Effect of Selenite on T-Cell Mitogenesis: Contribution of ROS Production and Apoptosis Signal-Regulating Kinase 1

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Received April 5, 2014; accepted May 16, 2014

Although supplementation with the selenocompound, sodium selenite, has been shown to stimulate the concanavalin A-induced T-cell mitogenic response, the mechanisms responsible remain unclear. This study was conducted to evaluate the relationships between the induction of apoptosis, formation of tumor necrosis factor (TNF)-alpha and reactive oxygen species (ROS), activation of apoptosis signal-regulating kinase (ASK) 1 and the thioredoxin (Trx) system when mitogenesis was stimulated by selenite. TNF-alpha was dose-dependently released by mouse splenocytes treated with selenite, and apoptosis was induced when TNF-alpha was added at the indicated concentrations. However, supplementation with selenite at low concentrations inhibited the accumulation of ROS with the increased expression of Trx reductase 1 and induction of apoptosis in wild-type splenocytes, and also at high concentrations in Trx-1-transgenic mouse splenocytes. The suppression of apoptosis was accompanied by a decrease in the expression of phospho-ASK1. These results suggest that the stimulation of T-cell mitogenesis by selenite may be partly attributed to the inhibited accumulation of ROS due to a reduced Trx-1/TR1 system, the inactivation of ASK1, and the suppression of apoptosis.

Key words selenium; mitogenesis; apoptosis; thioredoxin; apoptosis signal-regulating kinase (ASK) 1; tumor necrosis factor

Selenium is an essential trace element in mammals and has been shown to regulate many intracellular functions as a key component of selenoproteins.1 Well-known selenoproteins include selenium-dependent enzymes such as the glutathione peroxidase family (GPX1, GPX2, GPX3, GPX4, and GPX6) and thioredoxin family (TR1, TR2, and TR3), which have selenocysteine residues that serve as redox catalysts and function in response to oxidative stress,1–3 particularly in the immune system.4 Therefore, a selenium deficiency may cause the loss of immunological competence, which may be responsible for the pathogenesis of some disorders such as viral or bacterial infections. Selenium deficiencies have been implicated in accelerated disease progression and poorer survival among populations infected with human immunodeficiency virus,5 and improvements in immunocompetence have been reported when selenium-deficient and virus-infected hosts were administered dietary selenium.6

In cellular immune reactions, selenium has been shown to promote cell growth,7,8 protect against apoptosis,9,10 be involved in the production of cytokines,11 and induce transcription factor nuclear factor-xB (NF-xB).12 However, selenium has also been shown to inhibit cell growth,13,14 induce cell death15 or apoptosis,6,17 and suppress NF-xB.18,19 Although the essential roles of selenium in immune cell functions have already been reported, the functions and underlying mechanisms have yet to be elucidated due to these inconsistencies. Selenium has been shown to have anticancer effects at concentrations higher than nutritional requirements,20,21 and these may be mediated through cell cycle arrest, apoptosis, and/or the toxic effect. These effects have been attributed to the actions of selenium on the functions of many intracellular proteins important for signal transduction, and are dependent on the chemical form and concentration of selenium compounds.

The cellular thiol redox state is known to be crucial for signal transduction in immune cells and transcriptional processes for cytokines, and the fine balance between oxidizing and reducing conditions is essential for these functions as well as survival or apoptosis.22 Reactive oxygen species (ROS) have been identified as important second messengers in intracellular signaling23 and enhance the activation of NF-xB,24 which plays an important role in regulating the gene expression of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and other immune response factors. The reduced form of thioredoxin (Trx)-1, together with the TR system, serves as a critical regulator for the activation of NF-xB and promotes DNA-binding by converting essential cysteine residues in proteins into active thiol forms.25 This thiol redox regulation of transcription factors including NF-xB appears to be dependent on the intracellular redox balance between the production of ROS and their scavenging by the glutathione (GSH)/GPX1 and/or Trx-1/peroxiredoxin/TR systems in connection with the selenium status. Previous studies suggested that supplementation with the most popular selenium compound, sodium selenite, may not only induce selenoenzymes such as GPX1 and TR, but also cause the endogenous consumption of GSH26 and production of ROS27; therefore, the

The authors declare no conflict of interest.

