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Role of Thioredoxin System in Cell Death Caused by Toxic Compounds

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To my family

献给我的父亲，母亲，先生和即将到来的宝宝

ABSTRACT

Thioredoxin systems, comprising Trx, TrxR and NADPH, are one of the major disulfide reductase systems, which is crucial in maintaining cellular redox balance in mammalian cells. TrxR is a selenoprotein with a Sec residue in its C-terminal active site. The low pK_a value and the easily accessible property of the Sec residue make TrxR a target of many electrophilic compounds, including some clinically approved drugs. TrxR exert most of its cellular function by reducing Trx. Through the substrates of Trx, or its interacting proteins, Trx plays important roles in DNA synthesis, cellular defense against oxidative stress, regulation of transcription factors and cell death pathways.

There are two distinct Trx systems in mammalian cells, Trx1 system located in cytosol and Trx2 system located in mitochondria. In Paper I we found that treatment with brilliant green (BG) can cause a dramatic decrease of Trx2 in the mitochondria and subsequent cell death. The natural amount of Trx2 in Hela cells are much higher compared to that in fibroblast cells. Down-regulation of the amount of Trx2 by using an siRNA method in both cell lines can greatly sensitize Hela cells towards BG toxicity, but not fibroblast cells, suggesting the importance of Trx2 for some cancer cells.

Different from Trx2, which only have two Cys residues in the active site; Trx1 has three additional Cys residues, Cys62, Cys69 and Cys73. Previous studies about the function of Trx1 are mainly focused on the active site cysteines. However, accumulating evidence showed that the three so called structural Cys residues also play important roles in regulating Trx1´s activities and functions. In paper II and IV, we focused on studying the impact of the second disulfide (Cys62-Cys69) on Trx1 activity. We show that Trx1 with two disulfides can be found in cells under high oxidative stress, and although it is not a substrate of TrxR, but it can be reduced by the glutaredoxin (Grx) system at the expense of GSH. In addition the formation of the second disulfide or only the disulfide between Cys62 and Cys69 disturbed the ability of Trx1 to reduce oxidized Prx1, and sensitized SH-SH5Y cells towards arsenic compounds inducing cell death.

In Paper III we characterized that GSH plus Grx2 can be a backup of TrxR and can reduce both Trx1 and Trx2 when TrxR was inhibited. Overexpression of Grx2 in Hela cells can protect cells from cell death induced by the inhibitors of TrxR.

Apart from Trxs, we also explored the role of TrxR as a target of the clinically applied anti-cancer drug mitomycin C and mercury. In paper V, we proposed that targeting TrxR as a new mechanism of mitomycin C´s action. In Paper VI, TrxR was shown to be a target of mercury, and selenium can reactivated the TrxR treated with mercury by a substitution mechanism.

In summary, in the thesis we stressed the role of Trx and TrxR in the cell death induced by the toxic compounds which are targeting the Trx system.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AF	Auranofin
AIF	Apoptosis inducing factor
AP-1	Activator protein-1
ARE	Antioxidant responsive element
ASK1	Apoptosis signaling-regulating kinase 1
BG	Brilliant green
BSO	Buthionine sulfoximine
Cys	Cysteine
dNDPs	Deoxyribonucleoside diphosphates
FAD	Flavin adenine dinucleotide
GCL	Glutamate cysteine Ligase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
NHE	4-hydroxynonenal
Met	Methionine
MetSO	Methionine sulfoxide
MMC	Mitomycin C
Msr	Methionine sulfoxide reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NDPs	Ribonucleoside diphosphates
NF-κB	Nuclear factor-κB
Nox	NADPH oxidase
Prx	Peroxiredoxin
Ref-1	Redox factor-1
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBP2	Thioredoxin binding protein-2 (also known as Txnip)
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TNF	Tumor necrosis factor
Txnip	Thioredoxin interacting protein
VDUP1	Vitamin D3 upregulated protein-1 (also known as Txnip)

1 INTRODUCTION

1.1 CELL DEATH PATHWAYS

Cells are considered to be dead after they pass the first irreversible phase or the so called "point-of-no-return"[1]. There are two major pathways of cell death to be clarified: apoptosis and necrosis. These two types of cell death pathways are classified according to the difference in the cell's morphological and biochemical properties. Generally from the morphological point of view, apoptotic cells maintain a functioning membrane throughout the whole process, and the cell shrinks by losing water; necrotic cells, lose their membrane integrity at a very early stage and then the cell swells due to the influx of water, sodium and calcium[1].

Apoptosis or programmed cell death needs stimulating signals from either inside or outside of the cells. When the signals are from inside of the cells (the intrinsic pathway), mitochondria play an important role in this kind of apoptosis. The intrinsic signal, which can be DNA damage, reactive oxygen species (ROS), as well as growth-factor depletion, first reaches mitochondria. Then, the proapoptotic members of the Bcl-2 family proteins are activated and start to form pores in the mitochondrial outer membrane[2], which then can facilitate the release of proapoptotic proteins from mitochondria, such as cytochrome c and apoptosis inducing factor (AIF). Cytochrome c can promote the formation of the apoptosome, and subsequently activate the caspase pathway[3].

The apoptotic signal can also come from outside of the cells, in a manner called extrinsic receptor mediated pathway. The receptors which can receive such stimuli are belonging to the tumor necrosis factor (TNF) receptor superfamily[4]. Upon the stimuli, a complex called Death Inducing Signalling Complex (DISC) is formed in the membrane and subsequently actives caspase-8[5,6]. the activated caspase-8 can either directly active caspase-3 or activate Bid, a proapoptotic member of Bcl-2 family, and initiate the mitochondrial signalling pathways[7].

Apoptosis is considered as a natural process which could occur in both physiological and pathological conditions. It is also a part of the normal maintenance process for the multicellular lives to remove "unhealthy" cells, for example, cells undergoing carcinogenesis[8]. In contrast of apoptosis, necrosis is considered to be an

unprogrammed, energy independent and toxic process of cell death. It is worth to mention, new findings suggest that necrosis, even not the whole process, is also regulated in some way[9,10].

1.2 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are the inevitable byproducts of aerobic metabolism, including a variety of molecules and free radicals derived from molecular oxygen[11]. It was reported that about 2% of electrons leak from the respiratory chain and form reactive oxygen species[11]. There are three major types of ROS: superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). Superoxide is the precursor of other ROS, and is formed when oxygen gets one electron leaking from respiratory chain. Superoxide cannot pass through the lipid bilayer membrane of mitochondria. However, superoxide can be easily converted into hydrogen peroxide by the catalyzing of superoxide dismutase (SOD)[12]. Hydrogen peroxide is not a radical molecule, but it can pass through membranes and diffuse freely in the cell. Hydrogen peroxide can be easily converted into highly reactive hydroxyl radical through a Fenton reaction (Reaction 1); at the same time the metal (Cu^+ or Fe^{2+}) is oxidized. The oxidized metal can also react with superoxide and convert it into an oxygen molecule (Reaction 2). The net reaction is called Haber-Weiss reaction (Reaction3). Hydroxyl radical is high reactive and can react with DNA, lipids, amino acids and carbohydrates[13].

$$Fe^{2+}\ (Cu^{+}) + H_2O_2 \longrightarrow Fe^{3+}\ (Cu^{2+}) + OH^- + HO^{\cdot} \qquad (1)$$

$$Fe^{3+}\ (Cu^{2+}) + O_2^{\bullet -} \longrightarrow Fe^{2+}\ (Cu^{+}) + O_2 \qquad (2)$$

$$O_2^{\bullet -} + H_2O_2 \longrightarrow HO^{\cdot} + OH^- + O_2 \qquad (3)$$

Besides the respiratory chain, the other sources of ROS are NADPH oxidase (Nox), glucose oxidase, lipoxygenases, nitric oxide synthase, xanthine oxidase and flavoprotein reductases[14].

1.3 THIOREDOXIN SYSTEM

Thioredoxin system, composing thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, plays an important role in maintaining cellular redox homeostasis and protecting cells from oxidative stress[15]. Trx executes its function by reducing the disulfide in its target proteins (Reaction 4), or binding to its substrate as a regulator. Oxidized Trx can then be reduced by TrxR, and NADPH is the ultimate electron donor for the system[16] (Reaction 5). There are two distinct thioredoxin systems in mammalian cells: Trx1 system mainly in cytosol and nucleus, and Trx2 system in mitochondrial matrix[17].

$$\text{Trx-(SH)}_2 + \text{Protein-S}_2 \longrightarrow \text{Trx-S}_2 + \text{Protein-(SH)}_2 \qquad (4)$$

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{TrxR} \text{Trx-(SH)}_2 + \text{NADP}^+ \qquad (5)$$

1.3.1 Thioredoxin

Thioredoxin was first isolated and characterized by Peter Reichard and co-workers from *E. coli* as the electron donor for ribonucleotide reductase (RNR)[18]. Trx1 is a 12 kDa globular protein ubiquitously expressed in various species. Besides being an electron donor for RNR, which is the rate limiting enzyme in the DNA synthesis by reducing ribonucleotide diphosphates (NDPs) to deoxyribonucleotide diphosphates(dNDPs)[19], Trx1 can also reduce methionine sulfoxide reductase and peroxiredoxins in cytosol, thus plays important roles in cellular defense against oxidative stress and regulating H_2O_2 signaling[20,21]. Trx1 were also found to interact with several transcription factors such as NF-κB, Ref-1, and p53[17,22–24]. The regulation of its substrates and the transcription factors will be discussed in detail later.

Mitochondrial Trx2 was first cloned and expressed as a 18 kDa mitochondrial protein with an N-terminal extension of 60 amino acids as a translocation signal[25]. Prx3 is the primary substrate of Trx2 in mitochondrial matrix, which is at the forefront of defending oxidative stress by eliminating excess hydrogen peroxide (H_2O_2). In addition, Trx2 can bind to ASK1 and regulate its function in mitochondria-dependent apoptosis[26,27]. Trx2 is also important in keeping mitochondrial permeability

transition[2,28]. Both Trx1 and Trx2 possess critical role in cell survival, and the mice with homozygous knockout of Trx1 or Trx2 both showed early embryonic lethality[29,30].

Both Trx1 and Trx2 belong to thioredoxin fold protein family[31]. In both structure, there is a central core of five β-strands which are surrounded by four α-helices (Fig. 1)[32]. The highly conserved active site (-Trp-Cys-Gly-Pro-Cys-) at the N-terminal of helix 2 (Cys32 and Cys35 in orange in Fig. 1). Besides the active site cysteine residues, there are three additional cysteine residues in Trx1, Cys62, Cys69 and Cys73. Cys73 protrudes from the surface, and it is known that under oxidative stress Trx1 can form homodimer through a disulfide formed by Cys73 of two Trx1 molecules[33,34]. After dimerization, Trx1 will lose its activity due to the active site is not accessible to TrxR anymore[34]. Dimerization of Trx1 can also be found in cells under oxidative stress, but the physiological function of the dimer is not clear[35].

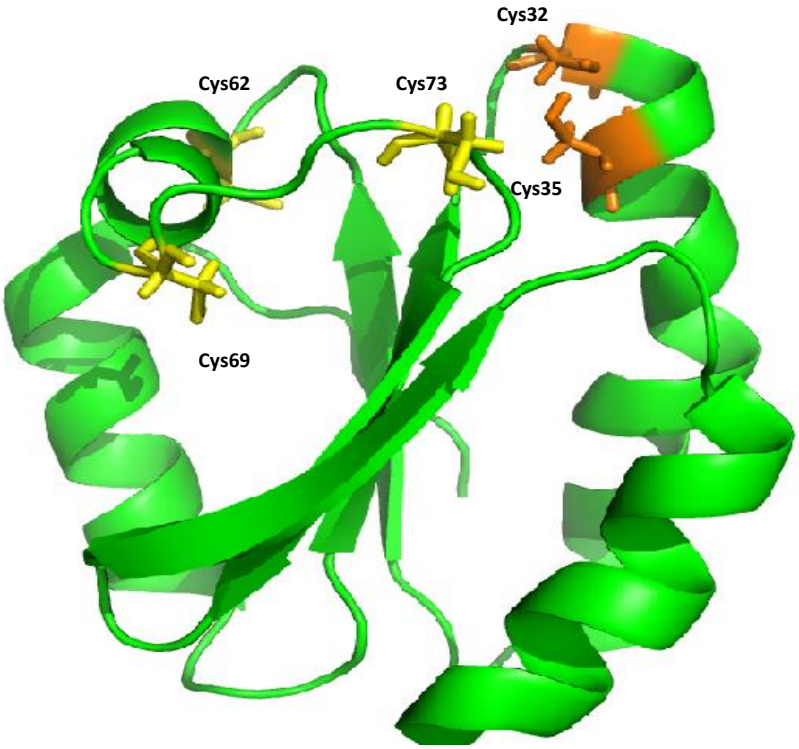


Figure. 1 Crystal structure of human Trx1[32]

Cys62 and Cys69 can form a second disulfide in Trx1, and accumulating evidence showed that the second disulfide may also play an important role in regulating Trx1´s function. Trx1 with two disulfide loses its function as a disulfide reductase and cannot be reduced by TrxR any more[36,37]. A recent finding in our group showed that Trx1 with two disulfides can be found in A549 cells under very oxidizing environment, and it is a

substrate of Grx system, both *in vitro* and in cells[38]. In our ongoing study (Paper IV), we found that the Trx1 with two disulfides or with only the disulfide between Cys62 and Cys69 is weakened the efficiency of reducing oxidized Prx1. All these findings suggested the biological function of the second disulfide in Trx1, although the exact mechanism is still unclear.

