Further insight into the impact of sodium selenite on selenoenzymes: High-dose selenite enhances hepatic thioredoxin reductase 1 activity as a consequence of liver injury

By: Jinsong Zhang, Huali Wang, Dungeng Peng, and Ethan W. Taylor


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Abstract:

Selenium (Se) at supranutritional levels can enhance the activity of glutathione S-transferase (GST), whose gene is a target of nuclear factor erythroid-2 related factor 2 (Nrf2). Recent studies indicated that the thioredoxin reductase 1 (TrxR1) gene could also be targeted by Nrf2. Thus, high-dose Se may stimulate TrxR1 provided it enhances GST activity. Indeed, one study found that Se at supranutritional levels transiently increased hepatic TrxR1 activity. However, another study reported that supranutritional Se had no such effect on hepatic TrxR1 activity. In view of this discrepancy, the present research investigated whether high-dose Se has any impact on hepatic TrxR1. Moreover, we investigated whether Se preferentially activates GST over TrxR1. We observed that when sodium selenite (SS) caused liver injury, both hepatic TrxR1 activity and hepatic GST activity increased. Further experiments indicated that SS increased hepatic GST activity at either toxic or high but non-toxic dose levels; however, increase in hepatic TrxR1 activity occurred only at toxic levels, suggesting that enhanced TrxR1 activity correlates with liver injury. To corroborate this, we showed that hepatotoxic agents, thioacetamide or carbon tetrachloride, caused marked increases in hepatic TrxR1 activity. In conclusion, high-dose SS indeed can enhance hepatic TrxR1 activity, but only on the condition that it causes liver injury. High-dose SS affects hepatic GST more readily than hepatic TrxR1. Thus, the cancer-preventive mechanism of Se at non-toxic supranutritional levels relies more on its modulation of GST rather than TrxR1, at least in liver tissue.

Keywords: Thioredoxin reductase | Glutathione S-transferase | Sodium selenite | Liver injury

Article:

1. Introduction

Mammalian thioredoxin reductase (TrxR), a ubiquitous enzyme present in all living cells, is an NADPH-dependent homodimer oxidoreductase with one FAD and one selenocysteine per subunit (Nordberg and Arnér, 2001). The major substrate of TrxR is oxidized thioredoxin (Trx).
Moreover, TrxR can directly reduce many endogenous and exogenous compounds such as selenium (Se) compounds, dehydroascorbic acid, lipid hydroperoxides and H₂O₂ (Arnér and Holmgren, 2000). There are three forms of mammalian TrxR isoenzymes: cytoplasmic TrxR (TrxR1), mitochondrial TrxR (TrxR2), and testis-specific TrxR with additional glutathione (GSH) reductase activity (TGR), all being selenoenzymes that share the same principal domain structure (Nordberg and Arnér, 2001). The Trx/TrxR system plays an important role in the redox regulation of multiple intracellular processes, including Se metabolism, DNA synthesis, transcriptional regulation, cell growth, and resistance to cytotoxic agents that induce oxidative stress and apoptosis (Kumar et al., 1992, Nordberg and Arnér, 2001).

There are two different regulation pathways for TrxR1. First, as a selenoenzyme, TrxR1 activity in Se-deficient subjects increases in response to nutritional Se supplementation (Hadley and Sunde, 2001, Reeves et al., 2005). Secondly, the TrxR1 gene contains an antioxidant response element (ARE) in the promoter region that can be targeted by nuclear factor erythroid-2 related factor 2 (Nrf2). Thus, TrxR1 can be induced by certain oxidative stressors or electrophiles which drive Nrf2 into nucleus (Chen et al., 2005, Conterato et al., 2007, Hintze et al., 2003, Sakurai et al., 2005, Zhang et al., 2003). Like that of TrxR1, the promoter region of another selenoenzyme, gastrointestinal glutathione peroxidase (GI-GPx), also contains an ARE. Consequently, the GI-GPx gene can be activated by electrophiles (Banning et al., 2005).