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effects of selenite on the activation of NF-kB may depend on the selenium status and remains controversial. We previously reported that physiological levels of selenite augmented NF-kB DNA-binding activity with enhancements in TR activity and the cell growth rate in the mitogenic response of mouse splenocytes with concanavalin A (Con A). These findings suggested that selenite may reduce Trx-1 depending on the expression level of TR. However, the effects of selenite on the expression of TNF-α following the activation of NF-kB, production of ROS, and induction of apoptosis, which may be triggered by the mitogenesis of splenocytes, remain unclear. On the other hand, the induction of apoptosis by TNF-α requires the activation of apoptosis signal-regulating kinase (ASK) 1, which has been identified as a mitogen-activated protein (MAP) kinase kinase kinase that activates c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways.

Although the reduced form of Trx-1 normally binds the N-terminal domain of ASK1 and inhibits multimerization and kinase activity, TNF-α can stimulate the production of ROS, which may result in the formation of the oxidized form of Trx-1, followed by its dissociation from ASK1 and subsequent activation.

Although selenite stimulates the Con A-induced T-cell mitogenic response, the underlying mechanisms have yet to be elucidated in detail. Therefore, the present study was designed to evaluate the relationships between the induction of apoptosis, formation of TNF-α and ROS, activation of ASK1, and the Trx system when T-cell mitogenesis was stimulated by selenite. These relationships were then compared between splenocytes prepared from human Trx-1-overexpressing (Tg) C57BL/6 mice and wild-type mice as the control.

MATERIALS AND METHODS

Chemicals and Animals Sodium selenite (Na₂SeO₃, 99.999%) and concanavalin A (Con A) were purchased from Sigma-Aldrich Japan (Tokyo). Male Trx-1-Tg mice were established as described previously and wild-type C57BL/6 mice were maintained under the same conditions of 25±1°C, 50±2% relative humidity and a light/dark cycle of 12 h each, and had access to sterilized water and pelleted rodent chow containing 135±47 ng Se/g. These mice were used in experiments at 6 weeks of age. The protocol used here met the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for the Ethical Use of Experimental Animals at Setsunan University. All efforts were invariably made to minimize animal suffering, reduce the number of animals used, and utilize alternatives to in vivo techniques.

T-Cell Mitogenesis The T-cell mitogenic response was induced as described previously, with a slight modification. Splenocytes were prepared by flushing with filter-sterilized RPMI-1640 medium containing cysteine instead of cystine plus 2-mercaptoethanol. The cells were dispersed into wells of a microtiter 24-well solid plate at 2×10⁶ cells/well in 2mL of the medium, which was supplemented by 5mM N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES), 50 mg/L potassium benzylpenicillin, 50 mg/L streptomycin sulfate, and 10% fetal calf serum (FCS). The cells were then incubated at 37°C in 5% CO₂ after the addition of T-cell mitogen Con A at 2μg/mL, selenite, or another additive at the specified concentration.

Determination of Apoptosis, ROS, and TNF-α Apoptosis was determined after 24h of the mitogen stimulation. Cells were washed once with phosphate buffered saline (PBS) after centrifugation at 500×g for 10min at 4°C. The packed cells were suspended in 100μL of the binding buffer of the ApoAlert Annexin V kit (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.), and 5μL of the fluorescein isothiocyanate (FITC)-conjugated annexin V solution for the detection of apoptosis and 10μL of propidium iodide (PI) solution for the detection of necrosis were added. After being incubated for 15min in the dark at room temperature, 400μL of the binding buffer was added, and the cells were analyzed on a CytoACE-300 flow cytometer (Jasco Co., Tokyo, Japan). The number of apoptotic cells was calculated by collecting PI-unstained cells.