1.3.1.1 Thioredoxin Substrates

1.3.1.1.1 Ribonucleotide Reductase (RNR)

RNR is the rate-limiting enzyme in DNA synthesis and repair by reduction of ribonucleotides (NTPs) to deoxyribonucleotides (dNTPs)[19]. Mammalian RNR is composed of two subunits: R1 and R2. R1 expresses constantly in the cells, while R2 only present during the S phase, which make it to be the rate limiting factor for enzyme activity. Each cycle of reduction of NTPs to dNTPs results in a disulfide between the active site Cys residues in R1 subunit. However, the narrow structure of the active site in R1 does not allow the access of Trx1 to reduce it directly[39]. But two Cys residues located in the mobile tail of the C-terminal of R1 can be reduced by Trx1 and then transfer the reducing equivalent to the active site disulfide[40]. Evidence from mutagenesis of *E. coli* R1 subunit suggest the critical role of C-terminal cysteines in reducing R1 active site[41,42]. Our result using the peptide containing 25 amino acid residues from C-terminal of R1 subunits also proved that it is the substrate of Trx1 (*unpublished data*).

Additionally, both Trx and Grx can provide electrons for RNR in mammalian cells, but through different mechanisms. By using recombinant mouse RNR, Trx1 and Grx2 showed very similar catalytic efficiency, but the Grx activity largely depended on GSH concentration, which suggest a GSH-mixed disulfide mechanism for Grx instead of the disulfide exchange mechanism for Trx[43].

1.3.1.1.2 Peroxiredoxin

Peroxiredoxin (Prx) was first identified as a substrate of Trx by Dr. Sue-Goo Rhee and his group in 1994[44]. Besides its ability of removing access H_2O_2 to protect cells from oxidative stress, Prx is also proposed to serve the function to control the redox signaling

through sensing and regulating H_2O_2[45–47], which was justified as a messenger molecule more than just evil oxidant. There are six Prxs in mammalian cells (Prx1 to Prx6), and they are classified into three groups: Prx1 to Prx4, which all possess an active site comprising conserved N-terminal cysteine and C-terminal cysteine, are classified into the 2-Cys subfamily; Prx5, also known as atypical 2-Cys Prx, which have very similar N-terminal cysteine sequence compare to the 2-Cys Prxs, but lack the C-terminal conserved sequence containing cysteine residue[48]. The Cys151 serves the role as the C-terminal cysteine in Prx5. Prx6, which only have one cysteine residue, is termed as 1-Cys Prx[49].

The typical 2-Cys Prxs are present as homodimers in mammalian cells. The Cys-SH in the N-terminal of one subunit is very sensitive to H_2O_2 and can be easily oxidized into Cys-SOH, which then reacts with the C-terminal Cys-SH of another subunit to generate an intermolecular disulfide. Then the disulfide can be reduced by Trxs. All four members of 2-Cys Prxs are substrates of Trxs[50]. In Prx5, which also exists in a dimer form, the N-terminal Cys residue is oxidized into Cys-SOH, which then react with the Cys151 in the same subunit and forms an intramolecular disulfide[48,51]. Prx5 can also be specifically reduced by Trx. Prx6, however, is not a substrate of Trx, because it cannot form disulfide upon oxidation[52].

1.3.1.1.3 Methionine Sulfoxide reductase

Both free methionine (Met) and methionine in proteins are easy to be oxidized into methionine sulfoxide (MetSO) under mild oxidative condition. According to different asymmetric forms, the oxidation of Met can result in either Met-(S)-SO or Met-(R)-SO. The oxidation of Met into MetSO affects the protein functions[53]. The methionine sulfoxide reductase (Msr) can reduce both the free and protein-bound MetSO residues. According to their different substrate specificities, there are two types of Msrs: MsrA which can reduce the Met-(S)-SO; and MsrB which reduces Met-(R)-SO[54,55].

The catalytic mechanism of Msrs is quite similar to atypical 2-Cys Prx. First, the "catalytic" Cys residues interact with the sulfoxide group of Met and form a sulfenic acid intermediate. Then the second Cys residue referred as "recycling" Cys residue comes into play and results in the formation of an intramolecular disulfide between the two Cys residues, which can then be reduced by Trx[54].

1.3.1.2 Transcription Factors regulated by Trx

The regulation of many transcription factors by Trx mainly depends on the activity of Trx to reduce the cysteine residues in the DNA binding domain of these transcription factors, directly or indirectly.

1.3.1.2.1 Nuclear Factor –κB (NF-κB)

Various oxidative stresses, such as UV and H_2O_2, and some toxic compounds such as cigarette smoke and lysophosphatidic acid can induce the activation of NF-κB[56,57], which controls several inflammation genes. Under normal condition, NF-κB binds with IκB as an inactive complex in cytosol. Under oxidative stress or other type of stimuli, the IκB is phosphorylated by IκB kinase. Upon the phosphorylation, IκB is degraded and NF-κB is released to be free. The free NF-κB can then translocate from cytosol to nucleus to exert its function as a transcription factor[58–60].

In nucleus, binding of DNA requires the reduced Cys62 in its p50 subunit. The reduction of Cys62 requires reduced Trx, on the other hand, oxidized Trx inhibits the binding of NF-κB to DNA[23,61]. In the cytoplasm, however, Trx plays distinct roles in regulation of NF-κB activation by preventing the dissociation and degradation of IκB from NF-κB[62].

1.3.1.2.2 Redox Factor -1 (Ref-1) and Activator Protein – 1(AP-1)

AP-1 controls the transcript of many gene involving in cell growth. The DNA binding activity of AP-1 is dependent on Trx's activity; however, Trx1 does not directly interact with the Cys residues in Ref-1, but exert its reducing power through a redox factor called Ref-1. *In vitro* experiments showed that Trx1 can form a heterodimer with Ref-1 through its active site Cys-32. Upon the dissociation of Ref-1 and Trx1, Ref-1 can reduce Cys residues in the DNA binding domain of AP-1 (Fos and Jun subunits)[63,64].

1.3.1.2.3 Tumor Suppressor p53

After activated by series of stress signals, such as oxidative stress, hypoxia, DNA damage agents, *etc.*, p53 controls the transcription of proteins related to cell cycle arrest, DNA repair, and apoptosis. The mutation of p53 will result in the loss of control of above mentioned cellular process, and leads to tumor genesis. P53 mutations are observed frequently in various types of human cancers[65,66]. The sequence-specific DNA binding ability relies on the reduction of the Cys residues in the DNA binding domain of p53[67]. Although there is no evidence showing the direct binding of p53 with Trx, many studies showed that Trx can enhance the DNA biding activity of p53 by itself, or through the activation of Ref-1[22,68,69]. In addition, deletion of TrxR in yeast strongly impaired p53 activity[70]. In mammalian cells, several electrophilic compounds which can damage TrxR activity also showed disruption of the function of p53[71].

1.3.1.3 Proteins binding to Trx

Besides above mentioned enzymatic activities, there are several proteins are known to bind to Trx. Two of the well-studied ones are Apoptosis Signal-regulating Kinase 1 (ASK1) and thioredoxin interacting protein (Txnip).

1.3.1.3.1 Apoptosis Signaling Kinase -1 (ASK1)

ASK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK). ASK1 first can activate MAP kinase kinase, which then activates two apoptosis pathways: c-Jun N-terminal kinase (JNK) and p38 MAP kinases pathways[72]. Trx inhibits the activation of ASK1 by directly binding to it and disturbing its homo-oligomerization[73]. The binding of Trx and ASK1 requires the involvement of at least one active site Cys residue (Cys32 or Cys35). A double mutant Trx1(Cys32S, Cys35S) cannot bind to ASK1[74]. The binding of Trx also promotes the ubiquitination and degradation of ASK1[74]. Besides Trx1, several studies have shown that Trx2 in mitochondria can also interact with ASK1, and inhibits its translocation and activation[26,75,76].

1.3.1.3.2 Thioredoxin interacting protein (Txnip)

Txnip, which was first found in HL-60 leukemia cells as a vitamin D3 up-regulated protein-1 (VDUP1)in 1995[77]. In 1999, Txnip was rediscovered as thioredoxin binding protein in a yeast two-hybrid system and termed as thioredoxin binding protein-2 (TBP2)[78]. The binding of Txnip with Trx is through formation of an intermolecular disulfide bond between Cys32 from Trx and Cys247 from Txnip[79,80]. The formation of this disulfide is redox-dependent, in order to archiving the binding, a reduced Trx and an oxidized Txnip are needed[79]. The binding of Txnip cause reduced Trx activity as a disulfide reductase, which results in elevated level of ROS in cells[81,82]. Txnip can also alter Trx's function as a competitive inhibitor and disturbs the interaction between Trx and its targeting proteins, such ASK1[27,82]. Recently, the structure of the complex of Trx and Txnip has been determined. The structure confirmed the disulfide formation between Txnip Cys247 and Trx Cys32, in addition, a disulfide bond switching mechanism was proposed to explain the structural rearrangement in Txnip[80].

1.3.1.4 Regulation of Trx activity/function in cells

The regulation of Trx activity/function can happen in different levels, including: expression, post-translational modifications and protein-protein interaction. Txnip is a well-known thioredoxin binding protein has its implications in regulating Trx activity and interaction with other proteins (see above).

1.3.1.4.1 Expression

Various stress, such as H_2O_2, O_2, hypoxia, UV, X-ray, *etc.*[83–86] and treatments of certain drugs such as arsenic trioxide and suberoylanilide hydroxamic acid (SAHA) [35,87]can increase the expression of Trx1 in mammalian cells. After analyzing the promoter region of human TXN1 gene, there are three types of stress-response elements were found in the promoter region, including: antioxidant responsive elements (ARE), oxidative response element and heat shock responsive element[88–90]. These findings can explain the induction of Trx1 expression under multiple stresses.

Under some conditions, such as hypertension, and the treatment of certain compounds such as cathepsin D, the amount of Trx is reported to be decreased may be due to the

impaired induction of Trx1 and the degradation of damaged protein during oxidative stress[91,92]. It is interesting to point out, for the treatment with the same drug, SAHA, which is the histone deacetylase inhibitor, contradictory results were given by different studies. In some study, expression of Trx1 was found to be positively regulated[87], while in another study, expression of Trx1 was shown to be negatively regulated[93]. These difference may due to the differences between cell types, the amount of the treatment, and maybe some other factors.

1.3.1.4.2 Post-translational modifications

Oxidative post-translational modifications of the Cys residues in Trx1 were investigated extensively, three types of modifications are the center of interest: oxidative modification of the Cys residues (through disulfide formation), *S*-glutathionylation and *S*-nitrosylation. The modification of each Cys residue is briefly summarized in Table 1.

There are five Cys residues in human Trx1, two of them are involved in the active site (Cys32 and Cys35) which are required for the activity of Trx and the interaction of Trx1 with its interacting proteins[74,79]. The rest three Cys residues, as known as structural cysteines, first caught the attention because they cause the aggregation and loss of activity in Trx1[94]. Although until now their physiological functions are still not clear, but accumulating evidence suggesting these cysteine residues may also involve in the redox signaling. Homodimerization of Trx1 is through the disulfide formed between two Cys73 residues from each molecule[33]. Trx1 dimer does not have any reduce activity because the active site was not accessible for TrxR[37,95]. Under oxidative environment, an extra disulfide can form between Cys62 and Cys69. Trx1 with only the disulfide between Cys62 and Cys69 can be reduced by TrxR because the disulfide can be transferred to the active site. Trx1 with two disulfide is inactive and cannot be reduced by TrxR[16,96], but is a substrate of Grx system[97]. Nevertheless, the formation of the second disulfide in Trx1 may provide a redox mechanism regulating its function and earns more time for the redox signaling transduction[38,96].