Modulation of carcinogen metabolism by phase 2 enzymes, such as glutathione S-transferase (GST), is one of the most effective strategies for protecting animals against the neoplastic effects of carcinogens (Hayes et al., 2005, Talalay, 2000). The gene of GST is a well-established target of Nrf2 (Ramos-Gomez et al., 2001). There is a large body of evidence indicating that Se at supranutritional level can increase detoxifying activity of GST (Ip and Lisk, 1997, Sohn et al., 1999, Wang et al., 2007, Xiao and Parkin, 2006), thus GST induction by Se is a potential mechanism of its cancer-preventive action.

Since expression of both GST and TrxR1 is regulated by Nrf2, it is plausible that Se may stimulate TrxR1 provided it increases GST activity. However, reports about the impact of Se at supranutritional levels on TrxR1 are inconsistent. One study found supranutritional Se transiently increased hepatic TrxR1 activity (Berggren et al., 1999), whereas another reported it had no effect on hepatic TrxR1 activity (Ganther and Ip, 2001). In view of this discrepancy, one purpose of the present study was to investigate whether high-dose Se affects hepatic TrxR1. The second purpose was to investigate whether Se preferentially activates GST over TrxR1.

2. Materials and methods

2.1. Materials

Nicotinamide-adenine dinucleotide phosphate (NADPH), hydroxyethyl piperazine ethanesulfonic acid (HEPES), insulin, guanidinium hydrochloride, Trx (E. coli), and SS were obtained from Sigma (St. Louis, MO, USA). Other reagents used were the highest grade available. Elemental Se at nanosize (Nano-Se, with an average size at 36 nm) was prepared according to the method previously reported by us (Zhang et al., 2001).
2.2. Animals

Male Kunming mice (weighing 22–24 g), male C57BL/6 mice (weighing 22–24 g), and their diet were all purchased from Shanghai Laboratory Animal Center (SLAC). Se content in the diet used in SLAC and the present study was 0.3 ppm, indicating the mice were Se-sufficient according to the study carried out by Hadley and Sunde (2001) or by Reeves et al. (2005). Male Sprague–Dawley rats (weighing 180–220 g) were purchased from the Animal Center, Anhui Medical University, Hefei, PR China. Animals were housed in plastic cages with controlled temperature (22 ± 1 °C) and humidity (50 ± 10%) and 12 h light/dark cycle. Animals were allowed *ad libitum* access to diet and water.

2.3. Animal treatments

Kunming mice were randomly divided into two groups with eight mice per group. Group I was intraperitoneally (i.p.) injected with saline as control and group II was i.p. injected with SS at 2 mg Se/kg for 14 days. All mice were sacrificed 24 h after the last Se administration.

C57BL/6 mice were randomly divided into five groups with eight mice per group. Saline (as control), SS and Nano-Se at 2 and 4 mg Se/kg were administered by gavage once daily for 28 days. All mice were sacrificed 24 h later after the last Se administration.

Sprague–Dawley rats were randomly divided into two groups with eight rats per group. Group I was i.p. injected with saline as control and group II was i.p. injected with thioacetamide (TAA) dissolved in saline at 200 mg/kg, twice weekly. All rats were sacrificed after 6 weeks of treatment.

Sprague–Dawley rats were randomly divided into two groups with eight rats per group. Group I was i.p. injected with corn oil as control and group II was i.p. injected with carbon tetrachloride (CCl4) dissolved in corn oil (CCl4:corn oil = 1:4) at 5 ml/kg twice weekly. All rats were sacrificed after 6 weeks of treatments.

Peripheral blood was collected and centrifuged at 3000 × g for 10 min to isolate serum. Livers were excised immediately and rinsed in ice-cold saline. The samples were stored at −30 °C before assay.

All protocols involved in animal experiments complied with the guidelines of University of Science and Technology of China for the care and use of laboratory animals.

2.4. Biomarkers

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were estimated by spectrophotometry using commercially available kits. Livers were homogenized with ice-cold saline and centrifuged at 15,000 × g at 4 °C for 15 min. The resulting supernatants were used for biochemical assessments. Protein level was determined by the Bradford dye-binding assay with bovine serum albumin as standard. Superoxide dismutase (SOD) was assayed using the system of xanthine–xanthine oxidase and nitroblue tetrazolium
(NBT). One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Data are expressed as U/mg of tissue protein (Sun et al., 1988). Glutathione peroxidase (GPx) activity was assayed by the method of Rotruck et al. (1973). The activity of GPx is expressed as units/mg protein; a unit is defined as 1 μmol of glutathione oxidized/min. GST activity was chemically determined by using CDNB. One unit of GST activity is calculated in terms of nmol CDNB changed/min/mg of tissue protein (Habig et al., 1974).

