Selenite Inhibits the c-Jun N-terminal Kinase/Stress-activated Protein Kinase (JNK/SAPK) through a Thiol Redox Mechanism*

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Hee-Sae Park‡§, Eun Park‡, Mi-Sung Kim‡§, Kwangseog Ahn§, Ick Young Kim§, and Eui-Ju Choi‡§¶
From the §National Creative Research Initiative Center for Cell Death and the ¶Graduate School of Biotechnology, Korea University, Anam-dong, Sungbuk-ku, Seoul, 136-701, Korea

Selenium, an essential biological trace element, has been shown to modulate functions of many regulatory proteins involved in signal transduction and to affect a variety of cellular activities including cell growth, survival, and death. The molecular mechanism by which selenium exerts its action on the cellular events, however, remains unclear. In our present study, we observed that selenium suppresses both the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and the p38 mitogen-activated protein kinase pathway in 293T cells. In contrast, selenium had little effect on the extracellular signal-regulated kinase pathway. Furthermore, selenite directly inhibited JNK/SAPK activity in vitro but not the p38 activity. The in vitro inhibition of JNK/SAPK by selenite was reversed by the addition of reducing agents such as dithiothreitol and β-mercaptoethanol. Replacement of cysteine 116 in JNK1 by serine abolished the inhibitory effect of selenite on JNK1 activity both in vitro and in vivo. Selenite also suppressed a c-Jun-dependent luciferase reporter activity stimulated through the JNK signaling pathway. Taken together, our findings strongly suggest that selenite differentially modulates the mammalian mitogen-activated protein kinase pathways and that it can repress the JNK/SAPK signaling pathway by inhibiting JNK/SAPK through a thiol redox mechanism.

Selenium, an essential trace element present in both prokaryotic and eukaryotic cells, has been linked to regulatory functions in cell growth, cell survival, cytotoxicity, and transformation (1–7). Selenium appears to modulate such cellular activities presumably by acting on proteins important for signal transduction (8–12). For instance, selenium and other selenium compounds have been shown to inhibit the functions of Na-K-ATPase, rat brain prostaglandin D synthase, a glucocorticoid receptor, nuclear factor-κB, and the transcription factor AP-1 by reacting with critical sulfhydryl groups of the proteins (8, 9, 11, 13–15). Selenium can also regulate the functions of certain proteins by becoming incorporated into the proteins (16). Such proteins include glutathione peroxidases, formate dehydrogenases, glycine reductases, and the 5′-deiodinases (16, 17). Nonetheless, the modulatory functions of selenium and other selenium compounds on intracellular signaling pathways are not fully understood.

The mitogen-activated protein kinase (MAPK) pathway is one of the most widely studied signaling pathways involved in the transduction of intracellular signals initiated by extracellular stimuli to the nucleus. MAPKs are serine/threonine protein kinases that play pivotal roles in a variety of cell activities, such as cell proliferation, differentiation, developments, and survival, in many cell types (18). The MAPKs have been subdivided into three subfamilies: ERK, JNK/SAPK, and p38 MAPK (19–24). ERK is often activated when cells are exposed to mitogens such as peptide growth factors (18, 25). The ERK pathway consists of ERK, upstream kinases that include mitogen-activated protein kinase/extracellular signal-regulated kinase, and Raf-1 (18, 25, 26). JNK/SAPK is activated in response to a variety of cellular stresses, including DNA damage, heat shock, or proinflammatory cytokines (20, 21, 23, 24). JNK/SAPK is activated by phosphorylation on threonine and tyrosine residues by SEK1/JNK kinase 1/MKK4 (27–29). SEK1, in turn, is activated by MEKK1 (30). Like JNK/SAPK, p38 MAPK can be also activated by various stresses (22). P38 MAPK is phosphorylated and activated by MKK3, MKK4, or MKK6 (28, 31, 32). The physiological functions of JNK or p38 MAPK are not yet clear, but they seem to be involved in stress-activated cellular events that under certain conditions can include cell death (23, 24, 33–37). The aim of the present study was to investigate the possible effects of selenite on mammalian MAPK pathways. We found that selenite exerts distinct effects on the ERK, JNK, and p38 MAPK signaling pathways. Selenite inhibits the JNK and p38 signaling pathways but not the ERK signaling pathway. Moreover, this study suggests that selenite suppresses JNK activity by a thiol redox mechanism. Differential regulation of the MAPK pathways by selenite may be important to understand the mechanism by which selenite can modulate the intracellular signaling cascades and, thus, exert its effects on various cellular events.

