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Selenium compounds are substrates for glutaredoxins: A novel pathway for selenium metabolism and a potential mechanism for selenium mediated cytotoxicity

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Running title: Glutaredoxins in selenium metabolism and toxicity

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Footnotes

Abbreviations: BSA, bovine serum albumin; BSO, buthionine sulfoximine; DL-Cys, DL-Cystine; DTT, dithiothreitol; FBS, Fetal bovine serum; GR, Glutathione reductase; GSH, Glutathione; GSSG, Oxidized glutathione; Grx, Glutaredoxin; HED, 2-hydroxyethyl disulfide; ROS, Reactive oxygen species; Se-DL-Cys, Seleno-DL-cystine; GS-Se-SG, Selenodiglutathione; Trx, Thioredoxin; TrxR, Thioredoxin reductase

Synopsis

The glutaredoxins (Grx) are oxidoreductases with a central function in maintaining the redox balance within the cell. In this study we have explored the reactions between selenium compounds and the glutaredoxin system. Selenite, selenodiglutathione and selenocystine were all shown to be substrates to human Grx1, implying a novel role for the glutaredoxins in selenium metabolism. During the last years, selenium has further evolved as a potential therapeutic agent in cancer treatment and a leading mechanism of cytotoxicity is generation of reactive oxygen species (ROS). Both selenite and selenodiglutathione were reduced by Grx1 and Grx2 in a non stoichiometric manner, due to redox cycling with oxygen, which in turn generated ROS. The role of Grx in selenium toxicity was therefore explored. Cells were treated with the selenium compounds in combination with transient overexpression and siRNA for Grx1. The results demonstrated an increased viability of the cells during silencing of Grx1, indicating that Grx1 is contributing to selenium toxicity. This is in contrast to thioredoxin reductase (TrxR), which previously was shown to protect cells from selenium cytotoxicity, verifying a diverse role between Grx and TrxR in selenium mediated cytotoxicity. Furthermore, selenium treatment led to a marked increase in protein glutathionylation and cysteinylation that potentially can influence the activity and function of several proteins within the cell.

Keywords

Cytotoxicity glutaredoxin, selenium metabolism, selenocystine selenite, selenodiglutathione

INTRODUCTION

Selenium compounds are known to decrease cell proliferation, cause DNA fragmentation and induce apoptosis in tumor cells at high concentrations [1]. Previous studies have also shown a high degree of selectivity towards malignant cells, where treatment with selenite (SeO_3^{2-}) demonstrated 80% cell death in primary malignant mesothelioma cells, while equimolar concentrations of selenite in the corresponding normal cells only had marginal effects [2]. Patient-matched pairs of primary normal and malignant prostate cells, selenite selectively killed the cancer cells, leaving the normal cells virtually unaffected [3]. One mechanism behind cancer specific cytotoxicity of selenite may be specific high uptake and accumulation in malignant cells due to an abnormally high reduced extracellular environment possibly connected to drug resistance [4]. In the intracellular environment reduced selenium in the form of selenide (RSe^-, HSe^-), undergoes redox cycling with oxygen and thiols, causing a rapid and massive non-stoichiometric ROS production [5, 6]. Unbalanced generation of ROS, such as superoxide, can in turn damage cellular constituents and consequently induce apoptosis [7]. The apoptotic response varies between different selenium compounds but is generally a caspase dependent process [8]. Selenium compounds have therefore been suggested as potent novel cytotoxic agents [9, 10]. Redox active selenium compounds are metabolized by GSH and the Trx-system. In these reactions HSe^- is formed for the syntesis of selenoproteins [11].

Glutaredoxins (Grx) are redox active proteins present in almost all living organisms [12]. In humans four glutaredoxins are presently known – Grx1, Grx2, Gx3 and Grx5 [13, 14]. Grx catalyze glutathione disulfide oxidoreductions via their active site consisting of two redox active cysteine residues. The glutaredoxin system is a coupled system comprised of glutathione (GSH) and glutathione reductase (GR) with NADPH as the source of reducing equivalents [12]. The activity of Grx is pH dependent as well as strongly affected by the intracellular redox state and more specifically on the GSH ratio (GSSG/GSH), which is the superior thiol buffer in the cell [15-19]. The oxidoreductions are either dithiol reactions with protein disulfides or monothiol reactions of mixed disulfides with GSH. The monothiol mechanism involves the ability to couple/uncouple protein cysteines with glutathione know as glutathionylation/deglutathionylation [20, 21]. Glutathionylation is rising as a key post-translational alteration, responsible for transducing oxidant signals and a sensor for the cellular redox balance [22, 23]. While protecting the cells from oxidative stress and maintaining the general redox balance, they are also involved in several other cellular functions, such as DNA synthesis [24], regulation of transcription factors [25, 26], regulation of cellular iron, including iron-cluster biogenesis [27] and apoptosis [28]. Grx has also been implicated in cancer with a clear upregulation in several tumors [9, 29-31].

The contribution of glutaredoxins in selenium metabolism and cytotoxicity, is largely unknown. Being a highly redox active protein catalyzing the reduction of disulfides and/or mixed disulfides makes it an ideal candidate. In this project we have used both pure *in vitro* systems and cell experiments to elucidate the role of glutaredoxins in selenium metabolism and toxicity.

