Rapid induction of cell death by selenium compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine

Karin Anestål and Elias S. J. Arnér

Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77 Stockholm

To whom correspondence should be addressed.
Tel: +46-8-728 69 83, fax: +46-8-31 15 51, e-mail: Elias.Arner@mbb.ki.se

Running title: Selenium compromised TrxR1 is a cell death factor
Abstract

Mammalian thioredoxin reductases are selenoproteins. For native catalytic activity, these enzymes utilize a carboxyterminal -Gly-Cys-Sec-Gly-COOH sequence (where Sec is selenocysteine) forming a redox active selenenylsulfide/selenolthiol motif. A range of cellular systems depend upon or are regulated by thioredoxin reductase and its major protein substrate thioredoxin, including apoptosis signal regulating kinase 1, peroxiredoxins, methionine sulfoxide reductase and several transcription factors. Cytosolic thioredoxin reductase 1 (TrxR1) is moreover inhibited by various electrophilic anticancer compounds. TrxR1 is hence generally considered to promote cell viability. Several recent studies have however suggested that TrxR1 may promote apoptosis and the enzyme was identified as a Gene associated with Retinoid-Interferon induced Mortality (GRIM-12). Transient transfection with GRIM-12/TrxR1 was also shown to directly induce cell death. To further analyze such effects, we have here employed lipid-mediated delivery of recombinant TrxR1 preparations into human A549 cells, thereby bypassing selenoprotein translation to facilitate assessment of the protein-related effects on cell viability. We found that selenium-deficient TrxR1 having a two-amino acid truncated carboxyterminal -Gly-Cys-COOH motif rapidly induced cell death (38 ± 29% apoptotic cells after 4 hours, p < 0.005 compared to controls). Cell death induction was also promoted by selenium compromised TrxR1 derivatized with either cis-diaminedichloroplatinum (II) (cisplatin, CDDP) or dinitrophenyl moieties, but not by the structurally related non-selenoprotein glutathione reductase. In contrast, TrxR1 with intact selenocysteine could not promote cell death. The direct cellular effects of selenium compromised forms of TrxR1 may be important for the pathophysiology of selenium deficiency as well as for the efficacy of antiproliferative drugs targeting the selenocysteine moiety of this enzyme.
Introduction

Three separate mammalian thioredoxin reductases are known. These include widely expressed cytosolic TrxR1, mitochondrial TrxR2, and a third isoform mainly located in testis (1, 2, 3, 4, 5). All of these enzymes are selenoproteins and carry a selenocysteine (Sec) residue in a carboxyterminal redox active motif having the amino acid sequence -Gly-Cys-Sec-Gly-COOH. The Sec residue in this motif is essential for catalytic activity (2, 6). Together with the neighboring cysteine it forms a redox active selenenylsulfide / selenolthiol motif (7) that receives electrons from a redox active -Cys-Val-Asn-Val-Gly-Cys- motif present in the N-terminal domain of the other subunit in the dimeric holoenzyme (8). This N-terminal redox active motif is also present in other enzymes of the pyridine nucleotide disulfide oxidoreductase family, such as glutathione reductase and lipoamide dehydrogenase; as in all of these enzymes is this motif receiving electrons from a flavin prosthetic group that in turn is reduced by NADPH (7, 8, 9).

Beside glutathione reductase (10), cytosolic TrxR1 is believed to be a most important enzyme for control of the cellular redox state, antioxidant defense and redox

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1 Abbreviations used in this study: Sec, selenocysteine; TrxR1, full length Sec-containing thioredoxin reductase; truncTrxR1, Sec-deficient two-amino acid truncated TrxR1; rTrxR1, recombinant TrxR1 prepartion; GR, glutathione reductase; GSSG, glutathione disulfide; AIF, apoptosis inducing factor; ASK-1, apoptosis signal regulating kinase 1; DNBC, 1-chloro-2,4,-dinitrobenzene; dnp-TrxR1, dinitrophenyl-derivatized rTrxR1 made by reaction with DNBC; CDDP or cisplatin, cis-diamminedichloroplatinum (II); CDDP-TrxR1, rTrxR1 derivatized by reaction with CDDP; IFN-ß, interferon-ß
regulation of cellular processes. These diverse functions mainly derive from cellular actions by its main substrate, thioredoxin 1 (Trx1) that has a remarkable range of cellular activities (11, 12). A main function of TrxR1 is hence to reduce Trx1 and many of the intracellular functions held by reduced Trx1 are known to promote cell viability. Among these functions are inhibition of apoptosis signal regulating kinase 1 (13), regeneration of peroxiredoxins (14) and methionine sulfoxide reductase (15), with the latter enzymes playing central roles for cell (16) and organism (17) survival or ageing. Moreover, TrxR1 is readily inhibited by a number of electrophilic agents used as anticancer agents in clinical use, including quinones (18, 19) and cisplatin (20, 21), and the drug auranofin that is used against rheumatic disorders (22). The potential importance of TrxR1 as a clinical drug target was recently reviewed (23).