S-glutathionylation is a reversible oxidative post-modification. Both *in vitro* and in cells study showed that Cys73 can be glutahionylated under oxidative stress. The glutathionylated protein is inactivated, but the activity can be regained automatically

when incubated with TrxR and NADPH, suggesting the glutathionylation process is reversible[98]. Although glutathionylation can happen during physiological conditions, Trx1 can only be glutathionylated in oxidative stress environment. Thus, the glutathionylation may play an protection role against irreversible oxidative modifications, such as dimer formation which leads to loss of activity[99].

Table1. Post-translational modifications of Cys residues in Trx1

Cys residue	Post-Translational modifications	Change of Trx1's activity / function
Cys32	disulfide formation	Loss of activity but can be reduced by TrxR
Cys35	disulfide formation	
Cys62	extra-disulfide formation	Loss of activity and cannot be reduced by TrxR
	S-nitrosylation	
Cys69	extra-disulfide formation	Loss of activity and cannot be reduced by TrxR
	S-nitrosylation	Stimulating Trx's activity and anti-apoptotic function
Cys73	homodimerization	loss of activity
	S-glutathionylation	loss of activity, but reversible
	S-nitrosylation	Trx can act as a trans-nitrosylation agent

Haendeler *et al* found that Trx1 can also be *S*-nitrosylation in 2002. They claimed that under physiological condition, Cys69 can be nitrosylated, and the nitrosylation of Cys69 stimulates Trx1's function in redox regulation and anti-apoptosis[100]. However, several following studies gave controversial results. In one study, the *S*-nitrosylation of Cys69 was detected and no *S*-nitrosylation of Cys73 was detected, in addition a disulfide between the active site Cys residues was observed[101]. In a structure study,

after treated with GSNO, both Cys69 and Cys62 were found to be nitrosylated. There is no nitrosylation on Cys73, and a disulfide bond was formed in the active site[102]. A study from our group suggested that the different results obtained from these studies maybe because the redox state of Trx1 was difference when the experiments were performed. Treatment of GSNO resulted in nitrosylation of Cys69 and cys73 and an additional disulfide in the active site in fully reduced Trx1. Treating Trx1 with two disulfides under the same condition resulted in the nitrosylation of Cys73, which can act as a trans-nitrosylation agents, and cause the nitrosylation of caspase-3 and subsequently inhibit apoptosis[101,103].

1.3.2 Thioredoxin Reductase

1.3.2.1 Classification

Thioredoxin reductase (TrxR) belongs to the family of dimeric flavoenzymes that catalyze the electron transfer from pyridine nucleotides to their substrates through flavin adenine dinucleotide (FAD) and the active site with redox active cysteine residues [104]. It is important to mention that there are two types of TrxRs which are classified according to the difference in the molecular weight of the subunit. The small TrxR or the low M_r type TrxR, comprising of two identical 35 kD subunits, which mainly exist in lower organisms such as bacteria, fungi, yeast and plants. The large TrxR or the high M_r type TrxR, comprising of two identical 55-60 kD subunits, which can be found in higher organism such as mammalians[104,105]. Besides the difference in the molecular weight, their catalytic mechanisms are also different.

E. coli TrxR is a well-studied small TrxR. Here we use it as an example to discuss the catalytic mechanism of small TrxR. In each cycle of the reaction, there is an unique rotation of the pyridine nucleotide-binding domain by 67 degree[106]. The structure before rotation facilitate the reduction of FAD by NADPH[107], while the structure after the rotation allows the transfer of electrons from reduced FAD to the active site disulfide[108].

Different from the small TrxR, the large TrxR comprises three functional domains in each subunit: FAD domain, NADPH domain and interface domain (Fig. 2). In each

subunit, there are also two redox active sites: one located at the C-terminal active mobile tail, the other one is close to the FAD.

The redox active site of the C-terminal tail is different from species to species. In some species, such as *plasmodium falciparum* (the malaria parasite) and *Drosophila Melanogaster* (fruit fly), the C-terminal redox active site is composed of two Cys residues; from *C.elegans*, the selenocysteine (Sec) replaces the last cysteine in the C-terminal tail. Compared to Cys, Sec has a lower pK_a value which makes it more active. The C-terminal tail is highly flexible and exposed on the surface of the enzyme upon reduction[109].

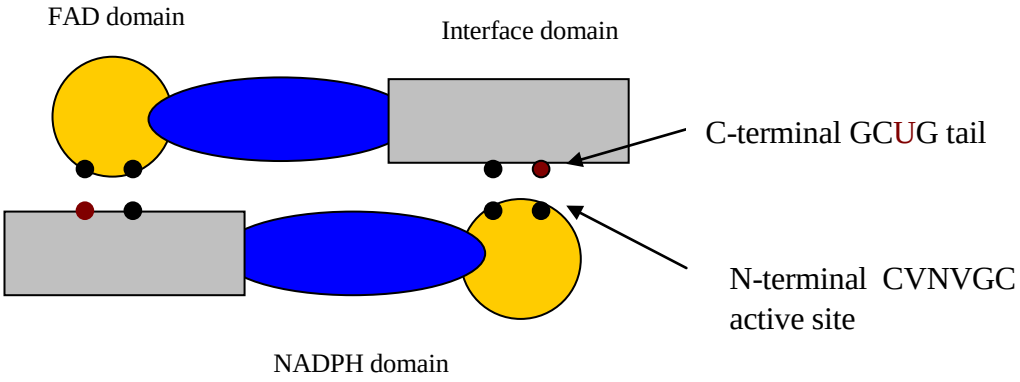


Figure 2. Schematic overview of TrxR structure.

1.3.2.2 Catalytic properties of mammalian TrxR

As shown in Fig. 2, the two TrxR monomers were arranged into a head-to-tail dimer. The two redox active sites are close to each other in space, although one of them is located in the FAD binding domain (Cys59 and Cys64), while the other is located in the C-terminal of the interface domain (Cys497 and Sec498). In each catalytic cycle, the fully oxidized TrxR is first reduced by NADPH and the FAD gains two electrons upon the reduction; then the reduced FAD transfers one electron to Cys59 and shares the other one with Cys64; after the stable charge transfer complex is formed in N-terminal active site, it can reduce the C-terminal of the other subunits; this reaction results in the reduced C-terminal selenothiol and a disulfide in the N-terminal active site; FAD can again reduce the N-terminal disulfide through transferring electrons from NADPH[109,110].

1.3.2.3 Isoforms of TrxR

In mammalian cells, there are three separate genes encoding three isoforms of TrxR: TrxR1, which is mainly present in cytosol; TrxR2, which is only present in the matrix of mitochondria; and thioredoxin glutathione reductase (TGR), which has a Grx domain in the N-terminal sequence, and mainly expresses in testes. TrxR1 and TrxR2 have similar kinetic properties[111], whereas TGR can reduce both Trx and GSSG, and also has a low Grx activity. But the Grx activity of TGR is not GSH-dependent since the C-terminal Sec residue may be able to transfer electrons to the Grx domain[112,113]. TGR can catalyze the formation of intermolecular disulfide bonds and protein isomerization, indicating its role in protein folding. In testes, TGR and its target protein GPx4 can serve as a disulfide bond formation system, including proteins that form the structural components of the sperm, suggesting its role in sperm maturation[114].

Besides these three isoforms of TrxR, there are many splicing variants of TrxR1 and TrxR2. For example, five different cDNA isoform of TrxR1 have been discovered which all have alternative N-terminal domains[115,116]. The biological functions of these variants are still under investigation.

1.3.2.4 Selenoproteins

Selenium is an essential trace element for human, and deficiency of selenium in dietary uptake can result in loss of immune function, weakened reproduction, depression and cardiovascular diseases[117]. One important application of selenium by mammals is the synthesis of selenoproteins. Until today, there are about 25 selenoproteins that have been identified in human by searching the mammalian genomes[118]. Most of these selenoproteins are found to be antioxidant enzymes, such as TrxR1 and TrxR2, methionine-*R*-sulfoxide reductase B1, GPx1 to 4 and 6, SelK, SelW and SelR. Other important selenoproteins are selenoprotein P (SelP) which functions in the Sec residue transport and storage due to the highly toxicity of free Sec residue; selenophosphate synthetase 2 (SPS2) functions in Sec synthesis, *etc*. The functions of some selenoproteins are still unknown.

The Sec is encoded by a UGA codon, which is in most cases recognized as a stop codon. In order to correctly recognize the codon and insert the Sec into the right position, a complex regulatory mechanism has been developed in mammalian cells. First of all, a secondary RNA structure called the SECIS element is used in the 3'UTR of the RNA message. In addition, a special translation machinery is required as well, including: a Sec specific tRNA, the specific elongation factor EFSec, SECIS binding protein 2 (SBP2), ribosomal L30 protein, and many other factors including some may be undiscovered factors[119].

1.3.2.5 Selenocysteine v.s. cysteine

Compared to Cys residues, the only difference of a Sec residue is the replacement of -SH by -SeH (Fig. 3). However, the insertion of one Sec is very expensive for cells, which suggests the irreplaceable advantages of Sec over Cys residue. Upon the replacement of sulfur by selenium, the pK_a value of the residue is changed from 8.3 in Cys to 5.2 in Sec. This change in the pK_a value result in an almost completely deprotonated selenolate at the physiological pH, which is more reactive comparing to thiol[120]. Actually under selenium deficiency condition, the Sec residue in TrxR was found to be replaced by a Cys residue in rat liver, resulted in a 10 fold decrease in TrxR activity[121].

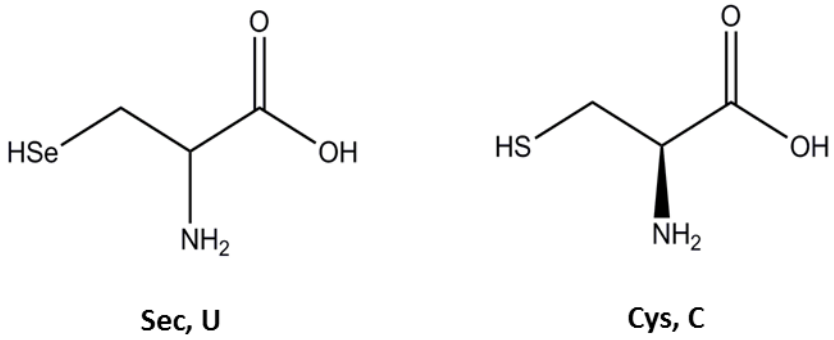


Figure 3. Chemical structure of Selenocysteine and cysteine.

1.3.3 Thioredoxin system in cancer

The ROS level in cancer cells was shown to be elevated compared to normal cells, which may be due to defects in the respiratory chain and disturbed redox balance[122]. A moderate elevated ROS is preferred by cancer cells because it can promote tumor

growth and survival by stimulating the expression of enzymes, such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) and cyclin D[123]. Trx and TrxR were also found to be overexpressed in many cancer cells, and they play important roles in cancer cell death[24,124]. Targeting Trx and/or TrxR can induce a massive increase in ROS level, and induces cell death through multiple pathways, such as activation of ASK1, translocation of cytochrome c from mitochondria to cytosol and induction of p53 expression, *etc*[27,29,125].These studies suggest the cellular redox pathways, such as Trx system, can be a promising target in anticancer therapy.

Table 2. Compounds used in the thesis

Chemical	Effects on TrxR/Trx	Paper
Auranofin	Inhibitor of TrxR	III
Arsenic trioxide	Inhibitor of TrxR	IV
Arsenic compound 6	Inhibitor of TrxR specific oxidation of Cys62 and Cys63 in Trx1	IV
Arsenic Compound 7	Inhibitor of TrxR Two disulfide formation in Trx1	IV
Brilliant Green	Inhibitor of TrxR(*in vitro* unpublished data) Cause Trx2 degradation	I
Ebselen	Super-fast oxidizer of Trx Substrate of both Trx and TrxR	II
4-hydroxynonenal	Inhibitor of TrxR	III
Mercury	Inhibitor of TrxR	VI
Mitomycin C	Inhibitor of TrxR	V

TrxR emerges as a new anti-cancer therapeutic target, and has a long list of inhibitors. The selenocysteine in the C-terminal active site is found to be a primary target of several electrophilic compounds, such as are anticancer drugs such as cisplatin, mitomycin C, doxorubicin, *etc*.[126–128] Several anticancer compounds, or clinically applied drugs have also shown the ability of interfere the expression level of thioredoxin or change its redox state[87,91,129]. Table 2 exhibits the compounds studied in this thesis.

1.4 GLUTAREDOXIN SYSTEM

Besides Trx system, glutaredoxin system composed of glutaredoxin (Grx), glutathione reductase (GR), glutathione (GSH) and NADPH is another major cellular protein disulfide reductase system. GSH is the most abundant small thiol-containing molecules in cells, which concentration can reach up to 10-15 millimolar levels. The reaction of protein-disulfide reduction catalyzed by Grx system has two distinct mechanisms. One is the thiol/disulfide exchange mechanism, which is similar to Trx system, called dithiol mechanism. The other one is the so called mono-thiol mechanism, in which only the N-terminal active site Cys residue is involved.