EXPERIMENTAL

Material

PEST and RPMI 1640 medium was obtained from Invitrogen Corporation. Sodium Selenite (Na_2SeO_3), Se-DL-cystine, DL-cystine dithiothreitol (DTT), HEPES, bovine serum albumin (BSA), Glutathione (GSH), Glutathione reductase (GR), buthionine sulfoximine (BSO) and NADPH were all purchased from Sigma. Selenodiglutathione (GS-Se-SG) was obtained from PharmaSe Inc. Thiolyte (MBB) was purchased from Calbiochem. *Escherichia coli* (*E. coli*) Grx1, Grx1C14S, Grx3C65Y and Grx3C14S/C65Y, were kindly provided by Dr. Vlamis-Gardikas. Recombinant human Grx1 and the specific Grx1 antibody were obtained from IMCO Corporation, and recombinant Grx2 was purchased from Histoline.

Glutaredoxin activity

The Grx activity was determined as previously described [32] by measuring the reduction of 0.7 mM 2-hydroxyethyl disulfide (HED). A fresh mixture of 1 mM GSH, 0.2 mM NADPH, 2 mM EDTA, 0.1 mg/mL BSA and 6 µg/mL yeast glutathione reductase was prepared in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0.

The activity was determined by the oxidation of NADPH monitored at A_{340} using a molar extinction coefficient of 6200 $M^{-1}cm^{-1}$ at 20 °C. Measurements of selenium compounds as substrates were performed under identical conditions, but with the replacement of HED for the selenium/sulphur compound tested. The assay was adapted to a 96-well plate with a final volume of the reaction mix to 100 µL. For selenite, the GSH content of the mixture was reduced to 50 µM, to achieve a minimal background and thereby enable monitoring of the reaction.

Superoxide production

Mitochondrial superoxide production mediated by selenium treatment was measured by MitoSOX™ Red mitochondrial superoxide indicator from Invitrogen. Briefly; Cells were seeded on slides (+) and incubated for 24 h, following treatment of selenium compounds for 4 h. Staining of cells was performed according to manufactures instruction. A flourescense microscope (LEICA DM IRBE) was used for ROS detection and pictures were captured and analyzed with software Open*lab*5 impro*vision*® and Volocity 5.

Cell line and cell culturing

Experiments were performed using the well established human large cell lung carcinoma H-157. Cells were grown in 75 cm^2 Sarstedt culture flasks with 1640 RPMI medium supplemented with 10% FBS, without antibiotics added to the medium, under conditions of 37 °C and 5% CO_2. Prior to treatment, cell confluence was assessed by light microscopy to approximately 70-80%, and upon treatment culture medium was exchanged with fresh medium prepared with different xenobiotics.

Viability assay

Cell viability was performed using the Cell Proliferation Kit II (XTT) (Roche Molecular Biochemicals) according to the manufacturer instructions. In brief, cells were seeded in

96-well cell culture plates and left to adhere over night prior to treatment. Upon treatment, medium was replaced with 100 µL fresh medium containing the xenobiotic of choice and incubated for additional 20 h. Absorbance was measured in a micro plate reader at 490 nm with the reference wavelength, 650 nm, subtracted (Power Wave HT, Microplate Spectrophotometer from BioTek). Each sample was performed in octiplets and the experiment was repeated three times.

Transfection

Transient overexpression of Grx1 was performed with the pIRES/Grx-vector [33] and the empty pIRES control-vector, both kindly provided by Dr. Cedazo-Minguez, Novum, Sweden. The plasmids were amplified in competent *Escherichia coli* TOP-10F´strain, extracted and purified using the Midi-prep kit from Qiagen. H-157 cells were seeded in a 6 wells plate in 2.5 mL growth medium and incubated for 24 h. Cells were transfected with 1 µg plasmid using 3 µL Lipofectamine™2000 (Invitrogen), pre-diluted in OptiMEM1, following the instructions provided by the manufacturer. To reduce cell death, the transfection reagent was removed after 4-6 h, and the cells were washed with PBS prior to the addition of fresh medium. The following day, cells were treated with selected selenium compounds for 24 h.

Three different commercial Grx1 siRNA primers (ID 109163, 10839, 117030) were initially used for optimization, together with a scrambled primer with randomized nucleotides as a control (all from Ambion). Analyzing suppression of Grx1 mRNA level after 24 h, primer 3 (ID 117030) showed to be the most effective, and hence our choice of construct in all experiments. H-157 cells were seeded in 6-well or 96-well plates, and directly transfected with 10 nM siRNA using 3 µL NeoFX (Ambion), as transfection reagent. All components were pre-diluted in OptiMEM1 (Invitrogen), and incubated for 24 h. The cell viability was not affected by the transfection with scrambled siRNA.

Preparation of total cell extracts

Cell pellets stored for enzymatic analysis were dissolved in cold PBS. The cells were sonicated at intensity 40, 3 x 5 seconds (Vibra Cell, Sonics & materials Inc.). The supernatants were cleared by centrifugation at 25200 x g for 30 min at 2 °C. Total protein concentration was determined by the Bradford method according to the manufacturer instructions (BIO-RAD).

RNA extraction

Total RNA extraction was performed according to the RNeasy Plus Mini kit protocol (Qiagen), with on column DNase digestion. RNA-quantification was measured on a Nanodrop (NanoDrop® Spectrophotometer ND-1000). Synthesis of cDNA was performed through reverse transcription according to the Omniscript Reverse Transcription Kit protocol (Omniscript), with 2 µg RNA and 0.1 µg/µL oligo $(dT)_{12\text{-}18}$ as primer.

Quantitative PCR (qPCR)

qPCR was performed on a BIO-RAD ICycler with 20 ng of cDNA/reaction in triplicates on 96-well plates using Platinum SYBR Green qPCR super mix (Invitrogen). The final volume for each reaction was 25 µL. The qPCR program, including primers and

concentrations, were performed according to Olm et al. [9]. Results were analyzed using the $2^{-\Delta\Delta CT}$ method. C_T-value cut-off was set to 32 cycles. The efficiency of the different primer sets were 95% +/- 5%.