In light of such studies as those referred to above, that all in essence identify the selenium-dependent TrxR1 and the thioredoxin system as important for normal cell function and viability, it is most interesting that TrxR1 has also been shown to function as a cell death promoting factor. A Gene associated with Retinoid-Interferon-induced Mortality (GRIM-12), subsequently shown to be TrxR1, was reported to mediate the cell death effects seen in tumor cell lines treated with a combination of IFN-β and all-trans-retinoic acid (24). The identification of GRIM-12 was elegantly made by use of an antisense cDNA library expressed under control of an interferon-induced promoter in HeLa cells, screened for induced resistance to retinoid- and interferon-induced cell death, cloned in E. coli and subsequently ascertained for protective effects when transfected into MCF-7 cells (24). Thus, antisense TrxR1 cDNA expression protected MCF-7 cells from cell death induced by the retinoid-interferon combination, suggesting that TrxR1 activity was a prerequisite for the cell death effect. Subsequent studies clearly indicated that the cell death was apoptotic, dependent upon wild type Trx1 and involved both caspase-8 and p53 (25, 26, 27). A link between Trx1 and p53 was known
previously (28) and GRIM-12/TrxR1 was shown to be a prerequisite for retinoid/interferon-induced cell death, but notably not for cell death induced by TNFα, etoposide or vincristine (24). This reflects a need for a functional thioredoxin system in induction of apoptosis through p53 upon treatment of cells specifically with the retinoid/interferon combination (24).

We were, however, intrigued by the fact that transient transfection with TrxR1 constructs were shown to directly induce cell death in MCF-7 cells, with 25% cell death 72 hours post-transfection compared to 8% in vector controls (27) or, in a subsequent study, up to 40% cell death 40 hours post-transfection with similar results in T47D, COS-7 and HeLa cells (29). How could this selenoprotein, hitherto considered essential for cell viability, display such potent cell death-promoting effects? Considering the well known inefficiency in the mammalian selenoprotein translation machinery, which also has been demonstrated using transfection constructs for TrxR1 (30), we hypothesized that these cell death-promoting effects of TrxR1 might have been due to the formation of truncated selenium-deficient protein species. This assumption was corroborated by the fact that the Selenocysteine Insertion Sequence (SECIS) element in the 3'-untranslated region, necessary for TrxR1 selenoprotein production (2, 30), had not been included in the constructs directly promoting cell death (27, 29). Furthermore, selenocysteine in TrxR1 is as in all selenoproteins co-translationally inserted at a UGA codon, which normally confers termination of translation (1, 2, 30, 31). In case of GRIM-12, this UGA codon was initially interpreted as a termination codon and replaced with a nucleotide sequence encoding a c-myc tag (24). That construct has, nonetheless, subsequently been utilized for transfection studies with results interpreted to show that “wild type” thioredoxin reductase may induce cell death (27, 29, 32). In an attempt to scrutinize whether the direct deleterious effects by TrxR1 on cell viability could specifically be attributed to selenium compromised forms of TrxR1, we have here
made use of a recently developed methodology for lipid-mediated delivery of intact proteins into mammalian cells (33). This approach was chosen to avoid any production of a mixture of full-length and truncated TrxR1 that may result from inefficient selenoprotein synthesis using transfection experiments, even using constructs including the selenocysteine-encoding UGA codon and a correct mammalian SECIS element. As reported herein, we found that selenium compromised TrxR1, either a selenium-deficient truncated form or an enzyme derivatized at the selenocysteine residue, indeed rapidly induced cell death, whereas such direct effect on viability could not be seen using the full-length selenocysteine-containing enzymatically active form of the enzyme.

**Experimental Procedures**

*Chemicals and reagents*

BioPORTER protein delivery reagent was obtained from Gene therapy systems, fetal calf serum (FCS) came from Biotech Line AS, whereas Dulbecco's modified Eagle medium, L-glutamine and phosphate-saline buffer (PBS) were from Invitrogen and antibiotics came from BIO-Whittaker Belgium. GR (purified from yeast), GSSG, 1-chloro-2,4-dinitrobenzene (DNCB), CDDP, 2'-[4-ethoxyphenyl]-5-[4-methyl-1piperazinyl]-2,5'-bi-1H-benzimidazole (Hoechst 33342), 5-iodoacetamido-fluorescein (5-IAF) and propidium iodide (PI) came from Sigma Chemical Co.

