BPB Reports

Report

Selenium Toxicity Accelerated by Out-of-Control Response of Nrf2-xCT Pathway

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Selenium (Se) is an essential biological element and selenite is used to supplement malnutrition of Se and may also have anticancer activity. However, Se is also known as a delicate micronutrient with a narrow window of useful dose and the mechanisms for the sudden toxic effect remain unclear. Recently, we reported elsewhere that selenite was incorporated into cells via xCT, a cystine/glutamate antiporter. xCT is regulated by a stress responsive nuclear factor erythroid 2-related factor 2 (Nrf2). Therefore, we hypothesized and preliminary substantiated that the Nrf2-xCT pathway underlies the toxicity mechanism of Se. Expression of xCT mRNA was remarkably increased in MCF-7 cells after Se treatment, which may further increase Se uptake and oxidative stress. Pretreatment with xCT or Nrf2 inhibitors prevented morphological changes by releasing cells from uncontrolled feedback on Se uptake. Paradoxically, Se-induced oxidative toxicity is promoted by a runaway of stress response in Nrf2-xCT pathway. The results imply a novel mechanism by which Se accelerates its oxidative toxicity through feedback via Nrf2-xCT. This mechanism explain the elusive toxicological properties of inorganic Se, including strong cytotoxicity, high sensitivity in cancer cells, and narrowness of pharmacological dose range.

Key words selenium toxicity, Nrf2, xCT, selenite

INTRODUCTION

Selenium (Se) is a rare element on Earth but an essential nutrient for animals. Se enters the food chain through plants, and the Se level in plants reflects a geochemically uneven soil Se distribution.[1] Insufficient Se intake has been estimated to affect up to 1 billion people worldwide.[2] In Se depleted areas, such as Qidong, Jiangsu Province, China, Se was directly supplemented using table salt fortified with sodium selenite.[3] On the other hand, chronic selenosis was identified in seleniferous areas, such as the northern Great Plains of USA, parts of Venezuela, Colombia, and China, especially in Enshi, Hubei Province.[4] Incidents of acute poisoning include industrial accidents, accidental ingestions, suicides, and attempted murder.[5]

In vitro studies revealed that subtoxic/supranutritional doses of Se exerted specific toxicity against cancer cells, suggesting a potential application to chemotherapy.[6] However, the major problem in applying Se as an anti-carcinogenic agent is the close proximity to toxic doses.[7] In between the risks of deficiency and toxicity, the dietary acceptable range of Se is one of the narrowest among all nutrients.[8] The dietary reference intakes for Japanese adult males were estimated to be between 25 and 450 μg/d.[9] This range needs attention, especially in infants and patients receiving long-term nutritional support.[10,11] Sodium selenite or sodium selenate is used to supplement Se malnutrition in infant formula and total parenteral nutrition. These inorganic Se compounds are considerably more toxic than organic Se compounds naturally found in breast milk and foods.[12]

Selenite forms selenotrisulfide with thiols.[13] Selenodiglutathione (GSSeSG) is formed in the presence of gluthathione (GSH) under physiological conditions.[14] GSSeSG may be an important metabolite because its toxicity is more enhanced than that of selenite.[15] The cytotoxicity of GSSeSG as well as selenite was found to be due to oxidative damage of cellular components such as DNA.[16] However, the mechanism for the toxicity of GSSeSG stronger than that of selenite was unknown. We recently identified evidence that GSSeSG enters into cells more rapidly than selenite through an amino acid transporter xCT.[17] xCT is coded on the *SLC7A11* gene and constitutes the cystine/glutamate exchange system x_c^- that supplies cysteine necessary for GSH biosynthesis.[18] Cells increase GSH synthesis by upregulating xCT expression to cope with reactive oxygen species (ROS).[19] Therefore, it is hypothesized that once cells incorporate ROS-generating Se, upregulate its transporter xCT, and again uptake more Se. We have investigated whether this destructive feedback loop mechanism is involved in the Se toxicity.