In a dithiol mechanism, Grx with two free thiols in the active site first reduces the disulfide in the substrate through a thiol-disulfide exchange mechanism, and results in a disulfide formation in the active site (Reaction 6). The disulfide in Grx's active site can then be reduced by gaining electrons from two molecule of GSH, results in the formation of one GSSG (Reaction 7). Finally, GSSH is reduced to two GSH by GR using electrons from NADPH (Reaction 8).

$$\text{Grx-(SH)}_2 + \text{Protein-S}_2 \longrightarrow \text{Grx-S}_2 + \text{Protein-(SH)}_2 \qquad (6)$$

$$\text{Grx-S}_2 + 2\ \text{GSH} \longrightarrow \text{Grx-(SH)}_2 + \text{GSSG} \qquad (7)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{GR} 2\text{GSH} + \text{NADP}^+ \qquad (8)$$

The monothiol mechanism is actually a deglutathionylation reaction[130]. First, GSH can form a mix-disulfide with protein thiol, and then reduced Grx can form a new mixed-disulfide between its N-terminal active site Cys residue and the GSH, and transfer the electron the protein thiol, which is reduced (Reaction 9). The resulting mixed-disulfide is subsequently reduced by another molecule of GSH, results in the fully reduced Grx and a GSSG (Reaction 10); this is the rate limiting step of the whole reaction[131] . Finally, GSSG is reduced in the same way as reaction (8).

$$\text{Grx-(SH)}_2 + \text{Protein-S-SG} \longrightarrow {}_{\text{(N-terminal)}}\text{GS-S-Grx-SH}_{\text{(c-terminal)}} + \text{Protein-SH} \quad (9)$$

$$\text{GS-S-Grx-SH} + \text{GSH} \longrightarrow \text{Grx-(SH)}_2 + \text{GSSG} \quad (10)$$

1.4.1 Glutaredoxin

Grx was first discovered in 1976 by Arne Holmgren as a GSH dependent electron donor for RNR in *E. coli* lacking Trx system[132]. Grx is a Trx fold family protein with conserved active site sequence -Cys-X-X-Cys-[133]. There are three different isoforms of Grxs in mammalian cell: Grx1 is located in cytosol, Grx2 is located in mitochondrial, and Grx5, which is a monothiol isoform and may be also target mitochondria[134].

Cytosolic Grx1 is about the same size of Trx1 (~12 kDa), which structure comprises a Trx fold with a central core of four β-sheets surrounding with five α-helixes. The conserved active site (-Cys-Pro-Tyr-Cys-) locates in the N-terminal part of helix 2 in mammalian Grx1[135]. The structure of reduced and oxidized Grx2 are very similar, only slightly changes around the N-terminal active site area[136]. The structure of *E. coli* Grx1 revealed a GSH binding site and a mixed-disulfide with GSH which can explain the high specificity and affinity of Grx to GSH[137]. In addition, Grx also has a hydrophobic surface area around the active site, which facilitates the interaction of Grx with its substrate[136]. The same as Trx1, Grx1 can also translocate into nucleus upon oxidative stress, where it may exert its role in regulating transcription factors.

Human Grx2 has two isoforms, Grx2a and Grx2c. Grx2a is located in mitochondria, while Grx2c is localized in nucleus, and cytosol of some tumor cells[138]. Under reducing condition, two molecule of Grx2 and two GSH form heterodimer through iron-sulfur

cluster[139]. The dimeric Grx2 is enzymatically inactive. But upon oxidative stress, Grx2 is dissociated and activated. This property of Grx2 suggests its role as a redox sensor, and the importance of it as a backup reductase under oxidative stress[140]. Compared to Grx1, Grx2 has a higher affinity to the *S*-glutathionylated protein substrates with lower turnover rates[141]. Grx2 can catalyze the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins, and plays important role in mitochondrial redox signalling and oxidative stress defense.[142].

Grx5 is named because it is a homologue of yeast Grx5. Grx5 is also located in mitochondrial but with only one Cys residue in its active site, and so far no redox activity has been reported[134]. Its role in mammalian cells is not clear yet , but in yeast Grx5 is found to be involved in the synthesis of iron-sulfur clusters and regulation of the activity of iron-sulfur enzymes[143].

1.4.2 Glutathione Reductase

GR is a flavoenzyme responsible for maintaining the reduced GSH pool in cells. The same as TrxR, GR is also belonged to the pyridine nucleotide disulfide oxidoreductase family. The active form of enzyme is a dimer with two identical subunit arranged into a "head to tail" pattern. Each subunit of GR also contains three domains: FAD binding domain, NADPH domain and interface domain[144]. The redox active site in the N-terminal (-Cys-Val-Asn-Val-Gly-Cys-) is homology to TrxR and is conserved in many species. When reducing GSSG, two electrons from NADPH was first transferred to FAD, and then transferred to the active site. Finally, GR reduces GSSG in a thiol/disulfide exchange manner. There is only one gene in mammalian cells encoding GR, although there are GR presenting both in cytosol and in mitochondrial. GR from different subcellular compartments has the same biological and chemistry properties[145]

1.4.3 Glutathione

GSH is a tripeptide (L-γ-glutamyl-L-cysteinylglycine), and is the most abundant thiol-based antioxidant in mammalian cells. The ratio between reduced GSH and oxidized GSSG (GSH/GSSG) is used as the indicator of cellular redox state. In a physiological state, the ratio can be above ten in the cytoplasm. The synthesis of GSH is a two steps ATP dependent reactions happens in cytosol: first is the rate limiting step of the

synthesis, γ-glutamyl-cysteine is synthesized from L-glutamate and cysteine through the catalysis of γ-glutamyl-cysteine synthetase (also called glutamate cysteine ligase, GCL)[146]. Then a glycine was added into the dipeptide by the catalysis of glutathione synthetase. The synthesis of GSH is regulated by many factors, such as oxidative stress which can enhance the activity of GCL by increase the holoenzyme formation; the availability of L-cysteine; and regulation of GCL and glutathione synthetase at transcriptional level[147].

After GSH is produced in cytosol, it can be transported into different subcellular compartments, providing different redox environment in different organelles in a cell. For example, GSH can be transported into mitochondria through both a carrier mediated pathway and diffusion[148,149]. Mitochondria contain a higher amount of GSH compare to cytosol or nucleus, and the pool of GSH in mitochondria has a longer half-time. When using buthionine sulfoximine (BSO), which is an inhibitor of GCL, to deplete cellular GSH, the GSH in mitochondria is much more resistant compared to cytosol[150,151]. These results suggest that GSH in mitochondria protects cells from oxidative stress when the pool in cytosol is low or oxidized. Another example is that in endoplasmic reticulum (ER), where the environment is much more oxidized than other parts of the cell, the ratio of GSH/GSSG is about 3:1. The oxidizing environment in ER can facilitate protein folding[152]. Besides the transportation within cell, GSH can also be transported outside cells, but mostly in oxidized form (GSSG). The extracellular GSH is much lower than intracellular, for example in plasma there is only about 2-20μM GSH, and the ratio between reduced GSH and oxidized GSSG in plasma decreases by aging, especially after 45 years of age[153,154].

Besides its role in protecting cells from oxidative stress, GSH participates in many cellular processes through *S*-glutathionylation, an important post-translational modification of proteins. Via *S*-glutathionylation, the target protein can either be activated or inactivated[155]. A large group of proteins can be regulated through glutathionylation, and involve in many important physiological pathways such as cell metabolism, growth and differentiation. Some transcription factors, such as AP-1 and NF-κB can also be glutathionylated and loss their ability of binding to DNA[156–158]. As mentioned before, Cys73 in Trx1 can also be glutathionylated, this modification may be able to protect protein being irreversibly oxidized, in the case of Trx1, forming inactivated dimer through Cys73[98,159].

In addition, GSH is implicated in the metabolism and detoxification of several toxic compounds. For example, after entering cells, pentavalent arsenic needs to be reduced in to trivalent arsenic in a GSH-dependent pathway, and then conjugated with GSH in order to be exported out of cells[160].

1.4.4 Cross-talk between Trx and Grx System

In mammalian cells, glutaredoxin system has some functions overlapping with thioredoxin systems. For example, both systems are electron donors for RNR with similar catalytic efficiency. With the present of 4 mM GSH, Grx1 showed a higher affinity compare to Trx1, but the later has a higher turnover number *in vitro*. Meanwhile, the catalytic activity of Grx system was strongly dependent on the concentration of GSH, and the catalytic reaction is more likely to follow the monothiol mechanism, which can be distinguished from the dithiol mechanism of Trx[43]. Other overlapping functions including their role in protecting cells from apoptosis, Grx1 can also negatively regulate ASK1's activity, though the binding site is different from Trx1[161]. Grxs are also reported to be able to regulate some transcription factors, such as NF-κB and nuclear factor I (NFI)[162,163].

Although there is much overlap in their functions, they cannot fully substitute for each other due to their difference in the selection of substrate groups and different reaction mechanism. Generally, Trxs reduce the disulfide in its substrates through a thiol/disulfide exchange mechanism, which is similar to the dithiol mechanism of Grxs. Prxs are specific substrates of Trx system, which is in the forefront of defending oxidative stress by elimination excess H_2O_2[45]. Grx system can exert its anti-oxidant function by providing electrons to glutathione peroxidases (GPxs), a family of enzymes which are also involved in balancing H_2O_2 homeostasis and multiple cellular signaling pathways including carcinogenesis, apoptosis, *etc.*[164]. In addition, Grxs can catalyze the reversible deglutathionylation reaction of its substrates through the so called monothiol mechanism as described previously in "Glutaredoxin" section.

Apart from the similarity and difference of their substrates and functions, Trx and Grx system can back up each other when the enzyme in one system does not function. Grx2 can be reduced by both GR and TrxR[141]. Trx can reduce GSSG in GR-deficient cells[165].

A study from our group showed that Grx system could work as a backup for TrxR and keep Trx1 in the reduced condition when TrxR1 was inhibited[166]. Moreover, the heavily oxidized Trx1 with two disulfides cannot be reduced by TrxR but can be reduced by Grx system at the expense of GSH[97]. Besides the finding in the cytosol, we also found that Grx2 is a very good back up for TrxR2 in mitochondria, and keep Trx2 in the reducing state when TrxR2 was inhibited. In Hela cells, which also express Grx2c in the cytosol, Grx2c can also protect Trx1 from being oxidized when TrxR1 is inhibited[167]. Under reducing environment, Grx2 is inactive and stored in dimer form together with two GSH through iron-sulfur cluster[139]. Upon oxidative stress, Grx2 will be released and activated to exert its anti-oxidant function. This fact together with its property of less sensitive to oxidative stress damages[103], make Grx2 very fit for being a backup for TrxRs.

2 AIM OF THE THESIS

As indicated in the title, our primary goal was to explore the role of Trx and TrxR in cell death caused by toxic compounds, as well as to investigate the potential possibility of Trx and TrxR as targets for anticancer therapy.

Specifically, in Paper I - IV we focused on studying the role Trxs in cell death and the properties of the protein.

- To investigate the role of Trx2 in some cationic triphenylmethanes causing cancer cell death.
- To characterize if Trx1 with two disulfides is a substrate of the Grx system and its possible role in redox signaling
- To characterize Grx2 as a backup system for both Trx1 and Trx2.
- To investigate if the oxidation of Trx1 can enhance the cytotoxicity of some arsenic compounds, and the role of Cys62 and Cys69 in regulating the function of Trx1

In Paper V and Paper VI, we focused on the inhibition of TrxR by toxic compounds or clinically applied anticancer drug.

- To investigate targeting TrxR as a new anticancer mechanism of mitomycin C.
- To study targeting TrxR as a new mechanism of mercury toxicity, and the role of selenium in recovery of the activity of TrxR inhibited by mercury.

3 PRESENT INVESTIGATIONS

3.1 METHODOLOGY

3.1.1 Cell Culture

For most cell based experiments described in papers I-VI, commercially available human cell lines from the American Type Culture Collection were used. Fibroblast cells in paper I was a gift from Dr. Laura Papp, Queensland Institute of Medical Research, Australian. Hela cell (human cervical carcinoma), A549 (human alveolar adenocarcinoma epithelial cell), SH-SH5Y (human neuroblastoma) and Du145 (human prostate cancer) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1 g/l glucose (VWR). HEK293t (human embryonic kidney cell) and Fibroblasts were cultured in RPMI 1640 medium with 1 g/l glucose (VWR).