*Preparation of different forms of recombinant TrxR1*

Recombinant selenocysteine containing TrxR1 (rTrxR1) was purified from an overproducing *E. coli* system as described previously (34) and full length TrxR1 was prepared from rTrxR1 using a recently developed technique based upon selective
binding to phenyl arsine oxide sepharose \(^2\). For fluorescence labeling, 5-IAF was conjugated to TrxR1 by prior reduction of rTrxR1 (1 µg/µl) in 50 µl 50 mM Tris-Cl pH 7.5, 2 mM EDTA, with addition of 11 µl 40 mM DTT in PBS buffer pH 7.4 at and incubation 4°C for 1 hour, followed by addition of 1 µl 80 mM 5-IAF incubation for additional 2 hours at 20°C. This reaction was stopped by addition of 5 µl 100 mM GSH. For DNBC derivatization (35), rTrxR (100 µg •1.8 nmol subunit) was preincubated with NADPH (250 nmol) 30 minutes at 20°C in 20 µl 50 mM Tris-CL, 2 mM EDTA, pH 7.5, whereupon 0.5 µl of 340 mM DNBC in ethanol was added and derivatization was allowed for 20 min at 20°C, hence producing dnp-TrxR1. Preparation of a DNBC control followed the same procedure as the preparation of dnp-TrxR1, except for excluding rTrxR from the reaction. CDDP-TrxR1 was prepared by incubating 20 µM rTrxR1 with 100 µM CDDP in DMSO in the presence of 1 mM NADPH in 50 µl 50 mM Tris-Cl, 2 mM EDTA, pH 7.5, for 20 min at 20°C. The CDDP control was prepared by the same procedure without addition of enzyme. To separate CDDP-TrxR1, 5-IAF-TrxR1 or dnp-TrxR1 from reductants, cofactor and unbound electrophilic reactant, the derivatized enzyme samples were run on Fast desalting PC 3.2/10 columns equilibrated with 50 mM Tris-Cl, 2 mM EDTA, pH 7.5 using the SMART/HPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein concentrations were determined by the Bradford method according to protocol from BioRad using bovine serum albumin as standard. The DNBC and CDDP controls were subjected to the same desalting procedures, with the fractions corresponding to the enzyme fractions used for the control cell experiments using equivalent volumes. To confirm labeling of TrxR1 with 5-IAF it was also analyzed on a Tris-Cl SDS-PAGE gradient 8-16% gel and fluorescent protein was detected by exposure of the gel to UV-light.

\(^2\) Rengby, Johansson, Carlson, Serini, Kårsnäs and Arnér; manuscript in preparation
Enzymatic assays

TrxR1 activities of different preparations were determined using the standard DTNB assay (36). NADPH oxidase activity was determined as described previously (35) by following A\textsubscript{340} in a reaction mixture with 500 µl 50 mM Tris-Cl, pH 7.5, 2 mM EDTA and 200 µM NADPH using 2.55 µM enzyme. The activity of GR was determined following the initial linear decrease of A\textsubscript{340} in a 500 µl reaction mixture containing 50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 200 µM NADPH and 2 mM GSSG. The NADPH oxidase activity of GR was determined according to the same protocol as for TrxR1.

Cell cultures and BioPORTER experiments

A549 (human lung carcinoma) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in an humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C. Cells were seeded in LabTec II chamber slides (0.7 cm\textsuperscript{2}/well) (Nunc, Denmark) 24 hours before incubation with BioPORTER, which was prepared according to the manufacturers protocol, shortly described as follows. Chloroform (250 µl) was used to dissolve the dried BioPORTER reagent present in one tube as delivered (catalog number BP502401, Gene Therapy Systems), which was vortexed 20 seconds and added in aliquots (2.5 µl) to the bottom of eppendorf tubes whereupon the tubes were left open in a hood to evaporate the chloroform. For our initial experiments, different quantities of each TrxR1 preparation (0.1 - 100 ng) were diluted in PBS to a final volume of 15 µL and were used to hydrate the aliquots with dry BioPORTER reagent by incubation 5 minutes at 20°C. In the experiments shown in Figure 4, only 100 ng of the TrxR1 preparations and 350 ng GR was used in each assay. Each tube was then briefly vortexed before addition of serum free cell medium to a final
volume of 250 µl. This medium preparation with BioPORTER/TrxR1 was added to the cells grown in chamber slides (10,000 cells per well), which had first been washed once with serum free media. Cells were subsequently incubated 4 hours at 37°C before microscopy assessment.