TrxR1 activity was measured based on the method of Holmgren and Bjornstedt (1995) with some modifications. A stock mixture contained HEPES buffer (0.25 M), NADPH (2.5 mM), EDTA (10 mM), and insulin (1 mM), with the final pH 7.6. In a 96-well plate, 7 μl stock mixture, 3 μl Trx (0.17 mM), 40 μl HEPES (50 mM, pH 7.6), and 10 μl sample (with 20–30 μg protein) were added into a well. The enzymatic reaction was maintained at 37 °C for 20 min, and then was ceased by adding 240 μl termination solution (0.5 mM DTNB/6 M guanidine hydrochloride in 0.2 M Tris–HCl, pH 8.0). For each sample there was a parallel non-enzymatic reaction in which Trx was substituted by saline, but all other components were exactly same as the enzymatic reaction. The 96-well plates were read at 412 nm. The A_{412} increase was calculated by subtracting the absorbance of the non-enzymatic reaction from the absorbance of the enzymatic reaction. A background control, which was the subtraction of absorbance with and without Trx in the absence of sample, was further subtracted from the A_{412} increase. TrxR1 activity unit was defined as A_{412} change × 1000/min (Ganther and Ip, 2001) and was expressed as U/mg protein.

2.5. Histopathological observations

Immediately after the sacrifice of the animals, livers were excised and rinsed with ice-cold saline. Then they were fixed in 10% neutral-buffered formalin solution, and embedded into molten paraffin wax. Tissue sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and observed under a light microscope by an experienced pathologist who was blind to the treatments.

2.6. Statistical analysis

Data are presented as the mean ± S.D. The difference between groups was examined by double sample analysis of variance followed by Student's t-test (two-tailed tests) or one-way ANOVA with post hoc Tukey test when multiple groups were compared. A p-value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. SS induces liver injury and enhances hepatic TrxR1 and GST activities

Mice were i.p. injected with SS at 2 mg Se/kg for 14 days. After the first 4 days of injections, SS significantly suppressed growth of the mice (Fig. 1A), thus indicating Se intoxication had occurred because growth suppression is recognized as part of the profile of Se intoxication in mice or rats (Thorlacius-Ussing, 1990, Gronbaek et al., 1995). Liver tissue is a major target of Se intoxication (Diskin et al., 1979), so we examined liver injury after 14 days of injections. SS
significantly increased serum ALT and AST activities (Fig. 1B) and caused moderate hydropic degeneration in most hepatocytes (Fig. 1C).

**Fig. 1.** SS induces liver injury and enhances hepatic TrxR1 and GST activities. Kunming mice were i.p. injected with SS at 2 mg Se/kg for 14 days: (A) growth curve; (B) serum ALT and AST activities; (C) photomicrograph of liver tissue in SS-treated mice. Original magnification: H&E staining 100×. Arrows indicate moderate hydropic degeneration. (D) Redox parameters in liver. The average GPx, SOD, TrxR1 and GST activities in the control were 2138, 26.2, 105.8 and 4123 U/mg protein, respectively. Data are presented as mean ± S.D. (n = 8).

Excess SS possesses pro-oxidant activity through its ability to catalyze the oxidation of sulphydryls, by which it can inactivate some enzymes (Spallholz and Hoffman, 2002) such as delta-aminolevulinate dehydratase (Barbosa et al., 1998). It can also promote the formation of reactive oxygen species (ROS) (Spallholz, 1994). We found SS decreased SOD and GPx activities (Fig. 1D), two enzymes that are paramount for defense against ROS. The production of ROS due to excess SS (Spallholz, 1994) along with compromised free radical scavenging capacity (Fig. 1D) together indicates a state of oxidative stress. Oxidative stress can induce TrxR1 (Chen et al., 2005, Conterato et al., 2007, Sakurai et al., 2005). Not surprisingly, we found SS significantly enhanced hepatic TrxR1 activity by 80% (Fig. 1D). TrxR1 plays an important role in defending against oxidative stress (Nordberg and Arnér, 2001). TrxR1 also reduces SS
into elemental Se with lower toxicity (Kumar et al., 1992). Cells over-expressing TrxR1 are more resistant to SS-induced cytotoxicity (Madeja et al., 2005). Thus, enhancement of TrxR1 activity may serve as an important adaptive response to cope with excess SS.