ROS production was determined in cells 72h after the mitogen stimulation according to the method of Bass et al., with a slight modification. Briefly, cells were washed once with PBS as stated above, and the packed cells were suspended in 500μL of PBS containing 5μm 5- and 6-carboxy-2’,7’-dichlorofluorescein diacetate (carboxy-DCFH-DA) and incubated for 10min at 37°C in the dark. After centrifugation at 500×g for 10min at 4°C, cells were rinsed with PBS three times and analyzed by flow cytometry. At least 10000 cells were examined in each sample. Fluorescence was measured on the FL1 channel of CytoACE-300, and the amplification scale was logarithmic.

The amount of TNF-α released from cells during mitogenesis was measured using the mouse colorimetric ELISA Kit (Pierce Biotechnology, Inc., IL, U.S.A.) for the culture medium 72h after the mitogen stimulation.

Western Blot Analysis The expression of murine TR1 and phosphor-ASK1 (Thr845) proteins was determined using an anti-murine TR1 rabbit polyclonal antibody (GeneTex, TX, U.S.A.) and anti-human phosphor-ASK1 (Thr845) rabbit polyclonal antibody (Cell Signaling Technology, MA, U.S.A.). Prepared splenocytes and cultured cells were washed once with PBS after centrifugation at 900×g for 10min at 4°C. The packed cells were suspended in lysis buffer containing 0.5% Nonidet P-40, 10mM Tris–HCl (pH 7.5), 150mM NaCl, 1mM phenylmethylsulfonyl fluoride, and 0.111 units/mL aprotinin, and the extracts were cleared by centrifugation. Equal amounts of protein estimated using the BCA Protein Assay Reagent Kit (Pierce Biotechnology) were electrophoresed on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and then electrophoretically transferred to a poly(vinylidene difluoride) membrane (ATTO, Tokyo, Japan). After blocking with 1% skim milk in Tris–HCl-buffered saline containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with the first antibody, and then with the peroxidase-linked anti-mouse immunoglobulin G (IgG) and the anti-rabbit IgG as the second antibodies (Cell Signaling Technology). β-Actin was used as a loading control with anti-β-actin (Novus Biologicals, CO, U.S.A.). Chemiluminescence was detected with an ECL Western blot detection kit (Amersham Biosciences). Immuno-reactive bands were quantified by volume densitometry using a Light-Capture and CS Analyzer (ATTO) and normalized to β-actin.

Statistical Analysis Results were analyzed statistically using a one-way ANOVA followed by Bonferroni’s multiple comparisons test using the Origin program (Northampton, MA, U.S.A.). Probability values <0.05 were considered sig-
RESULTS

**TNF-α Secretion by Selenite and Induction of Apoptosis during T-Cell Mitogenesis** The effects of supplementary sodium selenite on TNF-α secretion from lymphocytes during Con A-induced T-cell mitogenesis as well as the effects of inflammatory cytokines on the induction of apoptosis were tested using splenocytes prepared from wild-type C57BL/6 mice. As shown in Fig. 1, the amount of TNF-α released into the culture medium dose-dependently increased with the co-addition of selenite at concentrations from 0.1 to 50 µM with Con A (Fig. 1A). When TNF-α was added to the culture medium within the concentration range detected during the stimulation of T-cell mitogenesis by selenite (1–12 ng/mL), apoptotic cells dose-dependently appeared in spite of the absence of supplementary selenite (Fig. 1B). Data are given as means±S.D. (n=4). *,† p<0.05; **,†† p<0.01, significantly different from the control.