ELISA for Grx1

The assay was performed according to Lundberg et al [34] with some minor modifications. All steps were performed in a volume of 50 µL. The primary antibody (2 µg/mL) was diluted in carbonate buffer, pH 9.6 and plates were coated over night at 4 ºC. The secondary biotinylated antibody was added in a concentration of 1 µg/mL. Absorbance at 405 nm was measured using a Power Wave HT, Microplate Spectrophotometer from BioTek. Data were analyzed by the software Gen 5.

GSH depletion

To deplete cells from GSH cells were treated 24 h with 100 nM buthionine sulfoximine (BSO) prior to treatment.

Glutathione and cysteine determination

Samples for measurements of intracellular cysteine and GSH levels were prepared according to Cotgreave et al [35], and analyzed by the modified protocol by Luo et al [36].

Statistical analysis

Statistical analysis was performed for determination of significance, by using Wilcoxon matched pair test. For the analysis, Statistica (Statsoft® scandinavia AB) was used as software.

RESULTS

Selenium compounds are substrates to Glutaredoxins

In order to investigate whether Grx may reduce selenium compounds, specific activity of Grx was measured with various concentrations of selenite, GS-Se-SG, Se-DL-cystine and its sulphur analog DL-cystine. The reaction was initiated by the addition of Grx and NADPH consumption was monitored for four minutes. Grx1 reduced all substrates in a concentration dependent manner as shown in Fig. 1(A-D), where the background reaction of GSH is subtracted. While the reaction was stoichiometric with seleno-DL-cystine and DL-cystine (Fig. 1 C and D), the NADPH consumption with selenite and GS-Se-SG (Fig. 1A and B) continued beyond stoichiometric reduction, likely to selenide, caused by concomitant redox cycling with oxygen. The background reaction of the various compounds is illustrated in Fig. 2A, together with the reaction rate after addition of 1 µM Grx1. In order to detect superoxide and to investigate possible variations in ROS production caused by the different compounds (Fig. 2B), the cell line H-157 was treated for 4 h with selenite, selenodiglutathione and seleno-DL-cystine at IC_{50} concentrations prior to staining. Selenite and selenodiglutathione clearly triggered superoxide production, while seleno-DL-cystine did not. This also supports the *in vitro* findings where no redoxcycling with oxygen could be observed for seleno-DL-cystine.

Similar to Grx1, Grx2 also reduced GS-Se-SG (Fig. 3A) in a concentration dependent manner. The reduction by Grx1 and Grx2 was also dependent on the concentration of Grx (Fig. 3B-C), with the two proteins exhibiting similar reducing capacities. The activity of the human Grx1 was further compared with *E.coli* Grx1 and Grx3(C65Y) with similar activity observed between the two species (Table 1). To further study the mechanisms of Grx mediated reduction of selenium, *E.coli* Grx mutants were used. Two Grx mutants lacking the second cysteine residue in the active site, enabling them only to reduce mixed disulfides with GSH (Grx1C14S and Grx3C14S/C65Y) were used to distinguish between the two catalytic mechanisms of Grx, i.e. the monothiol and the dithiol. The NADPH consumption and activity was almost identical for the wild type and the active site mutants, demonstrating a monothiol reaction mechanism for the reduction of the selenium compounds (Table 1).

Differential toxicity among various selenium species

The IC_{50} for GS-Se-SG, selenite, selenate, Se-DL-cystine and DL-cystine were investigated using the cell line H-157 derived from human lung cancer. GS-Se-SG was the most cytotoxic compound with an IC_{50} at 3,5 µM followed by selenite at 4,5 µM and Se-DL-cystine at 100 µM (Table 2), after 24 h incubation. The sulfur analogues sulphite and sulphate which were also tested were at least 50 times less toxic (data not shown).

Selenite induces the expression of glutaredoxins

To explore the effects of selenite on glutaredoxins, H-157 cells were treated with 5 µM selenite over a period of 48 hours. RNA and protein fractions were collected at different time points for both treated and untreated cells. Grx1 and Grx2 mRNA levels (Fig. 4A and B), specific activities (Fig. 4C) and Grx1 protein levels (Fig. 4D) were upregulated at all timepoints examined. The mRNA levels for Grx1, Grx1as and Grx2b increased markedly after treatment compared to relative control up to 28 hours, (20-60 fold), while

Grx1 continued to increase even at 48 h. All other Grx variants decreased after 48 h. In the same manner, protein levels of Grx1 increased over time (Fig. 4D), as well as the amount of active protein, compared to the untreated cells (Fig. 4C).

Grx1 has a role in selenium cytotoxicity

To investigate the role of Grx1 in selenium cytotoxicity Grx1 levels in H-157 cells were altered by either siRNA or by transient overexpression. After optimization of conditions for knockdown of the gene, a decrease of Grx1 to 30% of normal activity after 24 h and 40% after 48 h, was accomplished (Fig. 5A). The overexpression of Grx1, resulted in an increase of the protein content of 16 fold at 24 h and 25 fold after 48 h, analyzed by ELISA (Fig. 5B). Transfected cells were treated with selenite, GS-Se-SG or Se-DL-cystine and viability was compared to mock treated cells (Grx1siRNA/scrambled, Grx1-vector/empty vector) respectively. Selenium treatment in combination with suppression of Grx1 resulted in a significant decreased cytotoxicity for all selenium compounds investigated (Fig. 5A). On the contrary, overexpression with Grx1 showed a tendency towards a slight increased cytotoxicity (Fig. 5B), although not statistical significant.