In our experiments, increased hepatic TrxR1 activity cannot simply be ascribed to nutritional Se supplementation because (1) diet with 0.1–0.3 ppm Se is sufficient to saturate selenoenzymes in animals (Hadley and Sunde, 2001, Reeves et al., 2005), whereas dietary Se content in the present study was 0.3 ppm and (2) TrxR1 can be saturated more readily than GPx in response to Se supplementation (Reeves et al., 2005) since TrxR1 ranks higher than GPx in view of the hierarchy of selenoenzymes (Berry, 2005). Given that enhanced TrxR1 activity was a result of Se supplementation, GPx activity should also increase. In contrast, we observed GPx activity significantly decreased (Fig. 1D).

In addition to TrxR1, we also found SS significantly increased hepatic GST activity (Fig. 1D). As indicated earlier, GST activity can be enhanced by Se at high but non-toxic dose levels (Ip and Lisk, 1997, Sohn et al., 1999, Wang et al., 2007, Xiao and Parkin, 2006). As to TrxR1, the reported results are inconsistent (Berggren et al., 1999, Ganther and Ip, 2001). Thus we next investigated the impact of Se on TrxR1 at high but non-toxic dose levels.

3.2. Enhancement of hepatic TrxR1 activity depends upon liver injury

Mice were orally administered with SS at 2 and 4 mg Se/kg for 28 days. SS at 4 mg Se/kg caused significant growth suppression (Fig. 2A) and liver injury indicated by either ALT activity (Fig. 2B) or histopathological alterations, predominantly in the form of inflammatory cell infiltration (Fig. 2C). SS at 2 mg Se/kg neither caused growth suppression (Fig. 2A) nor induced liver injury as evidenced by serum ALT activity (Fig. 2B) and histopathological observation (not shown).

SS at 4 mg Se/kg significantly increased hepatic TrxR1 activity, by 190%, whereas at 2 mg Se/kg did not affect its activity (Fig. 2D). The enhanced TrxR1 activity cannot be ascribed to nutritional Se supplementation because 2 mg Se/kg by daily oral gavage (a dose at least 20-fold higher than nutritional supplementation) for 28 days did not increase TrxR1 activity as compared with the control, demonstrating TrxR1 activity in the control had already been saturated.

Consistent with the notion that GST activity can be enhanced by Se at high but non-toxic dose levels, herein SS at 2 mg Se/kg, which had no toxicity, significantly increased GST activity (Fig. 2E). These data demonstrate that hepatic GST can be induced prior to liver injury, whereas hepatic TrxR1 induction correlates with liver toxicity. Based on this notion, we inferred that at an equivalent high dose, a Se compound with less liver toxicity would not affect hepatic TrxR1. To test this hypothesis, Nano-Se was used, because our previous studies indicate it possesses noticeably lower toxicity as compared with SS, selenomethione and methylselenocysteine. However, the bioavailability of Nano-Se at nutritional levels is similar to these other Se compounds (Zhang et al., 2001, Zhang et al., 2005, Zhang et al., 2007, Wang et al., 2007). Moreover, the impact of Nano-Se at supranutritional levels on GST activity is equivalent to methylselenocysteine (Zhang et al., 2007), and appears to be even higher than SS and selenomethione (Zhang et al., 2005, Wang et al., 2007). In parallel with SS treatment, mice were orally administered Nano-Se at either 2 or 4 mg Se/kg for 28 days. In contrast to SS, Nano-Se at
4 mg Se/kg neither suppressed growth (Fig. 2A) nor caused liver injury (Fig. 2B) to the animals. As predicted, hepatic TrxR1 activity did not change (Fig. 2D). It must be pointed out that the lack of toxicity or increment in TrxR1 activity cannot be ascribed to the possibility that Nano-Se is inert in this situation, because it increased GST activity more efficiently than SS at both 2 and 4 mg Se/kg doses (Fig. 2E).