3.1.2 RNA Silencing

The transfection of siRNA in Paper I and Paper III was performed according to the Dharmacon transfection protocol. Briefly, siRNA transfection reagent (Dharmacon, Lafayette, Co, USA) and siRNAs (Qiagen, Valencia, CA, US) were diluted in serum and antibiotic-free medium and left at room temperature for 5min. Then the siRNA transfection reagent and siRNA were mixed and incubated for 20 min. Complete medium were added into the mixture to achieve a final concentration of 50 nM siRNA in the culture medium. After 48 hours incubation, the medium was removed, and the cells were harvest for analysis or continue incubated with the desired compounds.

3.1.3 Cell Proliferation and Viability Assays

The most commonly used assay for cell proliferation and viability measurement in all six papers is the MTT assay. Because MTT assay can be affected by the alteration of mitochondrial metabolism, in order to confirm the results of MTT assay, we also used trypan blue exclusion and neutral red up take assay to investigate the effects of compounds we used on cell viability in some of our studies. Observations of the

morphology of cells by microscopy were always performed and recorded prior to each assay.

For MTT assay, cells were plated at a density of 1×10^4 cells/well in 96-microwell plate and allowed to grow about 24 hours to get confluence. Then the cells were treated with appropriate concentrations of compounds in 100 µl fresh medium for the appropriate time. After treatment, medium containing compounds were replaced by 100 µl of fresh medium, and 50 µl of MTT solution (1 mg/ml in PBS) was added to each well and incubated for 3h. Then the medium was carefully removed, and 100 µl of DMSO was added to each well. Plates were then put on a shaker for about 1 hour until all crystals were dissolved. Then the cell viabilities were determined by measuring the absorbance at 550 nm.

Neutral red up take assay was carried out following the previous described protocol[168]; cells were seeded and treated in the same way as in MTT assay. Neutral red working solution (40 µg/ml in culture medium) was incubated overnight at the same temperature as the cells, and then centrifuged to remove any precipitated dye crystals. The treatment medium was then removed and 100 µl of neutral red medium were added into each well. The plate was incubated for 2 hours at the appropriate culture conditions. Then the neutral red medium was removed and the cells were washed with 150 µl PBS carefully. Then 150 µl neutral red destaining solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was added into each well. The plate was then placed on a plate shaker until the neural red has been extracted from the cells. Then the cell viabilities were determined by measuring the absorbance at 540 nm.

For trypan blue exclusion assay, after the treated cells were collected, cells were centrifuged and resuspended in PBS. The density of the cells was determined using a hemocytometer. Then in every 1 ml cell suspension, 0.1 ml of trypan blue stock solution (0.4% trypan blue in PBS) was added. The numbers of the blue staining cells were counted right away, and the cells which took up trypan blue were considered non-viable.

3.1.4 Measuring TrxR activity using fluorescent method

Insulin reduction assay and DTNB reduction assay are two most commonly used method to measure TrxR activity in purified protein or in cell lysates. But in order to detect TrxR activity in precious biological materials, such as cell lysates from different subcellular organelles, or cell samples containing a low amount of TrxR, a newly developed fluorescent method was applied in the studies[169]. In the fluorescent method, insulin was replaced by isothiocyanate-labeled insulin (FiTC-insulin), which emits fluorescence at 520 nm after excitation at 480 nm. Upon the reduction of the disulfide by Trx, the fluorescence is increased. Compared to the conventional methods, fluorescence method is highly sensitive and stable[169].

Generally, in a 96-well black micro titer plates, appropriate amount of cell lysates were incubated with 20 µM of Trx and 0.25 mM NADPH in assay buffer (0.2mg/ml bovine serum albumin in 50 mM Tris-Cl and 1 mM EDTA, pH 7.5), the total volume was 90 µl in each well. After incubation 30 min at 37°C, 10 µl of FiTC-insulin was added into each well. The final concentration of FiTC-insulin in each well is 10 µM. Then the emission at 520 nm after 480 nm excitation was recorded for 60 min in room temperature. The rate of the reaction was calculated as the changes of fluorescence following time. In paper IV, due to the very low amount of TrxR in SH-SH5Y cells, an improved fluorescent method was used, in which FiTC-insulin was replaced by a new fluorescent substrate from the latest developed kit (FkTRXR-03-Star) by IMCO (www.imcocorp.se).

3.1.5 Redox Western Blot

To determine the redox state of Trx1 in cells, the experiment was performed based on the method described previously[38,166]. Cells were washed three times in cold PBS and then lysed in urea lysis buffer containing iodoacetamide (IAM) (10 mM IAM, 50 mM Tris-HCL, 1mM EDTA, 8 M urea, and pH 8.3). Then the proteins were precipitated and washed three times to remove the excess IAM with 1.5 ml of ice-cold acetone/HCl (98/2, v/v). The precipitate was then resuspended in urea lysis buffer containing 3.5 mM DTT to reduce the disulfides in Trx1. The free thiols of Trx were then alkylated with 30 mM iodoacetic acid (IAA) in urea lysis buffer. The samples were then

separated by PAGE containing 8 M urea and transferred with an Invitrogen transfer system.

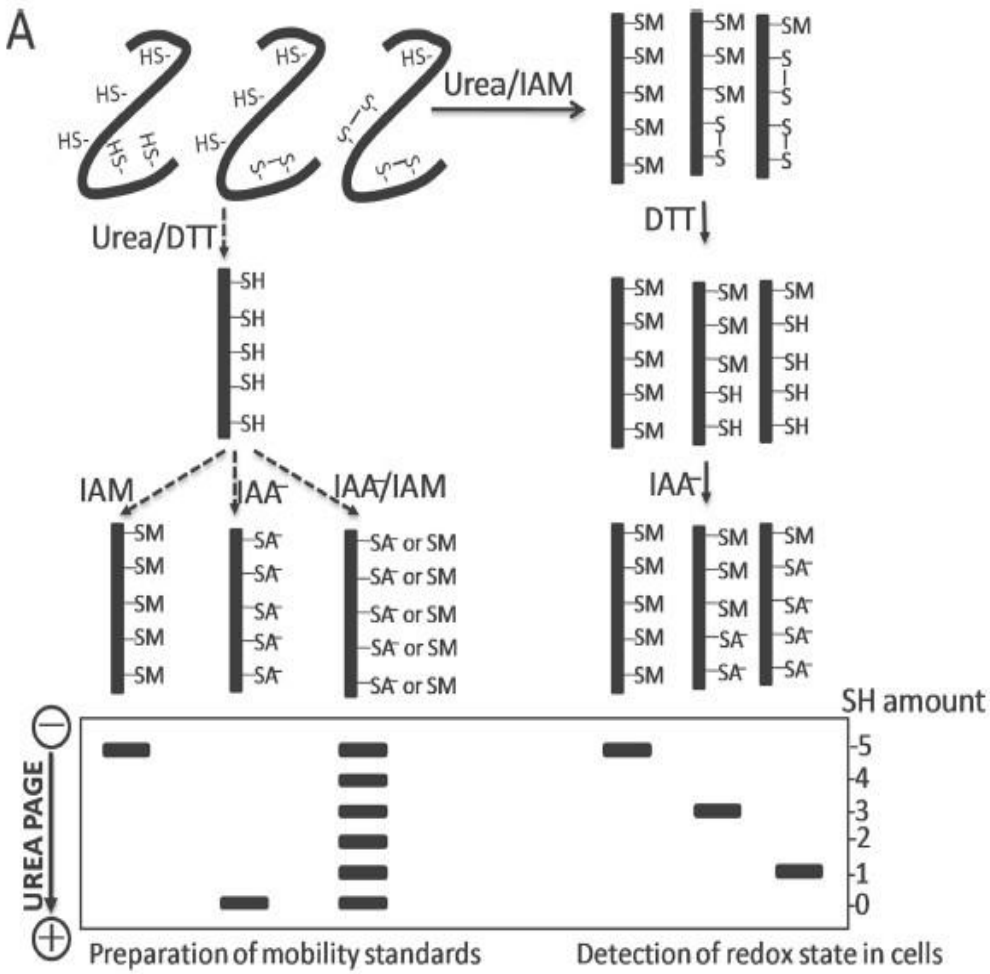


Figure 4. Principle of modified redox western blot (from paper II)

As exhibited in Fig. 4, in 8 M urea, proteins were all denatured and all the thiols were exposed. When first incubated with IAM, the free thiols in Trx1 were labeled with IAM, which will not give any extra charge to the protein. After removing excess IAM, the thiols which are oxidized into disulfide or bind to other proteins were reduced by 3.5 mM DTT, and were labeled by IAA. IAA is negatively charged, so the protein labeled with more IAA will migrate faster in the urea gel. In the end, Trx1 can be separated according to the free thiols they contained initially.

3.2 RESULTS AND DISCUSSIONS

3.2.1 Paper I

Xu Zhang, Yujuan Zheng, Levi E. Fried, Yatao Du, Sergio J. Montano, Allie Sohn, Benjamin Lefkove, Lars Holmgren, Jack L. Arbiser, Arne Holmgren, Jun Lu. Disruption of the mitochondrial thioredoxin system as a cell death mechanism of cationic triphenylmethanes. *Free Radical Biology & Medicine 50 (2011) 811-820.*

Trx2 system, comprising Trx2, TrxR2 and NADPH, catalyzes the disulfide reduction in mitochondrial matrix[25]. Prx3 is the main substrate of Trx2 in mitochondria, which can scavenge H_2O_2. In addition, Trx2 plays a key role in regulating mitochondria-dependent apoptosis by binding to ASK1 and Txnip, as well as regulating the mitochondrial permeability transition[2,26–28,170]. However, compared to Trx1 system, which is found to be over-expressed in many cancer cells and merging as an anticancer therapeutic target[24,129], the role of Trx2 system in cancer is less clear.

Cationic triphenylmenthane dyes, have been used by human beings for more than a century as antifungal and antibacterial agents[171–173]. Recently, some of them have also shown potent anticancer activity in mice and humans[174]. However the exact mechanism of these dyes remains unclear.

In this study, we found that Hela cells over expressing Trx2 in mitochondria compared to normal Fibroblast cells. Hela cells also showed to be more vulnerable upon BG treat compare to Fibroblasts. Down-regulation of Trx2 in Hela cells by siRNA technique resulted in a massive increase of cytotoxicity upon BG treatment, whereas, the same siRNA treatment in Fibroblast cells exhibited no significant change compared to untreated cells. These results suggest that the viability of Hela cells is more dependent on Trx2 than that of Fibroblasts.

We then performed fluorescence staining to detect the subcellular location of BG. The treatment of BG resulted in green fluorescence, which was overlapping with the MitoTracker red mitochondrial staining. Furthermore, we investigated the cellular response upon BG treatment in Hela cells and in Fibroblasts. Both Hela and Fibroblast cells were treated with various concentrations of BG (0.25 to 2μM) for 24 hours. There was only a marginal increase in Trx1 and TrxR1 protein levels, but no significant effects on TrxR activity in cell lysates. Interestingly, Trx2 protein levels were

dramatically decreased upon the treatment with low concentrations of BG (0.25 to 1.0 μM), both in Hela and in Fibroblast cells. Upon the treatment with 2 μM BG, the degradation of Trx2 was attenuated. RNR R1 protein level was not affected by BG treatment, whereas, RNR R2 protein, which is only expressed in S phase[175], almost disappeared upon 1.0 μM and 2.0 μM BG treatment. We applied a redox western blot method, which can detect the redox state of all the thiols in Trx1 or Trx2, to analyze the redox state of Trx1 and Trx2 as a result of BG treatment. Without any treatment, most Trx1 and Trx2 were in the fully reduced form. Upon 24 hours BG treatment, the reduced form of Trx1 or Trx2 decreased dramatically. By isolation of different cell organelles, we detected that the loss of Trx2 resulted in translocation of cytochrome c from mitochondrial to cytosol and shuffling of Trx1 from cytosol to nucleus.

In order to study how Trx2 is degraded upon treatment with BG, we used MG132, which is an inhibitor of both proteome and mitochondrial Lon protease, to treat Hela cells together with BG. The presence of MG132 can prevent the Trx2 degradation to some degree. We also found that upon the BG treatment, Lon protease mRNA was elevated. Thus we propose that mitochondrial Lon protease may mediate the Trx2 degradation process.

Discussion

The Trx1 and Trx2 system locate and exert their specific functions in different subcellular compartments. Trx1 is mainly located in cytosol. Trx1 is an electron donor for RNR, which is the rate limiting enzyme in DNA synthesis[19]. In mitochondria, where Trx1 only locates in the inter-membrane space, Trx2 is the most important enzyme to keep the redox balance in the matrix. Trx2 can provide electrons to Prx3, which can in turn remove the excess of H_2O_2 produced by oxidative respiration. In our study, under oxidative stress conditions, Trx1 and Trx2 system responded differently. Upon oxidative stress, Trx1 was found to translocate from cytosol into nucleus, where it can exert its function as a regulator of some transcription factors, such as Nrf2[124]. Upon the treatment of BG, the mRNA level of Trx1 and TrxR1 was significantly increased, where as, the mRNA level of TrxR2 was changed moderately and the mRNA level of Trx2 was almost unchanged.

