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REVIEW

Thioredoxin and Its Role in Toxicology

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Thioredoxins (Trx) are members of an evolutionarily conserved family of redox-active proteins containing a conserved active site dithiol motif. Trx supports diverse reduction reactions, including several of direct toxicologic interest, but relatively little information is available concerning the roles of Trx under specific toxicologic conditions. Accumulating evidence suggests that Trx serves a partially overlapping and highly complementary role to the glutathione (GSH) system in protecting against toxicity. GSH and Trx both function in the reduction of peroxides through the action of multiple GSH peroxidases and Trx peroxidases (peroxiredoxins), respectively. However, GSH is a small molecule that is present at millimolar concentrations, thereby providing a potential mechanism for elimination of alkylating electrophiles. In contrast, even though Trx is only present at micromolar or submicromolar concentrations, its dithiol motif makes it suited to reverse oxidative changes to proteins, including reduction of protein disulfides, methioninyl sulfoxides, and cysteinyl sulfenic acids. Moreover, Trx functions in redox-sensitive signal transduction, transcriptional activation of stress response genes, ribonucleotide reduction in synthesis of deoxyribonucleotides for DNA repair, and post-injury cell proliferation. Molecular studies show that the predominant cytoplasmic/nuclear form, Trx-1, and the mitochondrial form, Trx-2, both protect against oxidative stress, that both are essential for embryonic development, and that Trx-1 is inducible in response to oxidative stress. Because of the differences between GSH and Trx in distribution, catalytic activities and reactivities with electrophiles, particularly with the important role to be played by glutathione *S*-transferases, considerable research is needed to clarify their respective roles in protection against specific toxicologic conditions.

Key Words: **thioredoxin; glutathione; thiols; redox; toxicology; apoptosis.**

Introduction

Thioredoxin (Trx), a small protein first identified in *E. coli* and subsequently found to exist in most eukaryotic and prokaryotic species (Powis and Montfort, 2001), is used, as an abbreviation, to refer to any thioredoxin, or when studies are done on preparations without an explicit definition of the molecular species being examined (e.g., total activity in biologic systems). The molecular species discussed in this review are thioredoxin 1 (abbreviated as Trx-1), thioredoxin 2 (abbreviated as Trx-2), and thioredoxin-like molecules (abbreviated as $p32^{TrxL}$).

Trx has a redox-active dithiol in the active site that contains the highly conserved sequence, -Trp-Cys-Gly-Pro-Cys-Lys-. The cysteine moieties can be oxidized to the corresponding disulfide that is reduced, in turn, by thioredoxin reductase (TR), a NADPH-dependent selenoflavoprotein (Scheme 1). Trx exists in several forms with the cytosolic (Trx-1) and mitochondrial (Trx-2) forms being the most prevalent. Multiple thioredoxin reductases are also present, with a predominant cytosolic form, TR1, and a mitochondrial form, TR2.

A number of specific functions and cofactor activities have been identified for Trx (Arner and Holmgren, 2000) (Table 1). In addition, these proteins appear to have an important role in protecting cells from toxicants, especially oxidants (Nordberg and Arner, 2001) and electrophiles. Trx-1 acts as a cofactor, binding partner, and protein reductant. Each of these activities has an influence on cellular responses to toxic insults, particularly oxidative stress (Holmgren, 2000). As a cofactor for the peroxiredoxins, Trx-1 plays a direct role in reducing oxidative species, as has been illustrated for hydroperoxides (Rhee *et al.*, 2001). As a cofactor for ribonucleotide reductase and methionine sulfoxide reductase, Trx-1 contributes to DNA repair and the reduction of oxidized cellular constituents (Powis and Montfort, 2001). Binding of the reduced form of Trx-1 inhibits the activity of apoptosis signal-regulated kinase-1 (ASK1), a kinase involved in the initiation of apoptosis (Saitoh *et al.*, 1998). Finally, as a protein reductant, Trx-1 reduces disulfides and methionine sulfoxide groups in oxidized proteins. The net