EXPERIMENTAL PROCEDURES

Cell Culture and DNA Transfection—293T human embryonic kidney cells were routinely maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For DNA transfections, cells were plated in 100-mm dishes at a density of 2 × 10⁶ cells/dish, grown overnight, and then transfected with appropriate expression vectors using calcium phosphate (38). After 48 h of translec-

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† To whom correspondence should be addressed: Graduate School of Biotechnology, Korea University, Anam-dong, Sungbuk-ku, Seoul, 136-701, South Korea. Tel.: 82-2-3290-3446; Fax: 82-2-927-9028; E-mail: ejchoi@kuceunix.korea.ac.kr.

‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; DTT, dithiothreitol; β-ME, β-mercaptoethanol; NEM, N-ethylmaleimide; diamide, azodicarboxylic acid bis(dimethylamide); HA, hemagglutinin; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; SEK, stress-activated protein kinase/extracellular signal-regulated kinase kinase; MKK, mitogen-activated protein kinase kinase.
tion, the cells were treated with indicated agents and harvested for further experiments. When indicated, cells in culture were irradiated with 80 J/m² ultraviolet light for 1 h at 37 °C.

Immunocomplex Kinase Assays—Confluent cells were harvested and lysed in lysis buffer A containing 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 25 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Cell lysates were then subjected to centrifugation at 12,000 × g for 10 min at 4 °C. A soluble fraction was incubated with appropriate antibodies against the indicated protein kinases for 1 h at 4 °C. The immunocomplexes were then coupled to protein G-agarose during incubation for an additional 1 h at 4 °C, after which they were pelleted by centrifugation. The immunopellets were rinsed three times with buffer A and then twice with 20 mM Hepes, pH 7.4. Immunocomplex kinase assays were performed by incubating the immunopellets for 30 min at 30 °C with 2 μg of substrate proteins in 20 μl of reaction buffer containing 0.2 mM sodium orthovanadate, 10 mM MgCl₂, 2 μCi of [γ-³²P]ATP, 20 mM Hepes, pH 7.4. The reaction was stopped by adding 5 μl of 5× Laemmli's sample buffer and heating at 80 °C for 3 min. Phosphorylated substrates were then visualized by SDS-polyacrylamide gel electrophoresis and quantified using a Fuji BAS 2500 phosphorimager. Glutathione S-transferase (GST)-fusion proteins used as substrates were expressed in Escherichia coli using pGEX-2T (Amersham Pharmacia Biotech) and purified on glutathione-Sepharose as described previously (39). The antibodies used in the JNK1, p38, ERK2, and MEKK1 assays were as follows: mouse monoclonal anti-JNK1 (PharMingen), rabbit polyclonal anti-p38 (PharMingen), rabbit polyclonal anti-ERK2 (Upstate Biotechnology, Inc.), and rabbit polyclonal anti-MEK1 (PharMingen) antibodies, respectively. Protein concentrations were determined by the Bradford method (Bio-Rad).

Protein Kinase Assay for GST-SEK1—293T cells were transiently transfected with an expression vector, pEG, producing GST-SEK1 (27, 40). Transfected cells were harvested and homogenized in phosphate-buffered solution containing 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 7 μg/ml aprotinin. Cell lysates were clarified by microcentrifugation at 4 °C for 10 min and solubilized with 1% Triton X-100. The soluble fraction was then applied to glutathione-Sepharose resin. GST fusion proteins eluted from the resin were assayed for SEK1 activity by incubation with 2 μg of recombinant GST-SAPK65 in 20 μl of the reaction buffer, as described above for the immunocomplex kinase assay.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene). The following mutagenic primers were used: C79S, 5′-CAGAGTTATTTTTATGAAATCTTATGAAATCTTTAGTTAGTC-3′; and C116S, 5′-GATGCAAATCTTTcCCAAGTGATTCAGATGG-3′. The mismatched with the wild-type JNK1 template are indicated by lowercase letters. The mutation was verified by automatic DNA sequencing.

Luciferase Reporter Assay for c-Jun-dependent Transcription—Transcriptional activity of c-Jun was determined using the PathDetect luciferase reporter kit (Stratagene). 293T cells were transiently transfected with pFR-Luc, pFA2-c-jun, pFC-MEKK, and pcDNA3-vector. After 24 h of transfection, the cells were harvested, lysed, and subjected to microcentrifugation at 4 °C for 10 min. The soluble fraction was assayed for luciferase activity using a luciferase assay kit (Promega). The expression levels of the luciferase reporter protein in the transfected cells were normalized in reference to β-galactosidase activity by incubation with 2 μg of recombinant GST-SAPK65 in 20 μl of the reaction buffer, as described above for the immunocomplex kinase assay.