MATERIALS AND METHODS

Chemicals Selenium dioxide, sulfasalazine, and L-cysteine (Cys) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ML385 was obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Selenous acid (H_2SeO_3, 100 μM) was prepared as selenite by dissolving selenium dioxide in water. Cys was prepared as a 10 mM aqueous solution. Sulfasalazine was dissolved in dimethyl sulfoxide (DMSO) to form a 50 mM solution. ML385 was prepared as 2 mM DMSO solution and diluted with water to 400 μM; before

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use, the solution was confirmed to have no precipitation of acicular crystals. Other chemicals used were of the highest grade.

Cell Culture The human breast cancer cell line MCF-7 was from the European Collection of Authenticated Cell Cultures (Salisbury, UK) and was maintained in Dulbecco's Modified Eagle's medium (DMEM, low-glucose, Wako) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% penicillin/streptomycin (FUJIFILM Wako). Cells were incubated at 37°C under a humidified atmosphere equilibrated with 95% air and 5% CO_2. Before experiments, cells were seeded (10^6 cells/well) in 2 mL DMEM and incubated for 24 h. After washing with Dulbecco's PBS without calcium and magnesium (D-PBS (–)), cells were treated with chemicals in Hank's balanced salt solution (HBSS).

Real-Time Quantitative PCR Analysis Total RNA was isolated using a spin column (RNeasy mini kit, Qiagen N.V., Venlo, Netherlands) and 500 ng total RNA was used as a template for first-strand cDNA synthesis using a kit (ReverTra Ace qPCR RT Master Mix, Toyobo, Osaka, Japan), according to the manufacturer's instructions. Real-time RT-PCR analysis was performed using SYBR green reagent (LightCycler 480 SYBR green I master, Roche Diagnostics, Indianapolis, IN, USA). Primer pairs used for quantification of *SLC7A11* and *RPS18* (40S ribosomal protein S18) were as follows: *SLC7A11* forward, 5'-CCATGAACGGTGGTGTGTT-3'; *SLC7A11* reverse, 5'-GACCCTCTCGAGACGCAAC-3'; *RPS18* forward, 5'-GATGGGCGGCGGAAAATAG-3'; *RPS18* reverse, 5'-GCGTGGATTCTGCATAATGGT-3'. All reactions and data analyses were performed using a LightCycler 480 System II and its accompanying software (Roche Diagnostics). *SLC7A11* levels were each normalized to the *RPS18* levels and indicated by the fold-change relative to the control.

RESULTS AND DISCUSSION

In this study, we used an *in vitro* system of MCF-7 breast cancer cells as a model to assess the cytotoxicity of Se. The mRNA expression of the target gene xCT was measured by real-time PCR. The function of xCT and another target, Nrf2, was interfered by their inhibitors and the toxicological significances of these targets were evaluated by morphological changes using microscopic observation. Because Se-induced oxidative stress is expected to upregulate Cys uptake to increase cellular antioxidant GSH, we examined expression levels of xCT in cells treated with H_2SeO_3 and/or Cys by real-time PCR analysis (Fig. 1). H_2SeO_3 at 1 μM concentration did not affect xCT expression in MCF-7 cells. Cys at 100 μM slightly increased xCT mRNA expression. Since Cys is oxidized to cystine (Cyss), the xCT substrate, this increase may be a substrate-induced expression.[20] Simultaneous treatment of selenite and Cys increased xCT unequivocally. This strong synergy would be attributed to selenotrisulfide, which is formed during the co-treatment. The resulting compound, selenodicysteine (CSSeSC), is taken into the cell by xCT.[17] In the cell, CSSeSC undergoes sequential metabolisms into hydrogen selenide (H_2Se) by GSH and finally oxidizes to elemental Se, accompanied with ROS generation.[21] This oxidative stress induces protective cellular responses, including xCT upregulation, as observed in Fig.1.