Glutathione depletion sensitizes cells to selenite

Glutathione in H-157 cells was depleted for 24 h by BSO, which resulted in a more than 90 % reduction of total GSH levels, while cysteine levels remained intact (data not shown). The remaining pool of GSH in BSO treated cells was approximately 10 % more oxidized compared to control cells, while the cysteine/cystine ratio had a 10% shift towards the reduced pool (data not shown). Cells treated with selenite in combination with a pretreatment with BSO were significantly more sensitive to selenite (Fig. 6A) compared to control cells. BSO had no intrinsic effect on viability.

Selenite treatment increases the protein bound glutathione and cysteine levels

Intracellular glutathione and cysteine levels were determined by HPLC after 5 hours incubation with 5 µM selenite, and compared to untreated cells (Fig. 6B). The total levels of cysteine increased by 70% after treatment with 5 µM selenite, while glutathione remained unchanged. The oxidized form of both cysteine and glutathione increased after selenite treatment with 2/3 and 1/3 respectively, resulting in a redox shift to a more oxidizing ratio (cysteine/cystine and GSH/GSSG). Protein bound cysteine and glutathione markedly changed with a 5 fold increase, compared to untreated cells. Suppression of Grx1 with siRNA did not influence the results notably (data not shown).

DISCUSSION

Selenium compounds are known to generate ROS and cause oxidative stress, which in turn may cause cellular damage. The glutaredoxin system is one central cellular defence system against oxidative stress, by maintaining the redox balance within the cell through the reduction of thiols. As the mechanism behind selenium cytotoxicity is not thoroughly explored, we aimed to study the interaction between selenium compounds and the glutaredoxin system.

Selenite, GS-Se-SG, Se-DL-cystine and DL-cystine, were all shown to be substrates to Grx1. The reduction of the selenium compounds implies a novel and important role for the glutaredoxins in selenium metabolism [10]. The reduction of selenite and GS-Se-SG is one significant step in the selenium metabolism for enabling the incorporation of selenium in selenoproteins. Even though this step can be carried out by GSH, GSH is far less reactive with the selenium compounds in comparison to Grx. By comparing the oxidation of NADPH however, it is clear that the reaction of the Trx system is more efficient than the Grx system in the reduction of GS-Se-SG *in vitro* [37]. Selenite on the other hand is more readily reduced by the Grx system. The high efficiency of Thioredoxin reductase (TrxR) can with all likelihood be addressed to the more redox active selenocysteine in the active site of TrxR, where a mutation at this site markedly affects the catalytic activity of TrxR [31, 38]. In addition, a modified Grx consisting of a selenocysteine in the place of the redox active cysteine exhibited an almost 2-fold increased specific activity in the catalysis of thiol-disulfide exchange reactions [39]. The efficiency *in vivo* may also vary between the systems, depending on the redox state of the cell. Nevertheless, our data in combination with previous results [37], clearly demonstrate that both of the leading redox systems in the cell are involved in selenium metabolism.

Incubation of selenite and GS-Se-SG with Grx led to a non stoichiometric oxidation of NADPH, indicating the formation of HSe^- and redox cycling with oxygen and ROS formation as illustrated in Fig. 7A. Redox cycles of HSe^- and oxygen have previously been demonstrated by mammalian TrxR and the thioredoxin system [37]. Similar to the thioredoxin system, the initial reaction by Grx1 with selenite and GS-Se-SG was rapid, followed by a slightly slower reaction. The initial oxidation of stoichiometric amounts of NADPH is consistent with the cleavage of the compound, followed by the latter non stoichiometrical continous reaction. On the contrary, Se-DL-cystine, and DL-cystine, was reduced by Grx1 stoichiometrically with a one to one oxidation of NADPH, indicating complete reduction of the compounds. The superoxide production seen after treatment with selenite and selenodiglutathione was also absent in cells treated with seleno-DL-cystine, indicating that seleno-DL-cystine exerts its cytotoxic effects through a different pathway, which is mediated by other mechanisms not directly involving ROS production.

It has been proposed that selenocystine as well as cystine instead will redox cycle with intracellular thiols to make new S-S bridges with proteins (intracellular thiols) [40]. Selenocystine may also be random incorporated in the place of cysteine (Fig. 7B). This may affect tertiary structure and active sites of redox enzymes [41].

Glutaredoxins are able to catalyze the reduction of proteins and low molecular weight substrates via a monothiol or a dithiol mechanism [12]. By comparing glutaredoxins with mutations in the active site and/or GSH binding site [42], we examined the mechanism by which GS-Se-SG was reduced. No difference was however seen by the NADPH

consumptions monitored, confirming that GS-Se-SG was reduced by Grx through the monothiol mechanism.
From the selenium and sulfur compounds investigated in terms of cytotoxicity, GS-Se-SG proved to be the most potent, closely followed by selenite. Both selenite and selenate were more toxic than their sulfur analogues, implying that the highly reactive selenium atom causes the more pronounced toxic effects [11][43]. The results further supports the hypothesis that growth inhibition and cell death caused by selenium compounds is mediated via the more redox active forms, such as selenite and GS-Se-SG.