RESULTS

Selenium Represses the JNK and p38 Pathways but Not the ERK Pathway—To investigate a possible role of selenium in the regulation of intracellular signaling cascades, we examined whether selenium could modulate MAPK activation processes. In this study, we observed that exposure of 293T cells to 100 nM selenium resulted in suppression of the UV-stimulated activities of either JNK1 or p38 MAPK (Fig. 1), whereas it did not affect ERK2 activity. We then examined the effect of selenium on JNK1 and p38 MAPK after treating 293T cells with various concentrations of selenium. Selenium inhibited both the UV-stimulated JNK1 and p38 activities in a concentration-dependent manner (Fig. 2A). The inhibitory effect of selenium on either JNK1 or p38 MAPK activity was also time-dependent (Fig. 2B). Our findings thus suggest that selenium inhibits the two stress-regulated MAPK signals, the JNK and the p38 pathway, but not the ERK pathway. In the following experiments we investigated a possible mechanism of selenium suppression of the JNK signaling pathway.

Selenium Affects Neither MEKK1 Nor SEK1 Activity—The JNK signaling cascade is composed of JNK and upstream ki-
nases that include SEK1/JNK kinase 1/MEKK4 and MEKK1 (23, 24). To delineate a mechanism for the inhibition of the JNK signaling pathway by selenite, we examined whether selenite could inhibit the enzymatic activities of SEK1 or MEKK1 in cells (Fig. 3). To test a possible effect of selenite on SEK1 activity, we transiently transfected 293T cells with an expression vector encoding GST-SEK1. Enzymatic activity of the ectopically expressed GST-SEK1 was measured by immunocomplex kinase assay (37).

Selenite Inhibits JNK but Not p38 in Vitro—To test whether selenite could directly act on and thus repress JNK1 activity, we examined the effect of selenite on JNK1 activity. Treatment of JNK1 immunocomplex with 100 nM selenite resulted in a complete suppression of the JNK1 activity (Fig. 4A). Our data indicated that nanomolar concentrations of selenite were sufficient to inhibit JNK activity in vitro (Fig. 4B). In comparison, selenite at 100 µM did not affect the catalytic activity of either MEKK1 or SEK1 in vitro. Thus, JNK1 appears to be a primary target protein of selenite in the MEKK-SEK-JNK signaling cascade. Interestingly, selenite at 100 µM did not change enzymatic activity of p38 MAPK in vitro (Fig. 4A). These data suggest that selenite suppresses the JNK and p38 pathways by different mechanisms.

Thiol Agents Prevent Selenite from Inhibiting JNK1 Activity—There are many lines of evidence that selenite and other selenium compounds can modulate functions of target proteins by a redox mechanism (8, 9, 11, 13–15). One possible mechanism for the JNK inhibition by selenite could be oxidation of reactive sulphydryl groups present on JNK1. We tested this hypothesis by using reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol (β-ME) (Fig. 5). In vitro inhibition of JNK1 by selenite was completely reversed by either 10 mM DTT or 10 mM β-ME (Fig. 5A). Furthermore, JNK1 from selenite-treated cells was reactivated by DTT and β-ME (Fig. 5B). These findings suggest that selenite might suppress the JNK1 activity through a thiol redox mechanism and that the catalytic activity of JNK1 may require the reduced form of a critical sulphydryl group(s) present in the enzyme. The importance of the sulphydryl residue(s) in JNK activity is confirmed by the observation that JNK1 activity can be abolished by
pretreatment with thiol-oxidizing agents such as N-ethylmaleimide (NEM), azodicarboxylic acid bis-dimethylamide (diamide), or o-iodosobenzoate (Fig. 5C). NEM is a modifier of thiols, and both diamide and o-iodosobenzoate can oxidize vicinal dithiols (41–43). These thiol-modifying agents did not affect the p38 activity in vitro (data not shown).