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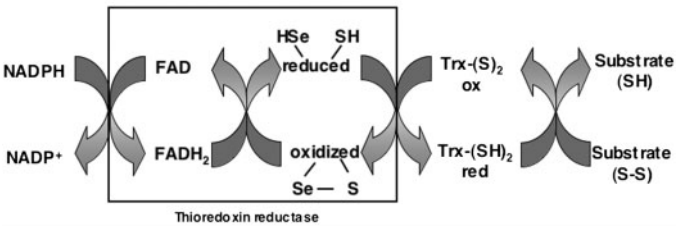
Scheme I



Scheme 1.

effect of Trx-mediated protein reduction may be to restore normal functions, or perhaps to modulate redox-signaling features of oxidized species. Although the roles of Trx in modulating the toxicities or signaling activities of xenobiotics are not yet well defined, an increasing amount of evidence supports critical roles for Trx in these processes. The purpose of this review is to summarize the current state of research regarding Trx as a cellular protectant. In particular, the specific mechanisms through which Trx may be exerting its modulating effects will be discussed.

Forms and Localization of Thioredoxin

As noted above, Trx exists in several forms. Human Trx-1 is a 12-kDa, 105-amino acid protein that exists primarily in the cytosol, but is also found in the nucleus and blood plasma. Trx-2 is found primarily in mitochondria. Trx-2 mRNA encodes a larger protein than that isolated from mitochondria. The protein is synthesized in a 166-amino acid, 18-kDa precursor form that includes a 60-amino acid N-terminal mitochondrial localization sequence that is cleaved upon import to yield the final 12.2-kDa Trx-2 protein. Trx-2 contains the conserved catalytic site, but lacks the other cysteine residues that are found in Trx-1. A third form of Trx, $p32^{TrxL}$, containing 289 amino acids was cloned from a human testis cDNA library (Miranda-Vizuete *et al.*, 1998). It contains the conserved Trx active site and is expressed in all human tissues, but appears not to be reduced by TR (Lee *et al.*, 1998). While $p32^{TrxL}$ is highly homologous to Trx-1 and also present in the cytoplasm, it is much larger and its function is not fully understood.

TABLE 1
Functions of Thioredoxin-1

Cell proliferation; growth factor
DNA synthesis
Ribonucleotide reductase
Redox signaling
Regulation of transcription factors (NF-kB, AP-1 [Ref-1], p53)
Detoxification; antioxidant
Reduction of H_2O_2; thioredoxin peroxidase, peroxiredoxins
Protein reduction
Methionine sulfoxide reduction
Protein disulfide reduction
Apoptosis
Reduced form of Trx-1 complexes with apoptosis signal-regulating kinase (ASK1); this prevents downstream signaling
Trx-1 likely affects other apoptosis pathways

Both Trx-1 and TR1 are found extra- as well as intracellularly (Rubartelli *et al.*, 1992; Soderberg *et al.*, 2000). In fact, a number of different cell types, including cancer cells, secrete Trx-1 (Powis *et al.*, 2000). One report has indicated that this secretion is not sensitive to oxidation (Tanudji *et al.*, 2003), but this needs to be confirmed. It remains possible that secretion is altered in response to various xenobiotics, including alkylating agents. Interestingly, studies in normal liver cells and the hepatocarcinoma cell line HepG2 have shown that, of these two types of cells, only normal cells secrete abundant Trx-1 (Rubartelli *et al.*, 1995). Secretion of Trx-1 by HepG2 cells, but not by normal hepatocytes, can be stimulated under reducing conditions (i.e., 80 μm to 1.4 mM 2-mercaptoethanol or 5 mM N-acetylcysteine), but these cells then undergo morphological changes and exhibit growth inhibition. Exogenous Trx-1 (100 nM) also inhibited cell proliferation in HepG2 cells, but did not induce the secretion of endogenous Trx-1. In contrast, 2-mercaptoethanol or N-acetylcysteine stimulated proliferation in a B-cell lymphoma line (Rubartelli *et al.*, 1995). These data indicate that secreted Trx-1 can have effects on cells, although this is apparently cell-line type dependent.