To further investigate the influence of selenium on Grx, the lung cancer cell line H-157 was treated with selenite for 48 h, and both mRNA and protein were extracted. Protein levels, as well as protein activity and mRNA levels for both Grx1 and Grx2 were all increased over time, after treatment compared to untreated controls. Both protein level and protein activity increased equally over time, which suggests that selenite does not decrease the protein synthesis of Grx1, nor inhibit the activity. The induction of translation and transcription of Grx is most likely due to the oxidizing effects of selenite and the redox sensory role of the glutaredoxins [44]. The effect of selenium compounds after suppressing Grx1 with siRNA increased the cell viability with up to 20 % compared to control cells (scrambled). In addition, the opposite trend was seen when overexpressing cells with Grx1 followed by the same selenium treatment, implicating that Grx1 contributes to selenium cytotoxicity. As the other Grx´s are still present in the cell, and in particular the proposed tumor specific Grx2c located in the cytosol, the effect of Grx1 might be less apparent as it has similar properties compared to Grx1. The increased viability after suppression of Grx1 might be explained by the diminished redox cycling of Grx with these compounds, generating less mixed disulfides and ROS. Grx might also contribute to selenium cytotoxicity by the reduction of cystine to cysteine intracellularly, and through this mechanism contribute to a more efficient uptake and consequently a higher cytotoxicity [4] . In addition, the mode of catalysis by Grx is dependent upon the redox environment, where Grx can act as a glutathionylating protein under oxidizing conditions and as a deglutathionylating protein when oxidative stress subsides [23]. Our results showing several fold increase in cysteinylated and glutathionylated proteins, by selenium treatment, are remarkable and may be an important mechanism in the toxicity of selenium.

In a previous publication, cells treated for suppression of TrxR, with the specific gold inhibitor Auranofin, resulted in a pronounced sensitization to selenite as shown by decreased viability [45]. Furthermore, TrxR overexpression leads to a pronounced increase in the tolerance to selenite [46]. As a result TrxR, in contrast to Grx instead provides a protective role of the cells against selenium mediated cytotoxicity, showing a diverse role between the two systems.
Many mechanisms have been suggested for the cytotoxic effects of selenite. Here we demonstrate that the glutaredoxin system reduces selenium compounds *in vitro* enabling redox cycling with oxygen, generating ROS and thereby contributing to the toxic effects observed by redox active selenium compounds. This study thus reveals a novel function of the glutaredoxins in the pathway of selenium metabolism.

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Table 1
Comparison of Glutaredoxin activity
NADPH (nmol) oxidized/min (ΔA_{340}) in reduction of selenodiglutathione by glutaredoxins

Glutaredoxin	**Selenodiglutathione**	
(1 µM)	**15 µM**	**25 µM**
human Grx1	2.78	4.23
human Grx2	2.71	4.21
E.coli Grx1	2.53	3.93
E.coli Grx1 C14S	2.47	4.23
E.coli Grx3 C65Y	2.86	4.62
E.coli Grx3 C14S/C65Y	2.86	4.21

Table 2
IC_{50} values for selenium compounds
Determined in H-157 after 24 h of treatment

Compound	IC_{50} (µM)
Selenite	4.5
Selenate	250
Seleno-DL-cystine	100
Seleno-di-glutathione	3.5
DL-cystine	100

FIGURE LEGENDS

Figure 1 Selenium compounds as substrates for human Grx
The reaction was performed in TE-buffer containing, 200 µM NADPH, 6 µg/mL GR and 1 mM GSH (reaction A with selenite, contained 50 µM GSH). All reactions were performed with 1 µM Grx1 in the sample cuvette and with an equal volume of TE buffer in the reference cuvette. The specific selenium compound, in varying concentrations was added in both the sample and reference cuvette and the reactions were initiated by the addition of Grx1. The background reaction has been subtracted. NADPH consumption was monitored at A_{340}. (**A**) Selenite (**B**) GS-Se-SG (**C**) Se-DL-cystine and (**D**) DL-cystine.

Figure 2 Reduction of selenium compounds and superoxide formation
Reduction of 25 µM Se-DL-cystine (square), 25 µM DL-cystine (triangle) or 15 µM selenite (diamond) and 15 µM GS-Se-SG (circle) by 1 µM Grx1. Both the background reaction of the mix with glutathione (black) and the total reduction after the addition of Grx1 (white) are displayed in the graph. B) Cells (H-157) treated with various selenium compounds for 4 h followed by detection of superoxide production with MitoSOX^{TM}. (a) Control, (b) 5 µM Selenite, (c) 3.5 µM GS-Se-SG, (d) 100 µM Se-DL-cystine

Figure 3 Reduction rate with varying concentrations of selenodiglutathione (GS-Se-SG) and Grx
(**A**) The reactions were performed as described in figure 1, but with 1 µM Grx2 and varying concentrations of GS-Se-SG (10-25 µM). Reduction of GS-Se-SG (15 µM) was monitored with 1 and 5 µM of (**B**) Grx1 and (**C**) Grx2.

Figure 4 Grx levels in selenite treated lung cancer cell
Cell line H-157 was treated with selenite for 48 hours. Cells were harvested and analyzed after 24, 28 and 48 h treatment. (**A**) mRNA levels of Grx1 and (**B**) Grx2 were measured using quantitative PCR. (C) The specific Grx activity was measured with the HED-assay and (**D**) Grx1 protein levels were detected by specific sandwich ELISA. The SE is derived from three independent experiments. Statistical analysis was performed by Wilcoxon matched pair test (** $p < 0.01$ * $p < 0.05$).

Figure 5 Selenium cytotoxicity after downregulation and transient overexpression of Grx
(**A**) Cell line H-157 was transfected with siRNA for Grx1 or with a scrambled primer nucleotide sequence for 24 h, followed by treatment with selenite, GS-Se-SG or Se-DL-cystine. (**B**) Cell-line H-157 was transfected with the pIRES/Grx1-vector (Grx1V) or the empty pIRES vector (EV), for 4 h, washed and incubated for additional 20 h, following treatment with selenite (5 µM), GS-Se-SG (3.5 µM) and Se-DL-cystine (100 µM) for additional 24 h. Values represents the mean of at least three independent experiments. Wilcoxon matched pair test was used for statistical analysis (** $p < 0.01$ * $p < 0.05$).

