cells.

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