The cationic triphenylmethane used in this study dyes belong to the lipophilic cations, which can easily pass through the lipid bilayers. The mitochondrial membrane potential (150-180 mV) is much higher than the plasma membrane potential (30-60 mV). This difference between membrane potentials drives the dyes to move into and accumulate in mitochondrial matrix[176]. This property of the dyes allowed them to interact with the Trx2 system in mitochondria, whereas the Trx1 system in cytosol was not affected directly. A remarkable founding in this study is that Trx2 was oxidized and degraded after BG treatment. Though we did not find any inhibition on TrxR both in cytosol and in in mitochondria, a purified TrxR protein can be inhibited by BG *in vitro* by reacting with the active-site cysteine/selenocysteine.

As a quality control enzyme, Lon protease has been found to be up-regulated under oxidative stress, and can specifically degrade oxidized proteins in mitochondria, such as aconitase[177,178]. Under the low concentration of BG treatment (below 1 μM), oxidized Trx2 will be degraded by Lon protease, however, when the concentration of BG treatment increased to above 2 μM, the degradation was diminished. This maybe because when Trx2 was highly oxidized, then it was unable to be degraded by Lon protease. The same property was also observed in the previous study, when aconitase was highly oxidized then it is difficult to be removed by Lon protease[177]. The existence of highly oxidized Trx2 in mitochondrial matrix will cause more dramatic cell death (above 2 μM treatment), compare to lower concentration treatment. This is also in line with the previous finding about the role of Lon protease in cell survival[179]. In addition, when over-oxidized Trx2 was not removed by Lon protease, the induction of HO-1 and NQO1 was also abolished.

In summary, the results in this paper demonstrate that the viability of some tumor cells is more dependent on the Trx2 system than normal fibroblast cells. When mitochondrial Trx system was disrupted by triphenylmethane dyes such as brilliant green, Trx2 was oxidized and degraded by Lon protease. However, over-oxidized Trx2 was not able to be removed by the cells. The results also shed light on the fact that mitochondrial Trx system can be a novel anticancer therapeutic target.

3.2.2 Paper II

Yatao Du, Huihui Zhang, **Xu Zhang**, Jun Lu, Arne Holmgren. Thioredoxin 1 Is Inactivated Due to Oxidation Induced by Peroxiredoxin under Oxidative Stress and Reactivated by the Glutaredoxin System *J. Biol. Chem. 2013, 288:32241-32247*

Hydrogen peroxide was long considered as only the by-product of respiratory chain and a major source of oxidative stress in cells. However, more and more studies have already proved the role of H_2O_2 as a second messenger in redox signaling[45,180]. Prxs can remove excess amount of H_2O_2 in living cells to protect cells from oxidative stress. On the other hand, Prxs may also be able to control the concentration of H_2O_2; the latter can exert its function as a signaling molecule[45]. By providing electrons to oxidized Prxs, Trx also plays important role in regulation of redox signaling. Trx1 with two disulfide (Cys32-Cys35, Cys62-Cys69) cannot be reduced by TrxR together with NADPH, and cannot provide electrons to Prxs anymore[96,181].

In our study, we found that in A549 cells, two-disulfide formed Trx1, with or without a thiol, can be found when cell were treated with a high dose of H_2O_2 (15 mM). In order to further investigate the formation of second disulfide (Cys62-Cys69), we prepared two types of Trx1: the active site double mutant Trx1 (SGPS) and Trx-S2, in which the active site cysteine residues was oxidized into disulfide by incubation with insulin. In both case, upon H_2O_2 treatment we can observe the formation of the second disulfide, but the present of Prx1 can strongly enhance the formation of the second disulfide. Ebselen is a substrate for both TrxR1 and Trx1, and it is a superfast oxidant for Trx1[182]. In Hela cells, the treatment of ebselen itself did not change the redox state of Trx1, however, upon the addition of BSO, Trx1 shifted into oxidized form dramatically. These results suggested that GSH system protected Trx1 from oxidation in cells. By using purified protein, we have found that GSH system can provide electrons and reduce two-disulfide form Trx1 (Trx1-S_4) or Trx1 (SGPS)-S_2. We also used *E. coli* Grx1C14S mutant to explore the mechanism of Grx system in reducing the non-active site disulfide of Trx1. Grx1C14S mutant also showed the capacity to reduce Trx (SGPS)-S2, which indicated that the reduction of non-active site disulfide by Grx1 followed the monothiol mechanism.

Discussion

Mammalian Trx1 has five cysteine residues, two of which locate in the active site (Cys32 and Cys35), and can reduce the disulfide in the target proteins. There are three so-called structural cysteines: Cys62, Cys69 and Cys73. Cys73 was known to be involved in the dimer formation, and Cys62 and Cys69 can form an extra disulfide, or so-called the second disulfide in Trx1. The formation of the second disulfide will affect its ability of reducing the disulfide in its substrates, as well as binding to its substrate such as ASK-1[103,183,184]. Trx1 with two disulfides was not a substrate of TrxR anymore, so it is important to investigate the regulation of the formation and reduction of the second disulfide. In our study, we found that GSH and Grx system can efficiently reduce Trx1 with two disulfide, which made the cycle of Trx1-$(SH)_5$ to Trx-S_4 is possible in living cells. Upon oxidative stress, Trx1 was oxidized into Trx-S4 form and the inactivation of Trx1 shuts down the electron flux from Trx1 to Prx1 to reduce H_2O_2, which may cause a transient increase of H_2O_2 amount, which in turn can exert its function as a secondary molecule. We also found that H_2O_2 alone was not very effective in terms of oxidizing Trx1. H_2O_2 was much more affinitive to Prx1 than to Trx1. Therefore, when the amount of H_2O_2 is enough, Prx1 will be oxidized and then it can oxidize Trx1 and give rise to formation of the second disulfide in Trx1. Similar mechanism was observed in endoplasmic reticulum (ER), where Prx4 can be oxidized by H_2O_2 and form a disulfide. Oxidized Prx4 can then transfer the disulfide to protein-disulfide isomerase thiols for the folding of newly synthesized proteins[45,185,186].

As a signaling messenger, H_2O_2 exerts its function by oxidizing and temporally inhibiting the effectors, such as protein-tyrosine phosphatases and tumor suppressor PTEN[45,187]. Prxs can remove excess H_2O_2 and get oxidized. There is a hypothesis called "floodgate hypothesis" that oxidized Prxs act as a peroxide floodgate, keeping peroxides away from its targets until the floodgate has to be open[180,188]. The inactivation of Trx1 by forming the second disulfide provides the possibility to inactivate Prxs, thus plays an important role in the regulation of oxidative signals.

3.2.3 Paper III

Huihui Zhang, Yatao Du, **Xu Zhang**, Jun Lu, and Arne Holmgren. Glutaredoxin 2 Reduces Both Thioredoxin 2 and Thioredoxin 1 and Protects Cells from Apoptosis Induced by Auranofin and 4-Hydroxynonenal. *Antioxidant & Redox Signaling. 2014 Feb 4. Epub ahead of print*

The Trx system, composed of Trx, TrxR and NADPH, and the Grx system, composed of GSH, Grx, GR and NADPH, are two major thiol-disulfide oxidoreductases systems in mammalian cells. They play critical roles in maintaining cellular redox homeostasis, DNA synthesis and regulating redox signaling of cell survival/apoptosis[189–191]. In resting cells, the two system work parallel due to their different substrate groups. In general, Trxs are more active in catalyzing the disulfide exchange reaction with its protein substrates. For example, Trxs will provide electron for peroxiredoxins, which is the major player in scavenging H_2O_2[45]. Trxs can also regulate several transcript factors, such as NF-κB, Ref-1; and apoptosis signaling factor such as ASK-1[124]. Grxs, on the other hand, are specifically active in catalyzing the deglutathionylation of their protein substrates at the expense of GSH[189]. Grx2, mainly located in mitochondrial matrix, but was also found in the cytosol of testis and some specific cancer cell lines[138]. Grx2 shares 34% sequence identity with Grx1, and has a higher affinity toward the *S*-glutathionylated substrates, but with a lower turn over[141]. Moreover, Grx2 is not inactivated by oxidation, thus can be a great back up for Trxs or Grx1 under oxidative stress condition[192].

Previous studies have proved the existence of cross talks between Trx and Grx systems. For example, Grx2 can receive electrons from both GR and TrxR[141], and Grx system can work as a backup for TrxR in cytosol and keep Trx1 in the reduced form even when TrxR1 was inhibited[166]. However, the backup system for Trx2 in mitochondrial matrix is still unclear.

In this study, we found that GSH alone could reduce oxidized Trx1 and Trx2 in the present of NADPH and GR, by adding Grx2, can greatly accelerate the reaction *in vitro*. In order to prove that Grx2 can also reduce Trx1 and Trx2 in cells, we used Hela cells which can stably overexpressing Grx2 to do further experiments. Both WT and Grx2 overexpressing Hela cells were treated with various concentrations of auronofin

(AF) and 4-hydroxynonenal (HNE), the Grx2 overexpressing cells were much more resistant to both treatments. By using redox western blot, we found that the treatment of HNE in WT Hela cells resulted in almost fully oxidize of Trx2, whereas, in the Hela cells overexpress mitochondrial Grx2 (M-Grx2) or cytosolic Grx2(C-Grx2), most Trx2 were still in the reduced form. The overexpression of M-Grx2 exhibited a better protection comparing to the overexpression of C-Grx2. Similarly, the overexpression of M-Grx2 and C-Grx2 produced the same positive effect in the protection of Trx1 from being oxidized by HNE treatment. On the contrary, the knockdown of Grx2 in Hela cells resulted in weakened cell viability upon AF or HNE treatment compare to WT Hela cells. The redox state of Trx1 and Trx2 showed an enhanced oxidation in Grx2 knockdown cells after treatment by HNE. The activity of Grx2 is not affect by HNE.

Discussion

Both Trx1 and Trx2 are crucial for cell survival. The homozygous knockout of Trx1 or Trx2 mouse showed embryonic lethality[29,30]. Meanwhile, certain cell lines with homozygous knockout of TrxR1 or TrxR2 can still survival[193,194]. These finding suggest that there must be a backup system for Trx1 and Trx2 besides TrxRs. The depletion of GSH would cause cell death in $TrxR2^{-/-}$ cells, implied the importance of GSH and Grx system as a backup system. In our study, we found that enzymatically, GSH can reduce the oxidized Trx1 or Trx2, but only when Grx2 was present, the reaction rate of Grx system as a backup is comparable to TrxR.

Grx2, but not Grx1 can form dimers through an iron-sulfur cluster together with two molecules of noncovalently bound GSH between the active sites of Grx2 monomer[139,140]. The Grx2 in dimmer form is not active as a reductase, however, upon oxidative stress, when the GSH pool became oxidized, the Grx2 dimer can be dissociated and catalytically active Grx2 will be released. Moreover, Grx2 is very resistant to oxidative stress compare to other thiol-based proteins[103]. Thus, under oxidative stress, when TrxR were inhibited, Grx2 can be a suitable backup.

In summary, our study provides a new insight into the new role of Grx2 in antioxidative and anti-apoptotic functions, by catalyzing the reduction of oxidized Trx1 and Trx2. In mammalian cells, both Trx system and Grx system are crucial for maintaining a reducing environment in cells and cell survival. In resting cells, these two systems work parallel, but under some circumstances, when TrxR was inhibited, Grx2

can catalyze the reduction of Trx1 or Trx2, thus, strengthens the cellular antioxidant capacity.

3.2.4 Paper IV

Xu Zhang, Jun Lu, Yatao Du, Panayiotis V. Ioannou and Arne Holmgren. Besides Inhibition of Thioredoxin Reductase, Oxidation of the Structural Cysteine residues in Thioredoxin by Certain Arsenicals Enhance Cytotoxicity to Cancer Cells. *Manuscript*

Arsenic is a widely existing metalloid in soil, water and air. Acute exposure to arsenic may cause acute poisoning, and chronic exposure may cause several diseases including cancer[195]. Paradoxically, arsenic trioxide (ATO) was discovered as a treatment for acute promyelocytic leukemia in 1990s, and now many studies showed the possibility of using ATO to treat solid tumors[196,197]. ATO treatment will cause depletion of GSH and inhibition of TrxR in cancer cells[129,160,198], and thus can damage the redox balance in cancer cells.