**Effects of Selenite on Apoptosis, Production of ROS, and Activation of ASK1 during T-Cell Mitogenesis Modified by Overexpressed Trx-1** The effects of the overexpression of Trx-1 on the induction of apoptosis, production of ROS, and activation of ASK1 during T-cell mitogenesis modified by selenite were investigated by comparing splenocytes prepared from both wild-type C57BL/6 and male Trx1-Tg mice. As shown in Fig. 2, although apoptosis was triggered by the Con A-induced mitogenic response in both splenocytes, the
apoptotic ratio was decreased by the co-addition of relatively low concentrations (0.02–1 µM) of selenite. During mitogenesis in wild-type splenocytes, apoptosis was significantly enhanced by more than 10 µM of supplementary selenite. However, apoptosis was less apparent when Trx-Tg mouse splenocytes were treated under the same conditions. As shown in Fig. 3, the Con A-induced production of ROS was also suppressed by the co-addition of selenite at low levels (0.02–1 µM) in both splenocytes. However, the co-addition of selenite at the higher concentrations (5–50 µM) strongly enhanced ROS production in wild-type mouse splenocytes. The production of ROS was marked reduced when Trx-Tg mouse splenocytes were substituted for wild-type mouse splenocytes. Figure 4 shows the expression of TR1 and phospho-ASK1 in wild-type and hTrx-1-Tg mouse splenocytes exposed to selenite at concentrations less than 10 µM indicating that the protein amount necessary for Western blotting can be collected. The expression of TR1 in both splenocyte types was relatively low in the control and enhanced by more than 0.05 µM of supplementary selenite. When the expression of phospho-ASK1 was normalized to β-actin, the relative expression indicated that the protein amount necessary for Western blotting can be collected. The expression of phospho-ASK1 was normalized to β-actin, the relative expression induced by the mitogenic response in both splenocyte types was reduced by the co-addition of selenite, except when wild-type splenocytes added at the higher concentrations of 5 and 10 µM. These results indicated that lower levels of selenite suppressed the induction of apoptosis, production of ROS, and expression of phospho-ASK1 during T-cell mitogenesis by Con A, depending on the expression of TR1.

DISCUSSION

We previously reported that physiological levels (0.02–1 µM) of selenite augmented NF-κB DNA-binding activity with enhancements in TR activity and the cell growth rate in the mitogenic responses of mouse splenocytes with Con A. These findings suggested that selenite reduced Trx-1 depending on the expression level of TR. However, the effects of selenite on the expression of TNF-α due to the activation of NF-κB, production of ROS, and induction of apoptosis, which may be triggered by mitogenesis in splenocytes, remain unclear. The induction of apoptosis by TNF-α has been shown to require the activation of ASK1. The reduced form of Trx-1 normally binds to the N-terminal domain of ASK1 and blocks its activation by TNF-α unless the oxidized form of Trx-1 is formed and dissociated from ASK1. Therefore, the present study was designed to evaluate the relationships among the induction of apoptosis, formation of TNF-α and ROS, activation of ASK1, and the Trx system under the stimulation of Con A-induced T-cell mitogenesis by selenite, and these relationships were compared between Trx-1-Tg mouse splenocytes and wild-type cells as the control. The co-addition of selenite at 0.1–50 µM including subtoxic doses with Con A to wild-type splenocytes led to the dose-dependent release of TNF-α (Fig. 1A), and the co-addition of TNF-α at the indicated concentrations, 1–12 ng/mL, with Con A induced apoptosis in spite of the absence of selenite (Fig. 1B). Similar findings have been reported in lymphocytes collected from selenium-supplemented animals, in which the gene expression of several cytokines including TNF-α was induced. These results suggest that the relatively high exposure levels of selenite promoted the secretion of TNF-α, which may be capable of inducing apoptosis through the production of ROS during T-cell mitogenesis.

As previously reported, Con A-induced cell growth rate in the presence of selenite at the concentrations of 0.02–0.1 µM is about 4 times higher than that without the co-addition. In the wild type cells, the viability after T-cell mitogenic response by Con A is not low as the percentage of apoptotic cells shows >20 (Figs. 1B, 2). However, the viability decreased when selenite was added at above concentration (data not shown) as well as the result of apoptosis in Fig. 2. When T-cell mitogenesis was compared between Trx-1-Tg mouse