Cys116 Is Involved in the Thiol Redox Regulation of JNK1—To further characterize the thiol redox regulation by selenite, we constructed the single mutants C79S, C116S, and C137S of JNK1, and examined the sensitivity of these JNK1 mutants to selenite. Cys79, Cys116, and Cys137 in JNK1 are the cysteine residues conserved among the JNK isoforms but not in ERKs or p38. We transiently transfected 293T cells with HA-tagged wild-type JNK1 or one of the single mutants and then examined the effect of selenite on enzymatic activities of the ectopically expressed JNK1 mutants (Fig. 6A). Enzymatic activities of all of the three single mutants (C79S, C116S, and C137S) were stimulated in response to transfected cells to UV irradiation. Selenite inhibited the UV-stimulated enzymatic activity of wild-type JNK1, JNK1(C79S), or JNK1(C137S). Interestingly, JNK1(C116S) was resistant to both in vitro and in vivo inhibition by selenite (Fig. 6A). The C116S mutant was also resistant to the inhibition by the thiol-oxidizing agents, NEM and diamide (Fig. 6B). In comparison, the C79S and C137S mutants were sensitive to the inhibition by NEM and diamide. Taken together, these data strongly suggest that Cys116 is a critical cysteine residue of JNK1 for the thiol redox regulation.

Selenite Suppresses c-Jun-dependent Luciferase Reporter Expression—c-Jun is one of the major substrates of JNK, and the c-Jun phosphorylation by JNK results in stimulation of the transcriptional activity of c-Jun (19). We, therefore, examined whether selenite could suppress the transcription-stimulating activity of c-Jun when this function of c-Jun was stimulated through the JNK pathway (Fig. 7). The transactivating activity of c-Jun was determined by measuring luciferase reporter gene expression. Whereas ΔMEKK1 induced the stimulation of c-Jun-mediated luciferase reporter activity (Fig. 7), exposure of cells to 100 nM selenite resulted in the inhibition of the ΔMEKK1-stimulated luciferase activity.

**DISCUSSION**

In the present study, we demonstrate that selenite can inhibit the JNK and the p38 MAPK signaling pathway. A major mechanism by which selenite suppresses the JNK pathway appears to be the direct action of selenite on JNK itself, although the possibility that selenite could also act on any site(s) upstream of JNK cannot be ruled out. Interestingly, in vitro inhibition of JNK by selenite can be reversed by reducing agents such as DTT or β-ME. These findings strongly suggest that a thiol redox mechanism may be involved in the inhibitory effect of selenite on JNK. Selenite and other selenium compounds have been shown previously to oxidize sulphydryl groups present in proteins, resulting in the formation of selenenolsulfide (44, 45). Thus, selenite could possibly inhibit JNK activity through interacting with redox active cysteine residue(s) on the enzyme. The importance of reactive sulphydryl residue(s) for JNK activity was confirmed by our data demonstrating that thiol-oxidizing agents such as NEM, diamide, or o-iodosobenzoate abolished the JNK1 activity in vitro. Furthermore, the site-directed cysteine mutant data in this study indicate that Cys116 is a critical cysteine residue of JNK1 for the inhibition by selenite and other thiol-oxidizing agents.

JNK1 and p38 MAPK respond similarly to a variety of cellular stresses (23, 24). Nevertheless, selenite did inhibit JNK1 activity, whereas it did not inhibit p38 activity in vitro. Furthermore, the thiol-oxidizing agents, which inhibited JNK1 activity, did not affect p38 activity (data not shown). At present, a mechanism by which selenite down-regulates the p38 signaling pathway has not yet been proposed. There could be some site(s) upstream of p38 that could be potential target(s) for selenium. In this context, our in vitro kinase assay data suggest that neither MEKK1 nor SEK1/JNK kinase 1/MKK4 are targets for selenite.

c-Jun is a component of the AP-1 complex, an important transcription factor involved in a variety of cellular events including cellular transformation (46, 47). It has been well documented that c-Jun is one of the major substrates for JNK, and c-Jun phosphorylation by JNK has been shown to result in higher transcription activation by c-Jun and to induce cellular transformation (20, 24). Interestingly, selenium compounds have been reported to inhibit AP-1 activity (8) and cellular transformation (5). Our findings in this study, therefore, suggest that the inhibition of JNK by selenite may be one possible mechanism by which selenium inhibits cellular transformation.
as well as AP-1 activity. It has been also suggested that selenium inhibits AP-1 activity through interacting with vicinal thiols present in c-Jun (8, 9). Thus, selenium compounds seem to repress the transcription-stimulating activity of c-Jun in at least two different ways. Selenite and other selenium compounds modulate a variety of cellular processes such as cell proliferation, survival, and transformation (1–3, 5, 48). The mechanisms underlying the functions of selenite are not yet fully understood, however. In the present study, we demonstrate that selenite exerts its modulatory function on the stress-activated MAPK signaling pathway may be important components of a mechanism by which selenite modulates intracellular signaling cascades that mediate various cellular functions.

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