Figure 6 GSH in selenium cytotoxicity
(**A**) Viability measured in H-157 cells after depletion of GSH with 100 nM BSO for 24 h and/or 5 µM selenite. (**B**) Intracellular levels of cysteine and glutathione were measured by HPLC, in H-157 cells after 5 h treatment with selenite (5 µM). (Black bars; untreated) (White bars: treated with 5 µM selenite). (T = total) (R = reduced form) (P = protein bound). Wilcoxon matched pair test was used for statistical analysis (* $p < 0.05$ in relation to control), (# $p < 0.05$ in relation to selenite).

Figure 7 Schematic overview of different selenium compounds illustrating their diverse reducing pathways. (A) Selenite and selenodiglutathione is reduced to selenide generating ROS products like superoxide, by cycling with oxygen. (**B)** Selenocystine as well as cystine, is reduced by intracellular thiols forming new S-S or mixed S-Se bridges within proteins.

Fig. 1 Wallenberg et al

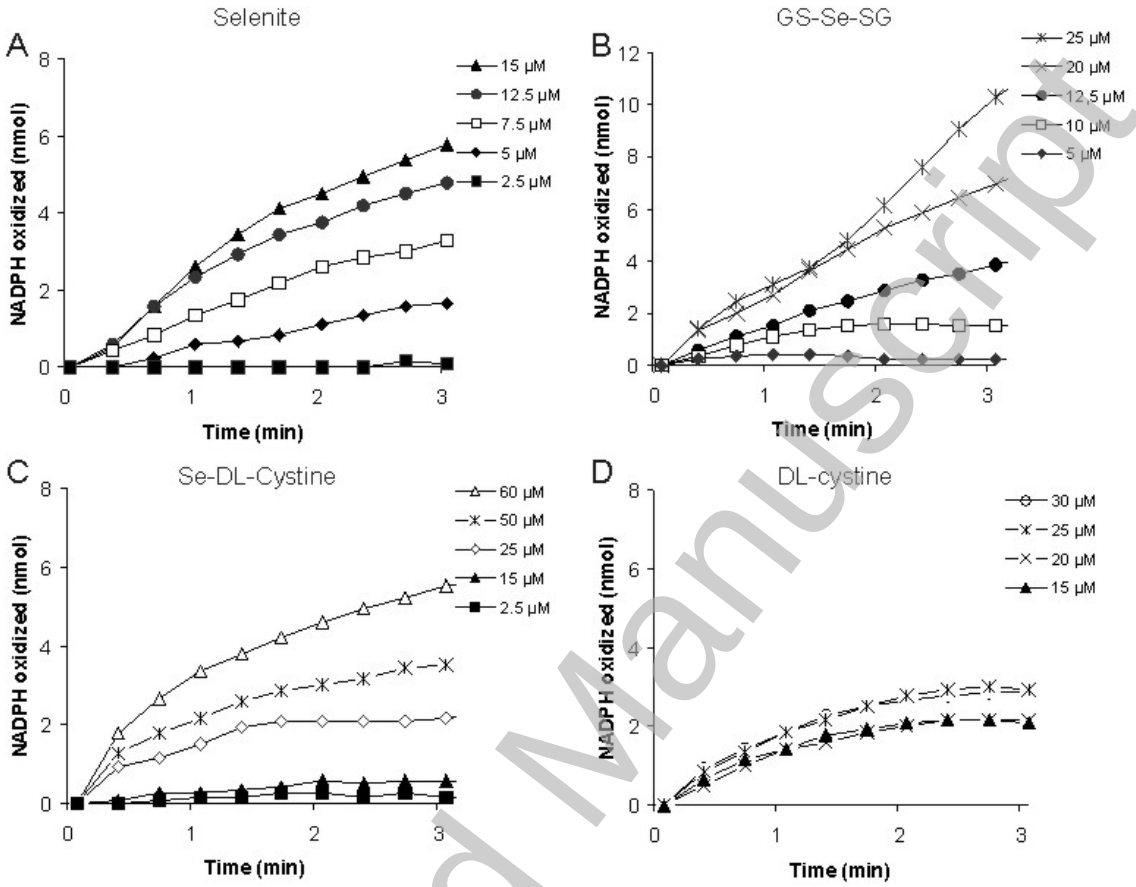


Fig 2 Wallenberg et al

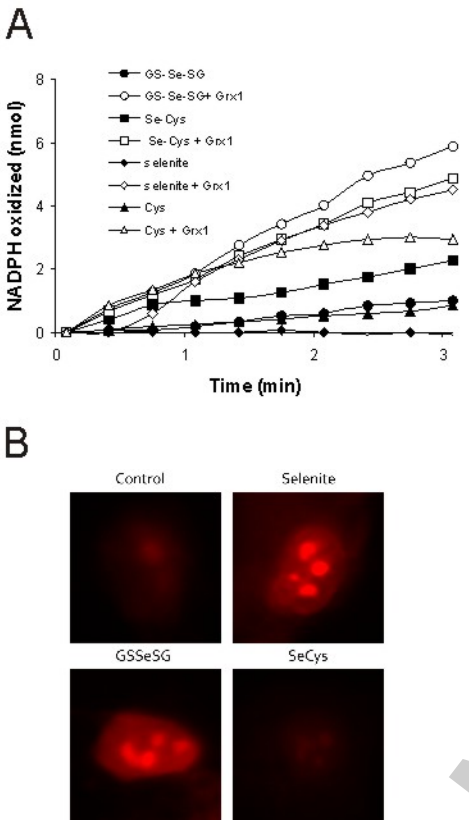
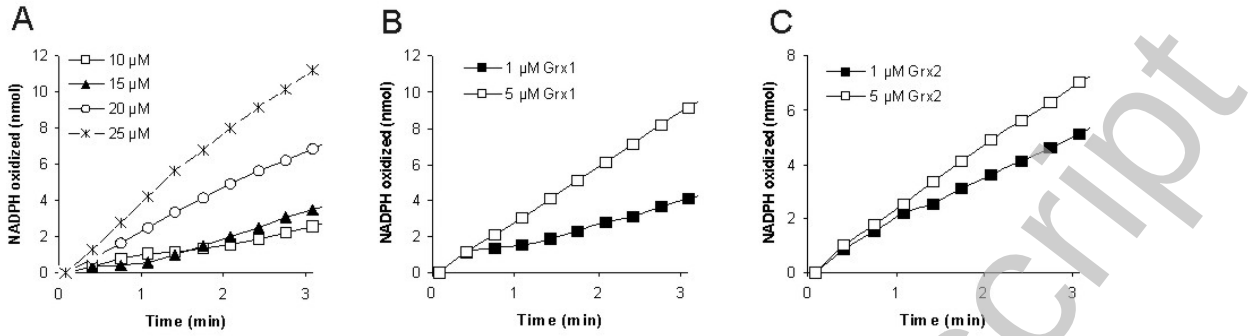


