Selenium uptake through cystine transporter mediated by glutathione conjugation

Takao Tobe, Koji Ueda, Akira Aoki, Yoshinori Okamoto, Nakao Kojima and Hideto Jinno

Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan

(Received November 18, 2016; Accepted November 23, 2016)

ABSTRACT — Selenium (Se) is an essential trace element and is regarded as a protective agent against cancer. In particular, antioxidant effects of selenoenzymes contribute to cancer prevention. Se can also produce reactive oxygen species and, thereby, exert cancer-selective cytotoxicity. Selenodiglutathione (SDG) is a primary Se metabolite conjugated to two glutathione (GSH) moieties. SDG increases intracellular Se accumulation and is more toxic than selenous acid (H$_2$SeO$_3$), but the mechanisms for importing Se compounds into cells are not fully understood. Here, we propose a novel mechanism for importing Se, in the form of SDG. Cellular intake of Se compounds was assessed based on Se accumulation, as detected by ICP-MS. SDG incorporation was decreased in the presence of thiols (GSH, cysteine or their oxidized forms, GSSG and cystine), whereas H$_2$SeO$_3$ uptake was increased by addition of GSH or cysteine. Cellular SDG uptake was decreased by pretreatment with specific inhibitors against gamma-glutamyl transpeptidase (GGT) or the cystine/glutamate antiporter (system $x_c^-$). Furthermore, siRNA against xCT, which is the light chain component of system $x_c^-$, significantly decreased SDG incorporation. These data suggest an involvement of SDG in Se incorporation, with SDG processed at the cell surface by GGT, leading to formation of selenodicysteine which, in turn, is likely to be imported via xCT. Because GGT and xCT are highly expressed in cancer cells, these mechanisms mediated by the cystine transporter might underlie the cancer-selective toxicity of Se. In addition, the system described in our study appears to represent a physiological transport mechanism for the essential element Se.

Key words: Selenium uptake, Anticancer activity, Micronutrient, Selenodiglutathione, System $x_c^-$, Gamma-glutamyl transpeptidase

INTRODUCTION

Selenium (Se) is the most recently discovered essential element, functioning as a component of mammalian enzymes such as glutathione peroxidase (Rotruck et al., 1973). In addition to its nutritional importance, studies demonstrated pharmacological effects of Se supplementation against tumor development (Corcoran et al., 2004; Chen et al., 2013; Moustafa et al., 2013; Wallenberg et al., 2014).

Se produces reactive oxygen species (ROS) during its reductive metabolism (Seko and Imura, 1997; Tobe et al., 2015). Se-induced ROS generation caused DNA strand breaks followed by cell cycle arrest and apoptosis, leading to suppression of cancer growth (Peyroche et al., 2012; Tobe et al., 2015). Such antitumor mechanisms of Se were attributed to pro-oxidative effects of inorganic Se compounds, rather than antioxidative effects of organic Se compounds (Drake, 2006; Ramoutar and Brumaghim, 2010).

Because cancer cells are more sensitive to Se-induced cytotoxicity than non-cancer cells (Husbeck et al., 2006; Jariwalla et al., 2009), Se may be useful for cancer-selective chemotherapy. Also, combined with anticancer drugs, sodium selenite reduced their side effects, such as renal injury, in rats (Okhawa et al., 1988; Park et al., 2015). It also prolonged efficacy of cisplatin treatment, likely by preventing development of cisplatin resistance, in ovarian tumor xenografts (Caffrey and Frankel, 2012).