Gene expression of xCT, as well as other enzymes for GSH biosynthesis, is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2).[22,23] Thus, we next examined the effect of

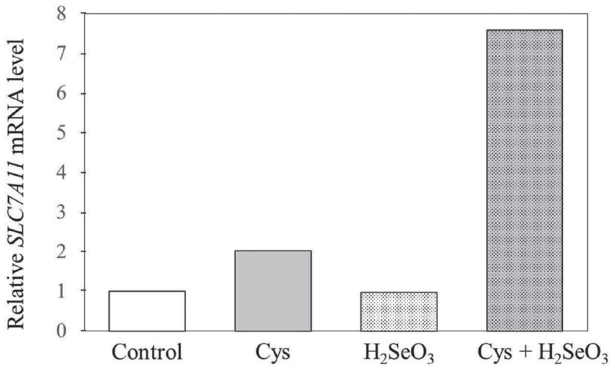

Fig. 1. Upregulation of xCT Gene Expression after Se Treatment

MCF-7 cells were incubated in HBSS for 5 h in the absence or presence of 100 μM Cys, 1 μM H_2SeO_3, or both. Total RNA was prepared and the mRNA amount of the xCT gene *SLC7A11* was quantified by real-time PCR. Expression levels are indicated as the fold change relative to the control.

pharmacological inhibition of xCT and Nrf2 (by sulfasalazine and ML385, respectively) on Se cytotoxicity (Fig. 2). Control cells showed normal shape in HBSS culture (Fig. 2A). H_2SeO_3 and Cys induced cell shrinkage (Fig. 2B), which is characteristic of programmed cell death.[24] However, pre-incubation with each inhibitor suppressed the shrinkage due to H_2SeO_3 and Cys (Figs. 2C and 2D). Inhibitors alone did not affect cellular morphologies, although they were harmful at higher concentrations. The xCT inhibitor, sulfasalazine, was reported to induce ferroptosis in cancer cells.[25]

Based on these results, we propose a novel mechanism whereby the toxicity of Se is exacerbated paradoxically by the stress response that is originally protective (Fig. 3). H_2SeO_3 interacts with GSH or Cys and forms GSSeSG or CSSeSC, respectively. GSSeSG is then converted to CSSeSC and taken up through xCT as a mimicry of Cyss. Incorporated CSSeSC is processed to H_2Se or HSe^- at the intracellular pH and generates ROS. Oxidative stress activates Nrf2 regulon containing GSH biosynthesis enzymes and xCT. Therefore, further Se is taken into the cell, more ROS is generated, and xCT is induced again. This dysregulated loop mechanism may damage the cell and finally result in catastrophic fate such as cell death.

The mechanism demonstrated here using the *in vitro* cell system needs to be evaluated *in vivo*. Constitutive expression of xCT is restricted to the central nervous system and the immune system, but is inducible in the thymus, spleen, and kidney in mice.[18] However, Se poisoning in humans include various acute symptoms involving cardiovascular, digestive, and neurological systems, and chronic toxicities are characterized by nail and hair changes as well as systemic weakness.[5] In contrast to the limited expression of xCT in normal cells, tumor cells stably express xCT to adapt to high levels of ROS generation and nutritional demand.[26] This difference in xCT level may explain how Se cytotoxicity is distinguished in normal and cancer cells and why Se is accumulated in tumors.[27] Also, individual differences in stress response may fluctuate threshold of toxic doses and thereby narrow usable range of Se.

In the present study, we have proposed a novel paradoxical mechanism for Se toxicity. This mechanism is driven by the originally protective Nrf2-mediated stress response system, where xCT functions as a gate valve at the confluence of Se and sulfur metabolisms. This mechanism provides a consistent