Microscopy and assessment of cell viability

The cells were washed three times with PBS prior to microscopy with a power Leica DMRB microscope equipped with Fluotar PL 10X and Fluotar PL 20X objectives with filters for DAPI, Rhodamine and FITC. A Hamamatsu digital camera 4742-95 was used to obtain the microscopic images. Cells treated with 5-IAF-TrxR1 were subjected to bright field and FITC-filtered images, whereas assessment of viability was performed with bright field, DAPI (for Hoechst staining) and Rhodamine (for propidium iodide staining) filters. For staining, the cells washed in PBS were first incubated for 15 minutes 20°C with Hoechst 33342 dye (5 µg/ml in PBS) whereupon propidium iodide was added (50 µg/ml from 5 mg/ml stock in PBS) and then cells were incubated for an additional period of 5 minutes. Cell morphology was assessed as follows: Viable cells were considered to have Hoechst-stained normal-sized smooth nuclei without red propidium iodide staining, while cells having red stained nuclei with either multiple bright specks of fragmented chromatin or one or more spheres of condensed chromatin with more compact nuclei than normal were judged to be apoptotic, whereas necrotic cells had red-stained smooth nuclei of roughly the same size as viable cells.

Results

The aim of this study was to introduce different forms of TrxR1 into mammalian cells and study effects on cell viability. As mentioned in the Introduction, use of a novel lipid-mediated delivery system (33), commercially available under the brand name BioPORTER, would by-pass inefficiencies in selenoprotein translation and thereby
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opens a possibility to study isolated effects of the selenium-containing compared to selenium-compromised forms of TrxR1. We wished to introduce into cells a) fully active selenocysteine containing TrxR1, b) truncated selenocysteine-deficient TrxR1 and c) TrxR1 derivatized with DNCB. The latter yields an enzyme devoid of the Trx1-reducing capacity but with an increased NADPH oxidase activity, producing superoxide (35). Furthermore, we also utilized TrxR1 derivatized with the clinically used antitumor agent cisplatin, which inhibits TrxR1 activity but does not induce its inherent NADPH oxidase activity (20). In order to have well-defined preparations of TrxR1 we made use of purified recombinant enzyme produced in E. coli either using a system tailored for heterologous production of the full-length selenoprotein or a construct without a SECIS element, thereby producing the two-amino acid truncated selenium deficient form of the enzyme (34). However, the system for production of recombinant selenoprotein results in a mixture of truncated and full-length enzyme (34). Therefore we have further isolated the full-length recombinant TrxR1 from the purified preparation, as described in Experimental Procedures. We also used the original preparation of rTrxR1 containing a mixture of full-length and truncated enzyme species. Furthermore, we analyzed effects of GR that is a non-selenoprotein enzyme closely related to mammalian TrxR1 in both structure (8) and primary sequence (2) but naturally lacking the C-terminal Sec-containing redox active center (2,7). We also found GR to have a greater inherent NADPH oxidase activity than TrxR1. The properties of the enzyme preparations used in this study are summarized in Table 1.

The BioPORTER technique has been shown to successfully introduce antibodies, dextran sulfates, phycobiliproteins, albumin, and functional enzymes like beta-galactosidase and caspases into living cells (33). However, it could not be used to introduce apoptosis-inducing cytochrome c (33), which contrasts the introduction into cells of cytochrome c using microinjection techniques (37). Therefore, we first used
fluorescently labeled TrxR1 in order to probe if the BioPORTER technique could be used for introduction of TrxR1 protein into living cells. Reportedly, the highest proportion of protein introduced into cells upon use of the BioPORTER technique was seen after four hours of incubation (33). In agreement with this, we could detect fluorescent TrxR1 in most cells after a four hour incubation period. A slight fluorescence at some cells also in the absence of BioPORTER seemed to derive from enzyme aggregating at the cell membrane, whereas inclusion of BioPORTER clearly led to a stronger and intracellular fluorescence in nearly all cells (Figure 1). We hence continued with our studies using the TrxR1 forms listed in Table 1, with the aim to analyze possible effects on cell viability.