Se must be taken up into the cell before exerting its cytotoxic effects. Eukaryotic Se transporters have not been identified (Rosen and Liu, 2009). However, in a study using yeast cells, selenite ion (SeO$_4^{2-}$) uptake was catalyzed by Jen1p, a transporter for monocarboxylates,

Correspondence: Koji Ueda (E-mail: cozy@meijo-u.ac.jp)
such as pyruvate and lactate (McDermott et al., 2010). In lung cancer cells, production of selenide (HSe⁻/H₂Se), a membrane permeable selenite metabolite, was dependent on extracellular thiols, primarily cysteine and cystine, whose turnover involves the cystine/glutamate exchange transporter, system x₀ (Olm et al., 2009). Such HSe⁻ permeation is related to the cancer-specific toxicity of Se because reported expression of the x₀ antiporter was higher in many cancer cells and was correlated with their drug-resistance (Huang et al., 2005; Takeuchi et al., 2013). Neurons selected for resistance to oxidative stress, induced by glutamate, expressed higher levels of the light chain component of system x₀, xCT (Lewerenz et al., 2006). xCT is encoded by the SLC7A11 gene and confers transporter activity (Sato et al., 2000; Sharma et al., 2010).

Our previous study demonstrated that a Se trisulfide conjugated with glutathione (GSH), forming seleno-diglutathione (GS-Se-SG, SDG), decreased viability of MCF-7 human breast cancer cells after 24 hr treatment. In contrast, selenite (selenous acid, H₂SeO₃), known to be the most pharmacologically potent chemical form of Se, did not. Moreover, we observed that SDG remarkably increased intracellular Se levels in MCF-7 cells during the 3 hr preceding cell death, whereas H₂SeO₃ had no such effect (Tobe et al., 2015). Thus, SDG is likely to be relevant in Se cytotoxicity, although its cellular uptake mechanisms remain unclear.

In our earlier study, we estimated production of H₂Se from H₃SeO₃ via SDG. However, we did not demonstrate whether Se was imported into cells as H₂Se. The unusually high cytotoxicity of SDG, compared with H₃SeO₃, could not be explained only by permeability of H₂Se, a product equally produced from both these agents.

In the present study, we investigated the SDG uptake mechanism by measuring intracellular Se accumulation. We compared effects of extracellular thiols on Se uptake from H₃SeO₃ and SDG. We then examined effects of specific inhibitors or RNA interference against relevant transporters, including xCT. Our findings identified a novel Se uptake pathway.

**MATERIALS AND METHODS**

**Chemicals**

Selenium dioxide (SeO₂; dissolved in water to prepare H₃SeO₃), 60% nitric acid, sulfasalazine, gamma-glutamyl transeptidase (GGT) inhibitor (GGsTop), GSH, L-cysteine (CSH) and L-cystine dihydrochloride (CSSC) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Oxidized GSH (GSSG) was from Sigma-Aldrich Inc. (St. Louis, MO, USA). SDG was synthesized by reaction of GSH with H₃SeO₃, at a molar ratio of 4:1 under acidic conditions, as reported previously (Tobe et al., 2015). Briefly, 100 mM GSH was reacted with 25 mM H₃SeO₃ in 2.5 mM HCl. SDG was purified by preparative HPLC using an octadecylsilica (ODS) column eluted with a linear gradient from 0.05% formic acid in purified water to 100% methanol, over 1 hr at a flow rate of 0.8 mL/min monitoring at 265 nm. Isolated SDG was quantified by ^1H NMR and stored at -80°C until use. 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 (Wako). Cells were incubated at 37°C under a humidified atmosphere equilibrated with 95% air, 5% CO₂.

**Intracellular Se determination**

MCF-7 cells were seeded (10⁶ cells/well) in 2 mL DMEM and incubated for 24 hr. After washing with Dulbecco’s PBS without calcium and magnesium (D-PBS (–)), cells were exposed to Se compounds in Hank’s balanced salt solution (HBSS) buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 for 3 hr and then harvested by trypsinization, followed by washing with DMEM and D-PBS (–). Collected cells were degraded in 100 μL nitric acid for 20 min at 60°C. Then cell lysates were diluted to be 2% nitric acid by purified water. Se was analyzed on an inductively coupled plasma mass spectrometer (ICP-MS; model 7500c; Agilent Technologies, Santa Clara, CA, USA) with operating parameters as described previously (Tobe et al., 2015).

**Gene knockdown by RNA interference**

MCF-7 cells were seeded (2 x 10⁵ cells/well) in 2.3 mL DMEM and cultured for 24 hr in the presence of transfection reagent and 5 nM siRNA designed against human SLC7A11 (FlexiTube GeneSolution GS23657 for SLC7A11, Qiagen, Valencia, CA, USA) or a siRNA control sequence not targeting any mammalian gene (AllStars Negative Control siRNA, Qiagen). Knockdown was then assessed by real-time RT PCR.