Trx1 has two cysteine residues in its active site (WCGPC), which can reduce the disulfide of target proteins by a thiol-disulfide exchange reaction. Besides, Trx1 also has three so called structural cysteines (Cys62, Cys69 and Cys73), and Cys62 and Cys69 can form an extra disulfide in Trx1. These additional cysteines caused more and more attentions, studies have shown that the disulfide between Cys62 and Cys69 is important for the regulation of Trx1 activity and its function in redox signaling[97,199].

In this study, we found that the structures of the arsenical compounds are closely linked to their ability to oxidize Trx1. Three out of eight compounds, which all have a resonance structure, have the ability of oxidizing Trx into dimer or higher oligomers. However, As5, which can oxidize Trx1 but lacks the ability of inhibit TrxR, cannot induce cytotoxicity in SH-SH5Y cells. Thus, the inhibition of TrxR is the basis of arsenic induced cell death. As6 and As7, on the other hand, can both inhibit TrxR and oxidize Trx, and exhibited the strongest cytotoxicity among all the arsenical compounds.

To further investigate why As6 and As7 had greater cytotoxicity, a redox western blot, which can identify the redox state of each individual cysteine in Trxs, was applied.

Combining with the results from DTNB titration, we confirmed treatment with As7 showed a gradually decreasing in the number of free thiols in Trx1 along the ratio between As compounds and TRx1 was increasing. The treatment of As6, on the other hand, can only cause two specific cysteine residues oxidized in Trx1. ATO showed no change of the number of free thiols in Trx1, even using as high as ten times of the concentration of Trx1. In order to determine the interaction between As6 and Trx thiols, we analyzed the redox state changes of Trx1, Trx2 and Cys62S/Cys73S double mutant after As6 treatment. The results showed that only the wtTrx1 can form a disulfide upon As6 treatment, which suggest the disulfide maybe formed between Cys62 and Cys69.

We also looked into the cellular response upon As6 or As7 treatment. SH-SH5Y cells were treated with various concentration of As7 for 24 hours, there were no significant changes of Trx2 or TrxR1 amount in protein level, however, the amount of Trx1 was dramatically increased at 5 μM and above treatment. The redox western revealed that before any treatment, a large part of Trx1 was in fully reduced form, after 8 hours of 10 μM As6 treatment and after 4 hours of 10 μM As7 treatment, the oxidized form of Trx1 became obvious. There was also Trx1 with loss of four thiols present in As7 treatment, especially after 24 hours treatment. Our in vitro experiment using Prx1 as the substrate of Trx system showed the fact that only As6 and As7 treatment affect the ability of Trx system to reduce Prx1, whereas ATO treatment, which can inhibit the activity of TrxR, can only partially affected the capacity of Trx to reduce Prx1.

Discussion

TrxR is by far one of the most well understood selenoproteins. The selenocysteine is located in the open C-terminal active site of TrxR which makes it easy to be accessed; and its low pK_a value makes it is highly reactive[200,201]. These characters make TrxR an easy target of many electrophilic compounds, such as mitomycin C, doxorubicin, and ATO as well[126,127,202]. In a previous study of our group, we found that ATO inhibits the C-terminal activity of TrxR, most likely by binding to the selenocysteine of TrxR. Studies have also shown that targeting the selenocysteine of TrxR can not only inhibit the activity of TrxR but can also turn TrxR into a NADPH oxidase and enhance ROS production in cells[128,203,204].

There is accumulating evident showing that the structural cysteine residues, especially Cys62 and Cys69 which can form an extra-disulfide in Trx1, may play important roles in regulating Trx1's function. Trx1 with 2-disulfide is not a substrate of TrxR anymore. Our recent study showed that the Trx1 with 2-disulfide can be reduced by Grx system, and we also found the existence of Trx1 with 2 disulfide in A549 cells treated with high dose of H_2O_2. In line with these studies, we have found that when Trx was oxidized into two-disulfide form, cell death happened massively. We have also discovered that oxidation of C62 and C69 into disulfide can affect Trx1's ability of reducing Prx1. H_2O_2 is not only an oxidative stress molecule as the by-product of respiratory chain, but also an important signal molecule in redox signaling[46,180]. Thus, the inactivation of Prx1 can cause a transient increase of H_2O_2, which make it is possible to induce oxidative stress dependent signals. Besides the ability of removing H_2O_2, Prx1 also plays important role in regulating cell death by interacting with the life span regulator protein p^{66shc} [205].

Cancer cells are found to be less tolerance to oxidative stress, most likely because they already have elevated ROS levels due to the impaired respiratory chain and defects in regulating of redox balance[122,123]. Cancer cells may prefer a moderate elevated ROS, because it can promote cancer cell growth and survival by activating a series of enzymes, such as MAPK, ERK and cyclin D1[27]. However, when extra ROS is induced in cancer cells, it can induce cell death through multiple pathways, such as activation of ASK1, release of cytochrome c from mitochondria and p53 induction, etc[27,124,125,206]. Trx1 and TrxR as anti-oxidative stress enzymes were found to be overexpressed in many cancer cell lines. But SH-SH5Y cells are one of the a few cancer cells which have lower expression of Trx1 and TrxR compare to other cancer cells and normal fibroblast cells. In line with its nature, the cellular environment of SH-SH5Y cells is more oxidized compare to Hela or A549 cells[97,206], because even in untreated cells, the Trx1 is not fully reduced. This property may make Trx system in SH-SH5Y cells is a more effective target for anticancer therapeutic treatment.

3.2.5 Paper V

Manuel M. Paz, **Xu Zhang**, Jun Lu, and Arne Holmgren. A New Mechanism of Action for the Anticancer Drug Mitomycin C: Mechanism-Based Inhibition of Thioredoxin Reductase. *Chemical Research in Toxicology. 2012 Jul 16; 25(7):1502-11*

Mitomycin C (MMC) has been used in combination chemotherapy since 1974, and has been used as an antibiotic for several surgical procedures[127,207,208]. The anticancer mechanism of MMC has been focused on its ability to bind covalently to DNA[207]. Recently, the hypothesis that MMC can induce redox cycling and increase ROS level in tumor cells has arose[209]. The protype of MMC is inert toward nucleophiles, but upon bioreduction, it became very reactive bis-electrophiles[210]. However, the exact mechanism of how MMC is reduced in cells is not clear. In recent studies, GRP58, a protein that containing thioredoxin-like domains, was proposed to be able to reduce MMC into its active form[211]. Studies have also shown that simple dithiols, such as DTT, could activate MMC by reduction. The activated MMC would then bind to the two sulfur atoms of the dithiol[212]. So we proposed that MMC could also act as an inhibitor of enzymes containing a dithiol active site.

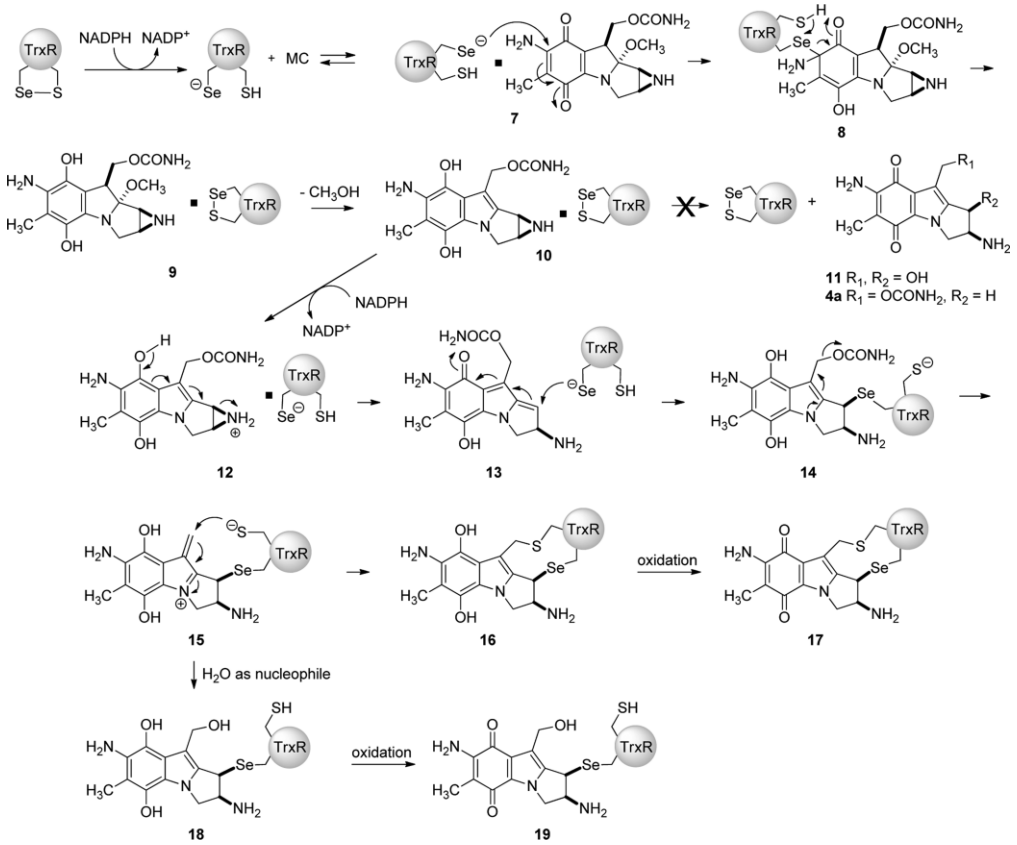
In our study, we have observed that TrxR was irreversibly inactivated by MMC, and the inhibition is both time and concentration dependent, but not pH-dependent. NADPH is required for the inhibition, which suggested that the inhibition involved the reduced form of the C-terminal active site of TrxR. But incubating MMC, TrxR and NADPH together would not cause consumption of NADPH, and no metabolites of MMC were found after the incubation, so MMC is not a substrate of TrxR. Addition of ethyl xanthante as external nucleophile did not influence the inhibition, suggested that MMC inactivated TrxR by binding to the active site. By using UV spectroscopy we can also characterize the complex of TrxR-MMC. By comparing the difference between unmodified TrxR and MMC inactivated TrxR, the spectrum of the mitosene derived from reductively activated MMC was shown.

We have also studied the toxic effect of MMC in different cell lines: Du145, Hela, A549 and MCF7, among all the cell lines, human prostate cancer cell Du146 cells were the most sensitive to the MMC treatment. MMC could inhibit TrxR in cells, and the loss of TrxR activity was consistent with the loss of cell viability. The cellular response of Trx system was also analyzed upon MMC treatment, and there was only a slight

decrease in the level of TrxR, so the loss of the TrxR activity was most likely due to the inhibition of TrxR by MMC but not the loss of TrxR at protein level.

Discussion

In the end, we proposed that MMC is a mechanism based inhibitor of TrxR because the inhibition fulfilled the requirements by which an inhibitor can be classified as a mechanism based inhibitor, as known as suicide inhibitor. The requirements are: a.) Time and concentration dependent; b.) irreversible; and c.) active-site directed, and d.) the conversion of enzyme-bound inhibitor to an active form. The exact mechanism was presented in scheme 1.



Scheme 1. Proposed mechanism for the mechanism based inhibition of TrxR by MMC (Paper V)

First, the selenolate from the Sec-498 of TrxR can form a conjugate to the quinone ring of the MMC to form a covalent complex intermediate (8). Then the intermediate undergoes an internal redox reaction involving the transfer of electron from Cys-497 to MMC to form a noncovalent complex 9. Then the hydroquinone of MMC (9) will undergoes a series of reactions (9,10,11,12) to generate the active intermediate. The

activated MMC can then react with the reduced C-terminal active site seleoate of TrxR when NADPH is present. (13 – 19)

The biological mechanism of action of MMC has mainly focused on its ability of crosslinking complementary strands of DNA[213] ever since it was first investigated in the 1960s. Considering the fact that bioactivitied MMC is highly reactive, other type of targets may exist. Recently, ribosome RNA was also found as a target of MMC[214]. In our study, we found that TrxR is strongly inhibited by MMC both in vitro and in cells suggest TrxR is a cellular target for MMC as well. TrxR is known as an emerging anticancer therapeutic target, and plays important roles in cancer development and progression[21,24,215,216]. TrxR was already known as a target of some other DNA alkylating agents such as: cisplatin, doxorubicin and ifosfamide[217–219]. Our finding support the ability of TrxR as a target for anticancer drugs, and MMC may function as a multi-target drugs.

3.2.6 Paper VI

Cristina M. L. Carvahlo, Jun Lu, **Xu Zhang**, Elias S. J. Arner, and Arne Holmgren. Effects of selenite and chelating agents on Mammalian thioredoxin reductase inhibited by mercury: implications for treatment of mercury poisoning. *The FASEB Journal 2011 Jan: 25(1):370-81*

Mercury is the only metal which is in liquid form and can evaporate in room temperature. Mercury toxicity induces damages in brain, kidney and immune system. Nowadays, people exposure to mercury mainly through the consumption of fish, the use of dental fillings containing mercury, and some vaccines with mercury compound as preservative, and acute exposure to mercury due to occupational reasons still happens[220,221]. Although the toxicity of mercury has been a highly interesting topic with a long history, the mechanisms of its toxicity and treatment were still not clear.