The ratio of protein to BioPORTER reagent may affect the dynamics of the delivery system (33). Therefore we used different amounts of each TrxR1 form (0.1 ng, 0.5 ng, 1 ng, 10 ng and 100 ng) incubated with a fixed number of A549 cells, constant amount of BioPORTER reagent and a four hour incubation time. We then assessed effects on cell morphology and viability using Hoechst staining and propidium iodide staining after incubation of A549 cells, in the presence or absence of BioPORTER, with full-length TrxR1, truncated TrxR1 or the irreversibly inhibited dinitrophenyl-derivatized TrxR1. Apoptotic morphology was denoted as condensed nuclei being slightly propidium iodide-colored, cells with large and intensely colored nuclei were considered necrotic, whereas cells lacking propidium iodide staining were considered live cells (Figure 2). As this distinction between apoptotic and necrotic cells, however, is somewhat capricious, and since in all cases the clear majority of the dying cells had apoptotic and not necrotic characteristics, the statistic evaluation was performed on effects on total cell death (apoptotic + necrotic). We could not find any clear dose response curve in the effects using any of the TrxR1 forms. Such lack of clear dose-response curves and a certain degree of inter-assay variability in effects using the
BioPORTER reagent has also been seen by others, analyzing BioPORTER-mediated introduction of nucleoside kinases (Magnus Johansson, personal communication), likely to be due to a variability in the reconstitution and hydration of the original lipid reagent, (see Experimental Procedures for the method) leading to a variability in the ratios of reagent to protein. Nonetheless, we found a striking difference in cell death-promoting capacity when comparing the effects of selenium compromised TrxR1 derivatives with the enzymatically active selenium-containing form of the enzyme. Figure 3 summarizes the results of these experiments, showing that full-length TrxR1 could not produce any significant increase in cell death as compared to controls, whereas introduction of truncated TrxR1 resulted in a cell death of 38 ± 29% which was highly significantly increased as compared to controls (Figure 3). Introduction of dinitrophenyl-derivatized TrxR1 resulted in a similar increase of cell death as truncated TrxR1. Control experiments with sole DNCB solution treated identically but without protein gave no effects on viability, which excluded that the cell death induction was due to traces of DNCB left in solution and should therefore be attributed to the derivatized enzyme. The mixture of full-length and truncated TrxR1, being the recombinant TrxR1 as produced, gave a tendency to induction of cell death intermediate between that of fully active enzyme and the non-active enzyme species.

We next analyzed if TrxR1 derivatized with cisplatin could induce cell death in a manner similar to truncated or dinitrophenyl derivatized TrxR1, which indeed was the case (Figure 4A). This suggested that the NADPH oxidase activity of the derivatized enzyme seemed not to be an essential factor for the cell death induction, since truncated TrxR1 or CDDP-TrxR1, in contrast to dnp-TrxR1, lacked a significant increase in the inherent NADPH oxidase activity (Table I). We also analyzed the effects of a glutathione reductase preparation that had higher inherent NADPH oxidase activity than
the utilized TrxR1 preparations (Table I). We found no induction of cell death using this glutathione reductase (Figure 4B).

Discussion

The studies presented here show that truncated selenium deficient TrxR1, or forms of the enzyme compromised at the selenocysteine residue with either cisplatin or dinitrophenyl moieties, may rapidly induce cell death. The enzymatically active full length enzyme did not hold this property. Remarkably, the only difference between truncated and full length TrxR1 protein is the presence in the latter of the two final carboxyterminal amino acids (-Sec-Gly-COOH). In case of truncated TrxR1, and also CDDP-TrxR1, use of BioPORTER was needed for cell death to be provoked which demonstrated that the cell death-promoting actions of these TrxR1 derivatives were intracellular (Figures 3 and 4A). The dinitrophenyl-derivatized enzyme gave a similar induction of cell death in the presence of BioPORTER, but there was also a certain induction of cell death by dinitrophenyl-derivatized TrxR1 in the absence of lipid reagent (Figure 3). This may possibly suggest that extracellularly, dinitrophenyl-derivatized TrxR1 can have access to reducing equivalents, perhaps by interaction with other reductive proteins at the cell surface, so that its increased oxidase activity (Table I) producing superoxide (35), may also induce cell death when present on the outside of cells. Glutathione reductase with a higher NADPH oxidase activity (Table I) could not provoke cell death under similar conditions, neither extra- nor intra-cellularly (Figure 4B). All other forms of NADPH oxidases can thereby not mimic the mechanism for induction of cell death by dnp-TrxR1 in the absence of BioPORTER; this could be indicative of specific functions of TrxR1 at the cell surface.