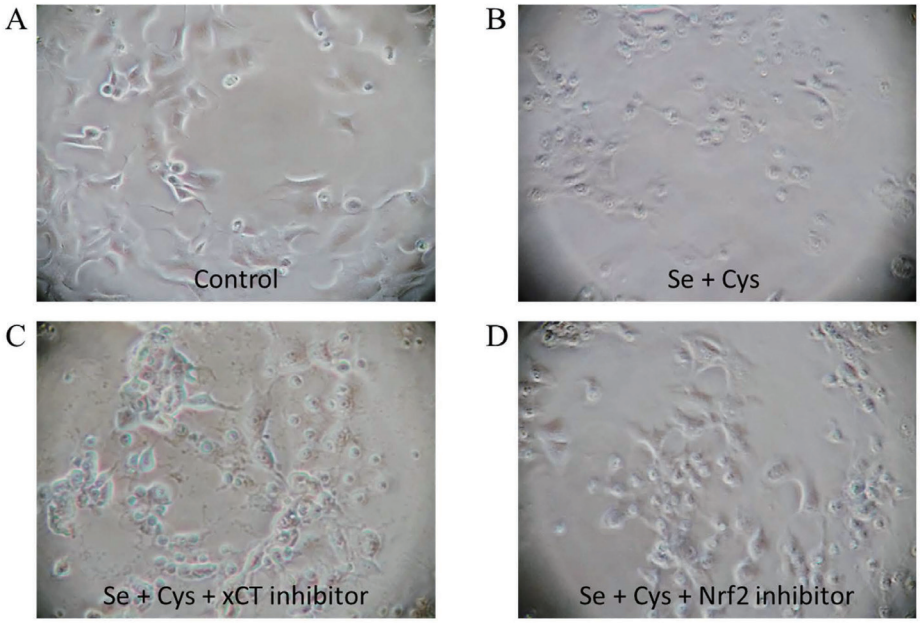

Fig. 2. Alleviation of Se Cytotoxicity with Inhibitors of xCT or Nrf2

MCF-7 cells in HBSS were pretreated for 1 h with 0.5% DMSO (A and B), 250 μM sulfasalazine (xCT inhibitor, C), or 2.5 μM ML385 (Nrf2 inhibitor, D), and then incubated for 8 h with 2 μM selenite (Se) and 200 μM Cys (B, C, and D). Cell morphologies were observed with an inverted microscope (Eclipse TS100, Nikon, Tokyo, Japan) at a magnification of 100X and photographed with a commercially available digital camera. The results shown are representatives of an experiment that was repeated more than four times.

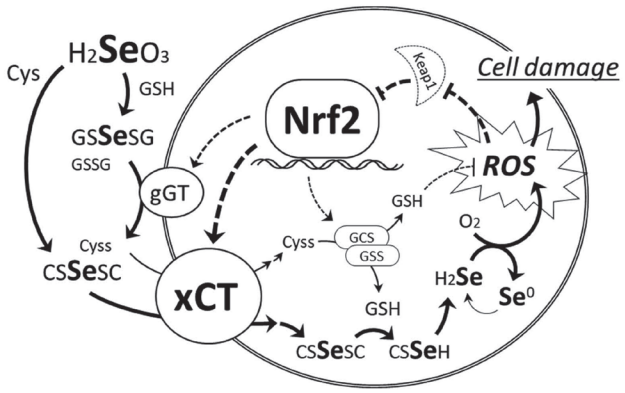

Fig. 3. Mechanism of Se Cytotoxicity Mediated by Nrf2-xCT Pathway

Possible mechanism for cytotoxicity of selenite is depicted. Se-induced oxidative stress activates the Nrf2-xCT pathway and forms a positive feedback loop that accelerates Se uptake. Detailed explanation is described in the Results and Discussion. Additional abbreviations are as follows: GSSG, glutathione disulfide; gGT, gamma-glutamyl transpeptidase; GCS, gamma-glutamylcysteine synthetase; GSS, glutathione synthase; GSSeH, GSH selenopersulfide; Se^0, elemental selenium; Keap1, Kelch-like ECH-associated protein 1.

explanation for the elusive toxicological properties of inorganic Se including strong cytotoxicity, high sensitivity in cancer cells, and narrowness of pharmacological dose range.

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Conflict of interest The authors declare no conflict of interest.

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