**Fig. 2.** Hepatic TrxR1 and GST activities at toxic and non-toxic dose levels of Se. C57BL/6 mice were orally administered with SS and Nano-Se at 2 and 4 mg Se/kg once daily for 28 days: (A) growth curve; (B) serum ALT activity; (C) photomicrograph of liver tissue in SS-treated mice. Original magnification: H&E staining 100×. Arrows indicate inflammatory cell infiltration. (D) Hepatic TrxR1 activity and (E) hepatic GST activity. Data are presented as mean ± S.D. (n = 8).

It is worth noting that a recent study reported that high but non-toxic doses of SS increased both TrxR1 and GST activities in liver (El-Sayed et al., 2006), whereas, under similar conditions, the present study observed only the latter. This discrepancy may be due to the assay method used for TrxR activity. We used the standard TrxR assay method with thioredoxin as substrate and oxidized insulin as the final electron acceptor (Holmgren and Bjornstedt, 1995). In El-Sayed's report, TrxR activity was estimated using the aurothioglucose-sensitive rate of reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) by NADPH. Furthermore, their data related to TrxR activity and mRNA were somewhat paradoxical. First, they used oltipraz, a chemopreventive agent, as reference. Oltipraz led to a fivefold induction of TrxR mRNA, but with no corresponding increase in TrxR activity. On the contrary, in their hands, SS did not induce TrxR mRNA, but
did produce an apparent increase in TrxR activity. Secondly, the control value of serum ALT activity in the mice they used was 44.43 ± 4.51 (mean ± S.E.M.); after oltipraz treatment, this increased to 61.53 ± 23.33. Although not significant statistically, the increase and the large S.E.M. of ALT in oltipraz-treated mice suggests that oltipraz had caused liver injury in a portion of the mice. Accordingly, TrxR mRNA markedly increased by fivefold due to liver injury. However, their observation that TrxR activity did not thereby increase seems unreasonable. Whether this was because of the assay method for TrxR activity is unclear. On the other hand, El-Sayed et al. claimed that SS at the prescribed doses and times did not cause liver toxicity, based only on ALT evaluation. The basal ALT activity in the mice they used was 44.43, which could be considered as a high basal value; although SS did not appear to significantly increase ALT activity beyond that level, one cannot totally exclude the possibility of liver injury, since according to our data, in mice with low basal ALT activity, SS only modestly but significantly increased ALT (from 20.8 to 34.7, Fig. 1B; from 23.8 to 33.8, Fig. 2B). However, a histological examination (which was not performed by El-Sayed et al.) revealed that the underlying liver injury was severe (Fig. 1, Fig. 2).

To further corroborate the hypothesis that the increase in hepatic TrxR1 correlates with liver toxicity, we next used the potent hepatotoxic agents TAA and CCl4 to generate liver injury in rats, to see if this was sufficient to produce a change in hepatic TrxR1 activity.

3.3. Both TAA and CCl4 enhance hepatic TrxR1 activity

Six weeks of TAA or CCl4 treatment caused severe liver injury, indicated by serum ALT and AST activities (Fig. 3, Fig. 4), as well as histopathological alterations, predominantly in the forms of coarse fibrous septa with inflammatory cell infiltration (TAA, Fig. 3B) and coarse diffuse steatosis (CCl4, Fig. 4B). In the mean time, both hepatotoxic agents significantly increased hepatic TrxR1 activity, by 86–100% (Fig. 3, Fig. 4).
Fig. 3. TAA enhances hepatic TrxR1 activity. TAA was i.p. injected twice weekly for 6 weeks: (A) serum ALT and AST activities; (B) photomicrograph of liver tissue in TAA-treated rats. Original magnification: H&E staining 100×. Arrows indicate coarse fibrous septa with inflammatory cell infiltration. (C) Hepatic TrxR1 activity. Data are presented as mean ± S.D. (n = 8).
In conclusion, although the genes of both GST and TrxR1 are targets for Nrf2, SS induces hepatic GST more readily than hepatic TrxR1. High-dose SS indeed can increase hepatic TrxR1 activity, but on the condition that it causes liver injury. The cancer-preventive mechanism of Se at supranutritional level thus is more likely to rely on its modulation of GST rather than TrxR1, at least in liver tissue.
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