How much TrxR1 derivative proteins were introduced into the cells using BioPORTER in these studies, in comparison to the endogenous TrxR1 levels in A549
cells? Based on TrxR1 determinations of others (38, 39) it can be estimated that A549 cells contain approx. 100 - 200 ng TrxR1 and 1.5 mg total protein per $10^6$ A549 cells. It was reported that BioPORTER may introduce 10 - 50% of the added protein into treated cells under optimal conditions (33). Since we utilized BioPORTER with 0.1 to 100 ng protein with 10,000 A549 cells, this results in the estimate that 0.01 - 50 ng TrxR1 could have been introduced into the cells. Endogenously 10,000 A549 cells contain approx. 1 - 2 ng TrxR1. Thereby the amount of TrxR1 derivatives introduced should have encompassed the endogenous TrxR1 levels with a wide margin both on the lower and upper end. Within the utilized range, however, we could see similar deleterious effects on viability using both the lower and higher amounts of selenium compromised TrxR1, albeit with a certain interassay variability. We believe this variability probably depended upon variable efficiency of protein delivery due to the BioPORTER technique, as further described in the Results section. However, since also substoichiometric amounts of TrxR1 derivatives, as compared to the endogenous TrxR1 levels, could induce cell death this suggests a rather specific signaling event.

The rapid induction of apoptotic cell death upon introduction of selenium compromised TrxR1 into the A549 cells within four hours is reminiscent of the 2- to 4-hour time-frame of apoptotic induction upon microinjection (37, 40, 41) or pinocytic loading (42) of cells with cytochrome c. This time frame should be compared to the cell death-promoting effects using transient transfections with vector constructs for GRIM-12/TrxR1, where 35% cell death was seen 40 hours post-transfection (29). As direct introduction of selenium compromised TrxR1 protein into cells by-passes the need for translation, it is natural that the cell death induction occurred faster compared to what is seen using transfection experiments.

What is the mechanism for cell death induced by different forms of TrxR1? From the published GRIM-12 studies, it seems clear that an intact thioredoxin system is a
prerequisite for a functional apoptotic machinery upon treatment of cells with a retinoid-interferon combination (24). The importance for an intact functional TrxR1/Trx system in induction of apoptosis after the retinoid-interferon combination clearly involved p53 modulation and caspase-8 activation (26, 27, 29, 43). The necessity of intact TrxR1 for p53 maturation was recently also demonstrated in a separate study showing derivatization of the selenocysteine in TrxR1 by electrophilic lipid derivatives, resulting in impaired maturation and function of p53 (44). The direct cell death-promoting effects of wild type TrxR1 that were proposed (27), however, were questionable to us and were the reason for the design of the here presented study. Since the transient transfection experiments with “wild type“ GRIM-12/TrxR1 shown to directly promote cell death (27, 29) in fact involved production of truncated TrxR1 (see the Introduction), some is already known about the death-promoting effects of truncated TrxR1. It seems that a preserved N-terminal redox active -Cys-Val-Asn-Val-Gly-Cys- motif is a prerequisite, because when this motif was replaced by a dipeptide -Gly-Ala- motif, the cell death-promoting effect was lost (29). Moreover, removal of the whole interface domain of TrxR1, i.e. the C-terminal third of the protein (2) governing subunit association (8), resulted in even further increased cell death-promoting effects as compared to solely truncated TrxR1 — called “wild-type“ in (27, 29) — while both the FAD and NADPH-binding domains (2, 8) were necessary to maintain in order to have a direct cell death-promoting effect (43).

It could be hypothesized that selenium compromised TrxR1 can interact with the endogenous selenium dependent thioredoxin system by formation of mixed complexes with endogenous Trx1 or TrxR1 subunits. If the endogenous thioredoxin system indeed would be inhibited in a dominant manner, apoptosis may be provoked both due to a reduced general antioxidant defense as well as direct activation of ASK-1, as proposed to be the possible downstream effects of general TrxR1 inhibition (12, 23). However,
induction of cell death by substoichiometric amounts (0.01 to maximum 0.1 ng
introduced into cells containing 1-2 ng endogenous TrxR1, at the lowest amounts
utilized; see above) suggests that selenium compromised TrxR1 can directly provoke
cell death with yet unknown mechanisms. One possibility is that selenium compromised
TrxR1 may function in a manner similar to (or connected with?) that of AIF. AIF is a
flavoprotein normally localized to the mitochondrial intermembrane space but which
when translocated to the cytosol and nucleus induces caspase independent apoptosis
(45, 46). Interestingly, both AIF (46) and TrxR1 (8) are 55-57 kDa flavoprotein
oxidoreductases with a structural fold similar to glutathione reductase. Moreover, the
importance of the native TrxR1 oxidoreductase activity for maintained cell viability (11,
35) is reminiscent of the fact that the redox activity of AIF also promotes cell viability
as a property separate from the apoptosis induction capacity (45).