Fig 3 Wallenberg et al



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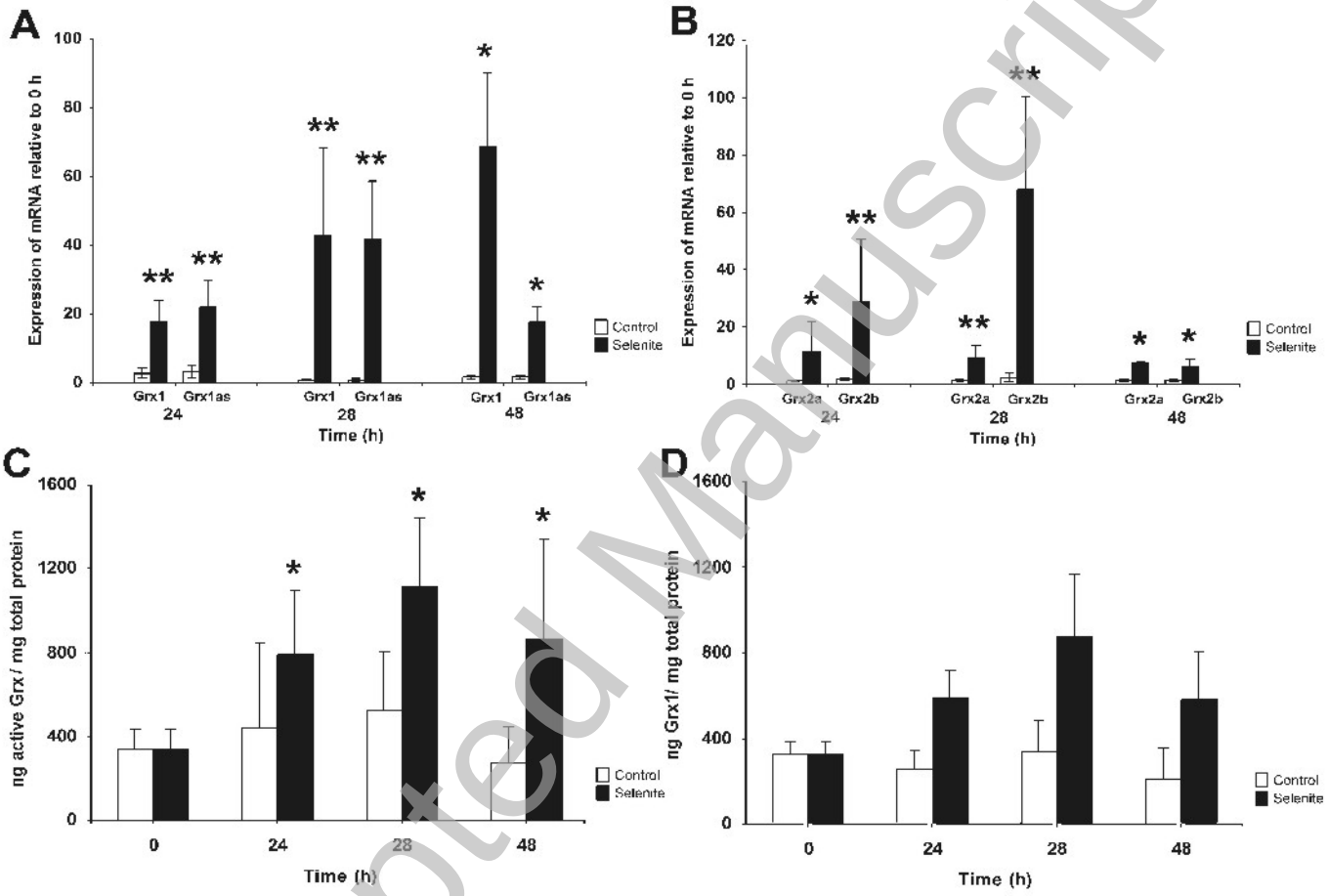