Selenium is an essential trace element for human, and one important use of selenium is the synthesis of selenoproteins. Deficiency of selenium in dietary up take can result in loss of immune function, weakened reproduction, depression and cardiovascular diseases[117]. Although the interaction of selenium with mercury has been extensively reported since it was first reported by Parizek and Ostadalova in 1967[222,223]. The exact mechanism is still unclear.

In our study, we have found that TrxR is a target of mercury, both organic MeHg and inorganic $HgCl_2$ can inhibit TrxR activity, but the inorganic form of mercury is more efficient. The presence of NADPH can largely enhance the inhibitory effect of both MeHg and $HgCl_2$, which suggested that the active site thiol and selenothiol of TrxR are involved in the binding. GSH, on the other hand, showed a protection effect in both the treatments with MeHg and $HgCl_2$, which is consistent with the previous finding that GSH can form complexes with both organic and inorganic mercury.

In order to further invest the interaction of mercury with TrxR, we used mass spectrometry method to compare the mass change of treated TrxR with untreated TrxR. Table 3 showed the interpretation of the mas spectrum result. Both the treatment of MeHg and $HgCl_2$ caused congregation of Hg with TrxR with maximum 8 Hg detected in 1 TrxR. Monovalent and bivalent mercury are both potent electrophiles, in our study, Hg^{2+} can bind to TrxR and damage its activity more effectively, whereas, the binding of MeHg showed less tendency of damaging its activity. Furthermore, in order to verify if selenocysteins in the C terminal active site of TrxR was involved in the binding of mercury, we used BIAM labeling method to detect the remaining Sec residue in TrxR after incubation with $HgCl_2$ and MeHg. Both the treatment of $HgCl_2$ and MeHg can cause the loss of detectable Sec residue in TrxR, but for $HgCl_2$, a ratio to TrxR of 4:1 can completely shelter the Sec residue; while for MeHg, the ratio needed to increase to 8:1 in order to get the same effect.

Table 3. MS analysis of $HgCl_2$ and MeHg binding with TrxR. (from Paper VI)

		Mass spectra data		
Protein	Activity in insulin assay	Mass (Da)	Mass difference (Da)	Comments
TrxR	100%	54,461; T	—	—
		54,666; FL	—	—
CH_3HgCl-treated TrxR, I:TrxR ~ 4)	+NADPH, 74 ± 3%	55,317	+651; FL	3× CH_3-Hg^+ 215.59 = 647
		55,530	+864; FL	4× CH_3-Hg^+ 215.59 = 862
	−NADPH, 95 ± 6%	55,744	+1078; FL	5× CH_3-Hg^+ 215.59 = 1078
		55,958	+1292; FL	6× CH_3-Hg^+ 215.59 = 1293
		56,173[a]	+1507; FL	7× CH_3-Hg^+ 215.59 = 1509
Saturated (20×) CH_3HgCl-treated TrxR	+NADPH, 1.1 ± 1.7% −NADPH, 100%			Up to 8 MeHg detected
$HgCl_2$-treated TrxR, I: TrxR ~ 4	+NADPH, 7 ± 1%	54,862	+401; T	2× Hg 200.59 − 4H = 397
		55,064	+395; FL	2× Hg 200.59 − 4H = 397
			+600; T	3× Hg 200.59 − 4H = 598
		55,265	+595; FL	3× Hg 200.59 − 4H = 598
	−NADPH, 23 ± 7%		+800; T	4× Hg 200.59 − 4H = 798
		55,464[b]	+798; FL	4× Hg 200.59 − 4H = 798
			+1003; T	5× Hg 200.59 − 4H = 999
Saturated (10×) $HgCl_2$-treated TrxR	+NADPH, 0 −NADPH, 0			Up to 8 Hg detected

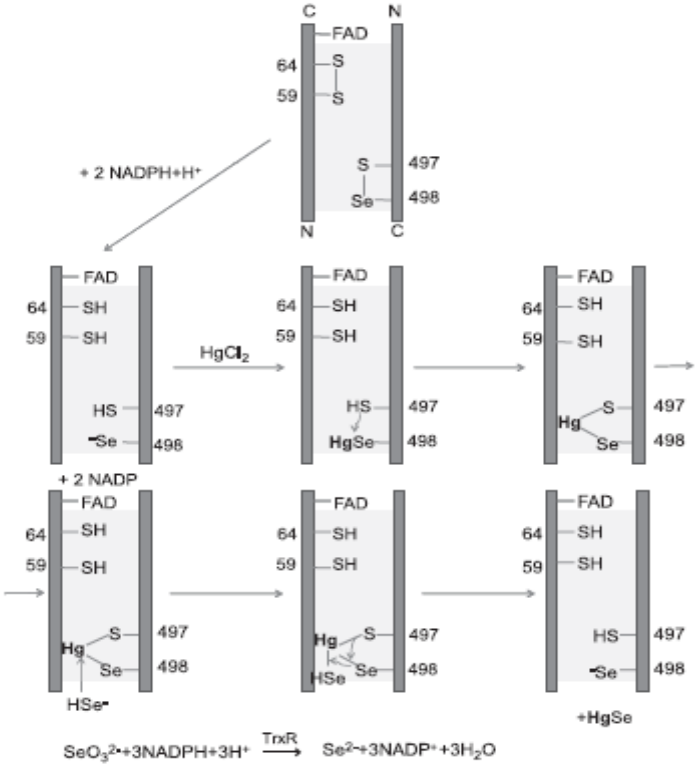
Because selenite is also the substrate of TrxR, so we tried to use selenite to measure the activity of TrxR after mercury treatment. To our surprise, with the selenite reduction assay, the activity of TrxR after MeHg treatment gave similar result as we observed in insulin reduction assay, while the activity of TrxR after $HgCl_2$ treatment was 100% recovered even when NADPH was in presence. Then we confirmed this result by using insulin reduction assay to make sure that selenite can recovery the activity of TrxR inhibited by $HgCl_2$. To further investigate the mechanism of the recovery, we performed mass spectrometry method to analyze the change of mercury biding to TrxR upon selenite addition. The result showed that selenite treatment resulted in new peaks, which coexist with the peaks appeared upon mercury treatment. These results suggested that the product of selenite reduction by TrxR, selenide, can remove Hg from the active site of TrxR. We have also compared the ability of reactivating TrxR by selenite with some clinical used chelating agents, such as BAL, DMPS and ALA. Selenite was as effective as any above mentioned chelating agents.

We have also examined the recovery effect of selenite in cultured HEK293t cells, consistent with *in vitro* data; selenite was able to remove both the inhibition of TrxR activity and the cytotoxicity caused by $HgCl_2$, but only at a relatively lower concentration (5 μM) and a shorter treatment time (9h). When the concentration of $HgCl_2$ was higher than 10 μM, or the treatment was longer than 24h, then the protection effect of selenite was abolished.

Discussion

There are in total 14 cysteine residues and 1 selenocysteine in human TrxR: Cys59 and Cys64 locate in the N-terminal active site; Cys497 and Sec498 locate in the C-terminal active site, and the rest 11 cysteine residue which are not directly involved in the activity of TrxR, but some of which expose in the surface of the enzyme and are also able to bind with mercury. This may explain in the MS results we can observe as many as 8 mercury molecules binding to one TrxR, while titration with $HgCl_2$, when NADPH was presence, a ratio between $HgCl_2$:TrxR was 2, was enough to block almost 100% of the activity.

Scheme 2 demonstrated the proposed mechanism for the inhibition of TrxR by mercury and reactivation of it by selenium. Briefly, bivalent mercury compound ($HgCl_2$) can target the reduced C terminal active site of TrxR, and binds to it to abolish its activity. Selenite can substitute the mercury molecule binds to the active site, and regenerate TrxR's activity.



Scheme 2. Schematic of the proposed mechanism for TrxR inactivation by mercury and reactivation by selenite. (from Paper VI)

3.3 CONCLUSION AND FUTURE PERSPECTIVES

In our first paper, we showed that the degradation of Trx2 upon BG treatment. Through comparing with fibroblast cells, we demonstrate the importance of Trx2 in cancer cells, such as Hela cells. However, the exact interaction of BG and Trx2 is not clear. Although we have *in vitro* data showed that BG can binds to thiols in small molecule such as dithiothreitol (DTT), but we did not find direct evidence of BG binding to Trx1 or Trx2 (*unpublished data*). In addition, we also found that BG is a strong inhibitor of TrxR1 *in vitro*, although we cannot find the inhibition effect on TrxR in cell lysates, this maybe because BG specifically accumulated in mitochondrial matrix, where it can only inhibit TrxR2. But even only considering mitochondria, where TrxR2 locates in the matrix and TrxR1 locates in the inter membrane space; the amount of TrxR2 is still much lower than that of TrxR1. So it is very difficult to obtain the data on TrxR2 in cells. However, the interaction of BG with Trx2 system should be further studied because it is very important for the investigation of the mechanism of degradation of Trx2. We have proposed mitochondrial Lon protease to be responsible for the degradation of Trx2, but this need to be further confirmed as well.

In paper II and IV, we investigated the importance of the second disulfide in regulating Trx1's activity and functions. In paper II, we proposed that when Trx1 with two disulfides cannot be reduced by TrxR, Grx system can reduce it through a monothiol mechanism. In paper IV, we further demonstrated that the formation of the second disulfide in Trx1 can sensitize SH-SH5Y cells towards cytotoxicity induced by some arsenical compounds. In addition, *in vitro* data showed that the formation of the second compounds can weaken Trx1's ability to reduce oxidized Prx1. However, because previous studies about Trx1's activity and functions were almost all focused on the active site Cys residues of Trx1, the data about the functions of the second disulfide in Trx1 is very limited. Although we have found that Trx1 with two disulfides can be generated in A549 cells under high dose of H_2O_2 treatment, there is no data showing its existence under physiological conditions. In my opinion, physiological process involving a high dose of oxidative stress should be paid more attention to, such as during inflammation. In addition, since Trx1 with two disulfides is not a substrate of TrxR, then the delay of reducing it may give a chance of transient inhibition of Prx1, and causes an increase of H_2O_2, which can subsequently exerts its function as a signaling molecule. Although there is no doubt that H_2O_2 is a messenger molecule more

than just an evil oxidant. Considering that it can diffuse freely in the cells, the mechanism how it can exert its function locally without spread the signal all over the cells is still not clear. In our Paper III, we investigated the role of Grx2 as a backup of TrxR, which can reduce both the oxidized Trx1 and Trx2 *in vitro* and in cells when TrxR is inhibited. It is interesting to think about the fact that Grx2 is more resistant to oxidative stress compare to Grx1, and it actually can be activated by ROS. This finding proposed a possibility that under some oxidative conditions, Grx2, not Grx1 reduces Trx1 with two disulfides. Considering the fact that the amount of Grx2 is low and its subcellular locations, maybe it plays a role in regulating the amount of H_2O_2 locally. Another factor that needs to be considered is GSH, because both Grx1 and Grx2 need the expense of GSH to reduce Trx, and the amount of GSH various in different cell organelles. On all accounts, the regulation and mechanism of the whole signaling pathway ($Grx1/Grx2 \rightarrow Trx1 \rightarrow Prx1 \rightarrow H_2O_2$) is not clear, more efforts should be spent on it, especially in a cellular model.

In our last two papers, we demonstrated TrxR as a cellular target of anticancer drug mitomycin C and the toxic compounds of mercury, and that selenium can act as a detoxification compound of mercury toxicity with a comparable recovery capacity of clinically used chelating agents. It is not surprising that the exposed highly reactive Sec residue in TrxR is a target of several electrophilic compounds and metals. However, by collaborating with experts who works in the chemical field and toxicology field, brought us new tools and different angels to study the interactions of compounds with Trx system, which can be applied in future studies.

In the end, it is worth to mention two new techniques which are developed and / or optimized by my colleagues during my PhD study, one is measuring TrxR/Trx activity by using fluorescent substrate, and another one is redox western blot. The new fluorescent method, which is very sensitive and stable, made it possible to measure the activity of TrxR/Trx in a very limited amount of cell samples, such as mitochondrial lysate; or cells with very low amount of TrxR/Trx, such as SH-SH5Y cells. The redox western blot made it is possible and reliable to analyze the redox state of each Cys residues in Trx1, thus it is a great tool to study the function of the structural Cys residues in Trx1. Moreover, it is possible to optimize the method and analyze the redox state of bigger molecules, such as peroxiredoxins. These two powerful tools will be applied more often in my future studies.

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