![Fig. 4. Expression of TR1 and Phospho-ASK1 after the Promotion of T-Cell Mitogenesis by Selenite in Wild-Type (A) and Trx-1-Tg Mouse Splenic Cells (B)](image-url)
Splenocytes and wild-type cells, apoptosis was found to be suppressed by the co-addition of relatively low concentrations (0.02–1 µM) of selenite in both splenocyte types. Furthermore, the supplementation with selenium at a concentration of more than 10 µM suppressed apoptosis more in Trx-1-Tg mouse splenocytes than in wild-type cells (Fig. 2). This result suggested that selenite may not only promote mitogenesis due to the inhibition of apoptosis at relatively low concentrations, but also induce apoptosis as a result of the extracellular release of TNF-α at subtoxic concentrations. The Con A-induced production of ROS was also suppressed by the co-addition of selenite at low levels (0.02–1 µM) in both splenocyte types, and supplementation with more than 5 µM selenium suppressed ROS production more in Trx-1-Tg mouse splenocytes than in wild-type cells (Fig. 3). As previously described, the selenoenzyme that was correlated with the promoting effects of T-cell mitogenesis by selenite in both cells was shown to be TR, not GPX1. This finding indicated that selenite suppressed the intracellular production of ROS induced by Con A, and this was dependent on TRx-1 expression and TR activity.

The expression of TR1 in both splenocyte types was dose-dependently promoted by supplementary selenite (Fig. 4). This result is consistent with our previous findings in which no significant difference was observed in TR activity between Trx-1-Tg mouse splenocytes and wild-type cells under the same conditions. Although the relative expression of phospho-ASK1 (Thr845) was induced by the mitogenic response (data not shown), the co-addition of relatively low levels of selenite (0.05–2 µM) significantly reduced its expression in both cells (Fig. 4). Moreover, the expression of phospho-ASK1 was significantly depressed in Trx-1-Tg mouse splenocytes at more than 5 µM, but was increased in wild-type splenocytes. ASK1 and its downstream stress-activated kinases p38 and JNK constitute an important mammalian signaling pathway that can promote cell survival, apoptosis, proliferation, or differentiation, depending on the cell type and/or cellular context. ASK1 can be activated by T-cell mutagenesis; thus, it is part of a redox-signaling pathway via the activation of NF-κB, expression and release of TNF-α, induction of oxidative stress and oxidation of Trx-1, and recruitment of TNF receptor-associated factor family proteins 2/6. Oxidative stress induces the phosphorylation of Thr845 in the activation loop of ASK1, which has been correlated with ASK1-signalsome formation and ASK1-dependent apoptosis. Taken together, on a physiological level of the selenite treatment, the activation of ASK1, which was partly induced by the treatment with Con A, may have been suppressed by the reduction of the locally oxidized Trx-1 due to the increased expression of TR1 and protection from oxidation of Trx-1 bound to ASK1, which may have stimulated the Con A-induced mitogenic response (Fig. 5). At higher levels of selenite, although the expression of TR1 increases, TR1/Trx-1 system cannot be resistible for the more enhanced expression of TNF-α and subsequent production of ROS, and thereby the activation of ASK1 and induction of apoptosis may consequently inhibit mitogenesis.

In conclusion, supplementary selenite inhibited the accumulation of ROS with an increase in the expression of TR1 and suppression of apoptosis at physiological levels in wild-type splenocytes, and even at high concentrations including subtoxic levels in Trx-1-Tg mouse splenocytes. The suppression of apoptosis was accompanied by a decrease in the expression of phospho-ASK1. These results suggest that the stimulation of T-cell mitogenesis by selenite was caused by the inhibited accumulation of ROS due to a reduced Trx-1/TR1 system, the inactivation of ASK1, and suppression of apoptosis, even though the release of TNF-α and production of ROS were induced by mitogenesis.
Acknowledgments  We thank Dr. Makiko Narita at Redox Biosciences, Inc. for providing Trx-I-Tg mice and Miss Sayaka Fujimoto at Setsunan University for her technical assistance. This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI 5590118) from Japan Society for the Promotion of Science.

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