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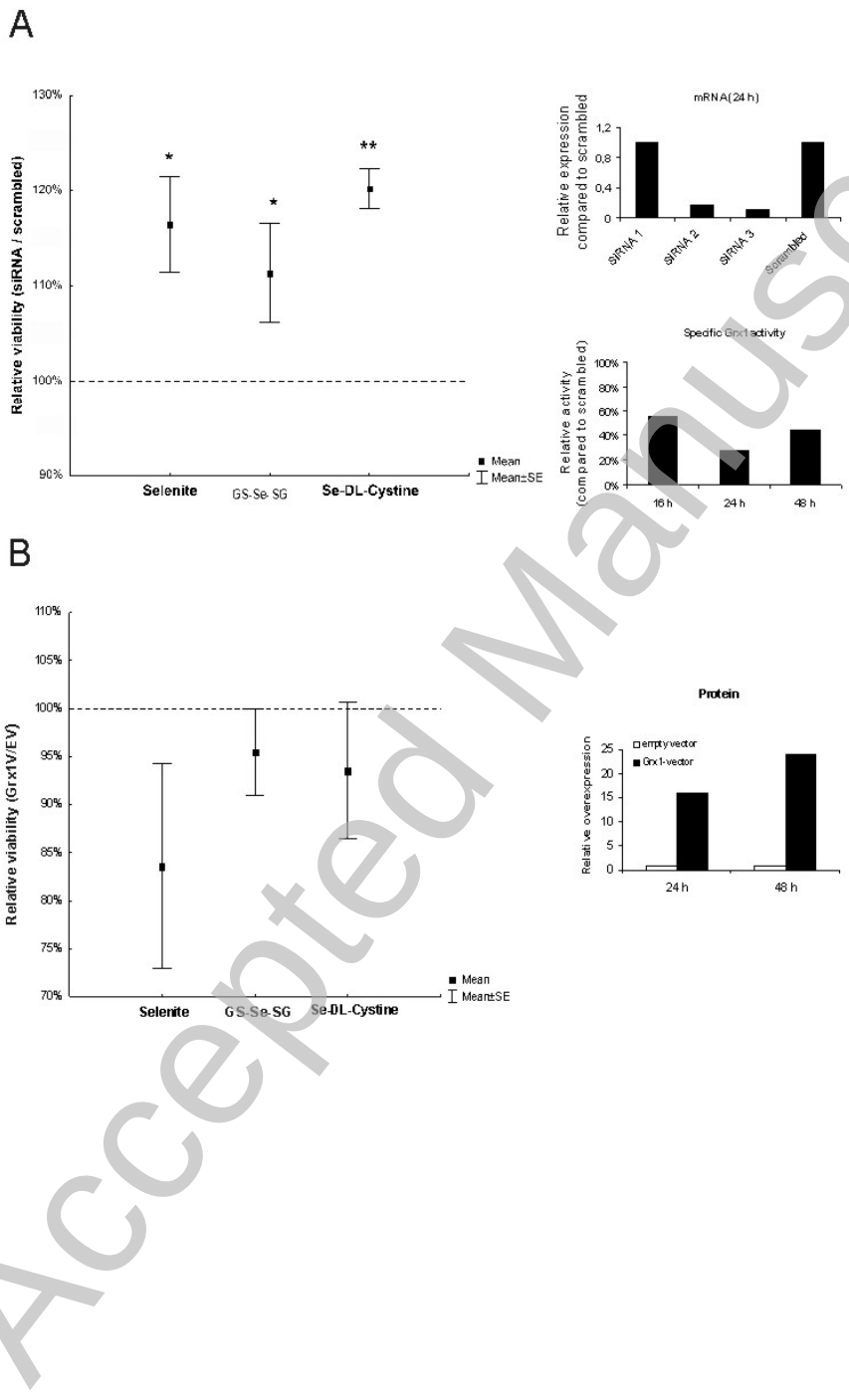

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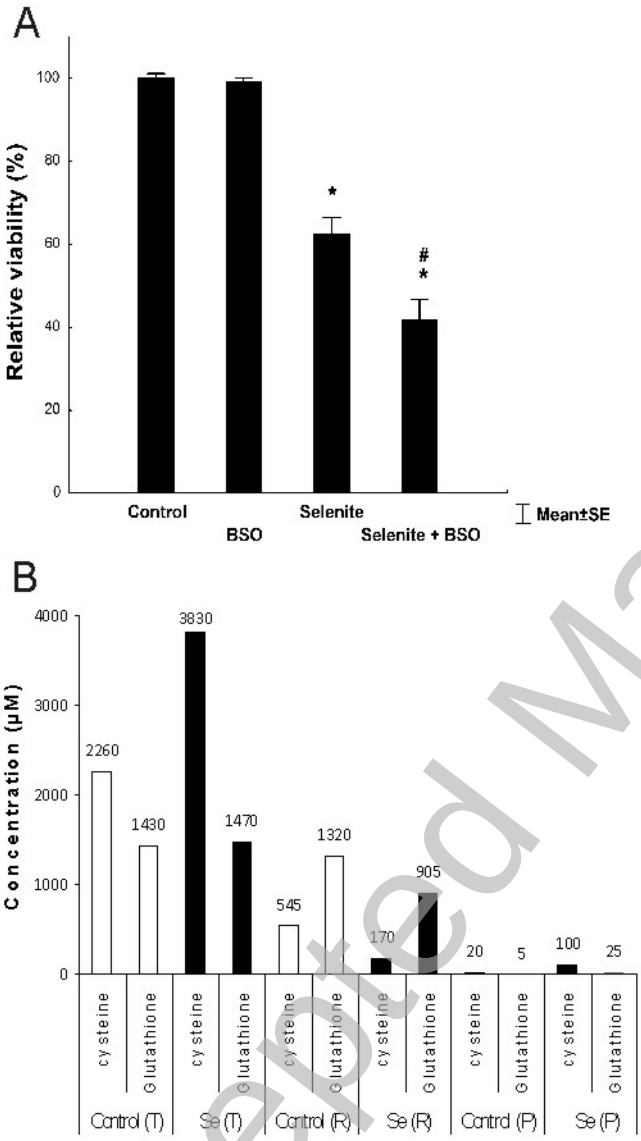

Fig 7 Wallenberg et al