We would like to conclude, based upon the findings presented here and a
thorough analysis of the design and results of the prior GRIM-12/TrxR1 experiments,
that we find no evidence that enzymatically fully active selenocysteine-containing
TrxR1 can have direct cell death promoting effects, as has been claimed (24, 25, 27, 29,
32, 43). In contrast, it is clear that truncated or derivatized selenium compromised forms
of TrxR1 may directly promote apoptosis. This fact is most interesting and may also
have medical relevance. It should be noted that truncated TrxR1 could possibly be
formed \textit{in vivo} under conditions of limited selenium supply, as has been shown in cell
lines (47). The direct cell death-promoting effects of truncated TrxR1 may thereby be
linked to some of the pathophysiology seen at events of selenium deficiency. Moreover,
as many chemotherapeutic drugs in clinical use inhibit TrxR1 (23), of which CDDP was
used in the present study, the possibility that such inhibition in a dominant manner
could directly induce cell death as a part of the therapeutic effect should be considered.
The importance for a functional thioredoxin system in maturation and activity of p53
and p53-mediated cell death (28, 44) requires an additional stimulus activating p53 for cell death execution to occur and is hence an alternative pathway in which the thioredoxin system is involved in apoptosis. Furthermore, the inhibition of ASK-1 and thereby inhibition of apoptosis by reduced thioredoxin (13) as well as antiapoptotic functions of peroxiredoxins being dependent on a functional thioredoxin system (16) result in a delicate balance between promotion of cell viability and induction of apoptosis, regarding effects mediated by the thioredoxin system. The different pathways for cell viability promotion or apoptosis induction that are affected by the status of the thioredoxin system are summarized in Figure 5. Further studies on the direct cell-death promoting effects of selenium compromised TrxR1 are certainly warranted and necessary to consider as an additional pathway for induction of apoptosis.

Acknowledgements

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References


### Table 1. Properties of the enzyme preparations used in this study

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<tr>
<th>TrxR1 preparation</th>
<th>Specific activity $^a$ [U/mg]</th>
<th>NADPH oxidase activity $^b$ [min$^{-1}$]</th>
<th>Estimated Se content [% of enzyme]</th>
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<td>TrxR</td>
<td>37.8 ± 2.6</td>
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<td>100</td>
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<td>0.31 ± 0.17</td>
<td>• 35 $^c$</td>
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<td>0.15 ± 0.09</td>
<td>0</td>
</tr>
<tr>
<td>dnp-TrxR1</td>
<td>&lt; 0.6</td>
<td>2.13 ± 0.83</td>
<td>• 20 $^d$</td>
</tr>
<tr>
<td>CDDP-TrxR1</td>
<td>&lt; 0.6</td>
<td>0.21 ± 0.01</td>
<td>• 35 $^e$</td>
</tr>
<tr>
<td>GR</td>
<td>159 ± 28</td>
<td>4.11 ± 1.5</td>
<td>0</td>
</tr>
</tbody>
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Enzyme activities shown are mean ± S.D. of three to four separate determinations.

$^a$ Specific activities of TrxR1 derivatives were determined using the classical DTNB assay, in which native enzyme homogeneously purified from mammalian tissues displays an activity of approx. 35 U/mg (36). The specific activity of GR was determined with 2 mM GSSG and 200 µM NADPH at pH 7.5, as described in Experimental Procedures.

$^b$ The NADPH oxidase activity was determined as described previously (35) and in Experimental Procedures.

$^c$ The rTrxR1 as purified from the bacterial production system is a mixture of full-length TrxR1 and truncated enzyme, with the Se content estimated from the specific activity. For further details, see (34).
d  Dinitrophenyl derivatization was performed with DNCB as described (35) using a preparation of rTrxR1 with 7 U/mg original specific activity as enzyme source.

e  CDDP derivatization was performed as described (20) using a preparation of rTrxR1 with 13.4 U/mg original specific activity as enzyme source.

f  Value derived from (48).
Figure legends

**Figure 1. Lipid-mediated delivery of TrxR1 into A549 cells.** The figure shows light microscopy (A) and fluorescence microscopy (B) images of cells incubated with fluorescent Alexa-derivatized TrxR1 in the absence (left) or presence (right) of *BioPORTER*. Note that fluorescence of TrxR1 was seen in nearly all cells upon use of the *BioPorter* reagent.