**Real-time quantitative PCR analysis**

Total RNA was isolated using a spin column
(RNeasy mini kit, Qiagen) 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, used as an internal standard) were as follows: SLC7A11 forward, 5’-CCATGAACGGTGGTGTGTT-3’; SLC7A11 reverse, 5’-GACCCTCTCGAGACGCAAC-3’; RPS18 forward, 5’-GATGGGCGGCGGAAAATAG-3’; RPS18 reverse, 5’-GGGTGATTCGTGACATGTTT-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 for the corresponding treatment.

Statistical analyses

Each experiment was repeated at least three times. For all quantitative analyses represented in histograms, values are means ± S.E.M. The significance of differences between or among mean values was assessed using one-way ANOVA followed by Tukey’s test or Student’s t-test.

RESULTS

Clear advantages of SDG for cellular Se uptake

To determine cellular uptake efficiency of SDG, we measured Se accumulation in SDG-treated cells using ICP-MS. For cytotoxicity assays, we used HBSS buffer instead of culture medium during treatment, conditions that we found increased SDG stability. For SDG accumulation studies, we used 500 nM or less because these levels did not affect cell viability. Under these conditions, Se accumulation should reflect SDG uptake in living cells. Se levels were increased approximately 3.5-, 6- and 10-fold of control values in cells treated with SDG at 125, 250 and 500 nM, respectively. In contrast, there was no significant increase in Se accumulation in cells incubated with H2SeO3 at the same concentrations. These results indicated that SDG is a preferable chemical form, over H2SeO3, for cellular uptake of Se.

Interaction between SDG and thiols in Se uptake

Olm et al. (2009) showed that reductive metabolism by extracellular thiol compounds increased selenite uptake in lung carcinoma cells. These investigators concluded that selenide is the important chemical form for Se uptake, but they did not consider SDG, which is formed during the production of selenide. Although our results (Fig. 1) demonstrated increased Se uptake with SDG treatment, it is possible that Se uptake was influenced by cell surface microenvironment localizing thiol compounds. Therefore, we measured effects of added extracellular thiols on Se uptake in SDG-treated cells. MCF-7 cells were treated with excess concentrations (0.1 and 1 mM) of thiols (CSH, CSSC, GSH or GSSG) along with 500 nM H2SeO3 or SDG (Fig. 2). CSH and GSH increased Se accumulation when given with H2SeO3, but decreased Se accumulation with SDG. These results

Fig. 1. Selective uptake of SDG. MCF-7 cells were treated with H2SeO3 or SDG for 3 hr and Se contents measured by ICP-MS. Each column and bar represents the mean ± S.E.M. of at least three independent reactions. *p < 0.05, ****p < 0.0001 (vs. Control).
showed that SDG itself, rather than its reduced metabolites such as selenide, was important for cellular uptake of Se. CSSC and GSSG also decreased Se accumulation after SDG treatment, suggesting competitive inhibition based on their structural similarity to SDG (GS-Se-SG).

**SDG as a substrate for the cystine transport system**

Because CSSC and GSSG were likely to compete with SDG in cellular uptake, we investigated involvement of system x_c^- and GGT, by which extracellular oxidized thiols are transported into cells. GSSG is cleaved by GGT and dipeptidase sequentially, on the cell surface, to produce cystine, which is then imported via system x_c^- (Lewerenz *et al*., 2013). We examined whether SDG could be a substrate for GGT and system x_c^-; Se accumulation was decreased by specific inhibitors of xCT (the light-chain catalytic subunit of system x_c^-) and GGT (Fig. 3A). We also performed siRNA-mediated knockdown of SLC7A11, the gene coding for xCT. Real-time RT-PCR revealed an approximately 80% decrease in xCT expression by siRNA-SLC7A11, compared with the nontargeting siRNA (Fig. 3B). Consistent with this, Se accumulation in siRNA-treated cells was decreased to half the levels in untreated cells (Fig. 3C). These results demonstrated that SDG was processed at the cell surface by GGT and, subsequently, taken up into the cell through the cystine transporter system x_c^-.