**Figure 2. Assessment of cell death-promoting effects.** For each experiment, summarized in Figures 3 and 4, cells with Hoechst-stained nuclei were counted and judged as either viable (lack of propidium iodide staining), apoptotic (Hoechst-stained condensed nuclei with propidium iodide stained spots) or necrotic (large nuclei intensely stained with Hoechst and propidium iodide stained). This figure illustrates such morphology assessment, with some of the cells judged to be apoptotic indicated with arrows, some of the viable cells indicated with open arrow heads and a necrotic cell indicated with an “n”. For the differences in induction of cell death by each form of TrxR1 or by GR, see Figures 3 and 4.

**Figure 3. Cell death-promoting effects by different forms of TrxR1.** A549 cells were incubated for 4 hours without addition of protein (“controls”), or with addition of fully active recombinant selenocysteine-containing TrxR1 (“TrxR1”), a less active recombinant TrxR1 preparation (“rTrxR1”), selenium-deficient two-amino acid truncated TrxR1 (“truncTrxR1”) and dinitrophenyl-derivatized TrxR1 (“dnp-TrxR1”) in the absence (“-BP”, open bars) or presence (“+BP”, filled bars) of *BioPORTER* reagent. For each experiment, all attached cells with Hoechst-stained nuclei within a microscopical field were counted and judged as either viable, apoptotic or necrotic as described in Figure 2. In cases of extensive cell death, cells also became more spherical and started to detach from the surface. Nonetheless, in each individual experiment 200 -
600 attached cells were evaluated. As described in the text, we found no clear dose-response curve using 0.1 – 100 ng of protein with fixed number of cells and amount of BioPORTER reagent. This graph therefore summarizes the combined results from 2 – 3 experiments for each dose of TrxR1 derivative, resulting in a total of fourteen separate experiments performed for each form of TrxR1 (i.e. in total 2800 - 8400 cells evaluated per treatment). All evaluations were performed by the same person, who at the time of assessment was unaware of the treatment the cells had been exposed to. The figure summarizes the total cell death (horizontal bars indicate mean ± S.D) for each form of treatment. Two-tailed heteroscedastic Student’s t test was used for a statistical evaluation in comparison to the cell death occurring in the relevant control cells (in presence or absence of BioPORTER). There was no significant difference in cell death between the two groups of control cells (p = 0.056). Statistically relevant increases in cell death are indicated in the figure with asterisks: * = p < 0.01; ** = p < 0.005. n = 14 for each treatment.

Figure 4. Effects of cisplatin derivatized TrxR1 and glutathione reductase on cell viability. In A), A549 cells were incubated with rTrxR1, truncTrxR1 or cisplatin derivatized TrxR1 in the presence or absence of BioPORTER and subsequently analyzed for viability and evaluated as described in the legend of figures 2 and 3. The CDDP control shows the effects of a control sample treated identically to “CDDP-TrxR1“, except that no enzyme had been added in the original sample. In B), an experiment comparing the effects of truncTrxR1 with fully active GR (see Table I) is shown. * = p < 0.01; ** = p < 0.005; n = 4 for each treatment in both A and B.
Figure 5. Summary of pathways affecting cell viability or apoptosis that involve the mammalian thioredoxin system. An enzymatically fully active thioredoxin system composed of intact TrxR1 and Trx1 (circled) normally promotes cell viability (a). It is moreover important for maturation and activity of p53 mediated apoptosis, which also requires an additional stimulus activating p53 (b). If thioredoxin is oxidized, as in excessive oxidative stress, ASK-1 may be activated and initiate apoptosis (c). As shown in the present study, selenium compromised forms of TrxR1 — formed either by derivatization at the Sec residue with electrophilic agents or possibly by formation of truncated enzyme at events of selenium deficiency — may also promote apoptosis in a direct and dominant manner (d). For references and further discussion, see the text.
Figure 1 Anestål & Arnér

<table>
<thead>
<tr>
<th>TrxR-Alexa - BP</th>
<th>TrxR-Alexa + BP</th>
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<tbody>
<tr>
<td>A</td>
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Figure 4 AnestáI & Arnér

A

B

Cell death (%)
Figure 5. Ancițăl and Arnér

Antioxidant systems
Redox regulation
Cell viability
Cell growth

TrxR1/Trx1

a) p53 stimulates
b) oxidative stress

c) oxidized Trx1

Apoptosis

p53

Selenium compromised

TrxR1

Selenium deficiency?

electrophilic compounds (BNCl, CDPD, etc.)

ASK-1

?
Rapid induction of cell death by selenium compromised thioredoxin reductase 1 but not by the fully active enzyme containing selenocysteine
Karin Anestål and Elias S.J. Arnér

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