**DISCUSSION**

The possibility that SDG uptake would be increased by extracellular thiol compounds was suggested by a report proposing a mechanism for H_2SeO_3 uptake assisted by extracellular thiols (Olm *et al*., 2009). However, instead, we found that SDG uptake was decreased by extracellular thiols. The reason was the consumption of SDG by its reactions with thiols, indicating involvement of SDG itself in the Se uptake mechanism. Although oxidized thiols do not react with SDG, both CSSC and GSSG also decreased SDG uptake. We attributed this result to competition between SDG and CSSC or GSSG in the process of cellular uptake. SDG uptake was, in fact, decreased by an inhibitor or siRNA against the CSSC transporter, xCT. Thus, we found that SDG was at least partially imported through system x_c^-; We also considered enhanced uptake of SDG into cells with higher xCT activity. High xCT activity was described in many kinds of cancer cells (Huang *et al*., 2005). Cancer cells were reported to increase GSH synthesis to balance their increased oxidative stress (Chandel *et al*., 2000; Narang *et al*., 2003; Huang *et al*., 2005). In addition, xCT expression, in most cases, has been associated with tumor growth and drug resistance (Banjac *et al*., 2008; Diaz *et al*., 2008; Jamali *et al*., 2015). In this manner, SDG would be expected to exert cytotoxicity selectively against cancer cells, especially those that are highly malignant.

Although we have proposed that the SDG import mechanism involves system x_c^-, a direct uptake of SDG
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through system x_c is unlikely because SDG is structurally quite different from CSSC, the originally reported xCT substrate. We, therefore, hypothesized that there was extracellular metabolism of SDG by GGT. GSSG is processed at the cell surface by GGT to form CSSC (Schulman et al., 1975; Hiraishi et al., 1994; Hanigan, 2014). If SDG were digested by GGT, the resulting metabolite, selenodicysteine (SDC, Cys-Se-Cys), would be structurally similar to CSSC. Our data using a GGT inhibitor supported the proposal that SDG is imported only after its conversion to SDC. GSH conjugation mediates not only detoxification but also toxicity (Monks et al., 1990). For example, uptake of GSH-conjugated methylmercury in the renal proximal tubules was significantly suppressed by a GGT inhibitor (Wang et al., 2012). It was suggested that various GSH-conjugated metals could be imported through several transport systems (Bridges and Zalups, 2005). The involvement of multiple transporters would explain why xCT inhibition produced only partial suppression of SDG uptake in our experiments.

Imai et al. (2014) demonstrated involvement of GSH in Se efflux from hepatoma cells, presumably by SDG formation. These investigators proposed that SDG-mediated Se excretion contributes to Se distribution, detoxification and homeostasis in the body. In our study, we added yet another role for SDG, in Se uptake.

In conclusion, Se is incorporated into cells via system x_c, in the form of a glutathione-conjugate that is first modulated by membrane-associated GGT. Identifying and characterizing Se uptake mechanisms should help develop pharmacological uses of Se-containing agents against cancer. Our findings should advance discovery of systems for delivering various pharmacologically promising elements to target organs. Although the nutritional significance of Se, an essential element, is clear, the regulation mechanisms influencing its metabolism remain to be elucidated. Our findings provide new insights into Se uptake mechanisms of Se, thus contributing to an overall understanding of Se metabolism.

ACKNOWLEDGMENTS

We are indebted to Dr. Kazuo Itoh and Dr. Yoshitaka Odo for use of ICP-MS. We would like to express gratitude to all the laboratory members who participated in this study for their technical support, especially Mr. Masayuki Hayashi, Ms. Shino Matsuura, and Ms. Mai Okawa. This work was supported by a Grant-in-Aid for Young Scientists (B) (grant number 23790161) and the Research Institute of Meijo University to K. U.
Conflict of interest--- The authors declare that there is no conflict of interest.

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