There have as yet been no studies to determine if secreted Trx-1 might provide protection from xenobiotic or oxidant stresses. However, extracellular Trx-1 appears to play a role in mediating responses to inflammation. Plasma Trx is increased in several diseases including HIV (Nakamura *et al.*, 2001), rheumatoid arthritis (Jikimoto *et al.*, 2002), asthma (Yamada *et al.*, 2003), hepatitis-C infection (Sumida *et al.*, 2000), and steatohepatitis (Sumida *et al.*, 2003). Secreted Trx-1 acts as a chemotactic factor for neutrophils, monocytes, and T cells (Bertini *et al.*, 1999). In another study, Trx-1 inhibited neutrophil chemotaxis initiated by endotoxin and mediated by the chemokines KC/GRO-alpha, RANTES, and MCP-1 (Nakamura *et al.*, 2001). This discrepancy may be explained by a variable balance between oxidant-induced chemotaxis and the antioxidant activity of Trx-1.

Trx-2 has been cloned from a rat heart cDNA library (Spyrou *et al.*, 1997), from a human liver cDNA library (Damdimopoulos *et al.,* 2002), and from human osteosarcoma cells and human embryonic stem cells (Chen *et al.*, 2002). Expression of Trx-2 is ubiquitous. Ejima *et al.*, (1999) showed that Trx-2 is present in mitochondrial fractions of human placenta while Bodenstein and Follmann (1991) found Trx-2 in pig heart. In brain, expressions of Trx-2 message and protein are high in several regions, and are inducible in selected regions by dexamethasone (Rybnikova *et al.*, 2000). In human heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, peripheral blood leukocytes, and

brain, Trx-2 mRNA levels generally paralleled mitochondrial contents (Chen *et al.*, 2002).

It is unknown whether Trx-2 may be transported out of mitochondria, or even whole cells under different conditions, and whether like Trx-1, this may affect cell function. Expression of Trx-2 without a mitochondrial localization sequence results in a cytosolic localization, and both the mitochondrially targeted and cytosolic forms of overexpressed Trx-2 protect against cell injury from oxidative stresses (Chen *et al.*, 2002).

Several truncated forms of Trx have been identified (Powis *et al.*, 2000). These may be formed subsequent to proteolytic activity. For example, a 10-kDa form appears to be secreted and bound to the outer plasma membrane of human MP6 (Rosen *et al.*, 1995) and U937 (Balcewicz-Sablinska *et al.*, 1991) cells. In addition, alternatively spliced forms of Trx mRNA have been documented (Hariharan *et al.*, 1996) including Trx80, a monocyte mitogen that is present in human plasma (Pekkari *et al.*, 2000).

Although it contains no recognizable nuclear localization or nuclear export sequences, Trx-1 can translocate into the nucleus in response to a variety of stimuli. Nuclear translocation has been shown in cell culture by western blotting of nuclear fractions and by immunostaining of cells that were treated with hydrogen peroxide (Makino *et al.*, 1999), hypoxia (Ema *et al.*, 1999), phorbol esters (Hirota *et al.*, 1997, 1999), tumor necrosis factor (Hirota *et al.*, 1999), ultraviolet irradiation (Didier *et al.*, 2001; Hirota *et al.*, 1997), ionizing radiation (Wei *et al.*, 2000), interleukin-1β, (Wiesel *et al.*, 2000), lipopolysaccharide (Wiesel *et al.*, 2000), and cisplatin (Ueno *et al.*, 1999). Nuclear translocation of Trx has also been documented in animal models including ischemia-reperfusion injury in the brain (Takagi *et al.*, 1998), and free radical-mediated kidney toxicity (Tanaka *et al.*, 1997). The reason for the increase in nuclear Trx in response to stress is unknown, but it may be related to its antioxidant and repair functions. Also, the increase in nuclear Trx-1 may provide the reducing environment required for DNA binding by a number of transcription factors.

Redox Activity of Thioredoxin

Thioredoxins evolved as chaperone-like proteins that function in maintenance of the dithiol/disulfide structure of proteins (Powis *et al.*, 1997). The highly conserved amino acid sequence contains 2 cysteines at the active site. These cysteines, present in the sequence Trp-Cys-Gly-Pro-Cys-Lys (at residues 32 and 35 of human Trx-1, and at residues 90 and 93 of human Trx-2) are oxidized to a disulfide through the transfer of two reducing equivalents from Trx to a disulfide-containing target protein. The resulting active site disulfides of Trx-1 and Trx-2 are reduced by TR1 and TR2, respectively, using electrons from NADPH (see Scheme 1). However, Trx-1 has 3 additional cysteine residues in addition to the 2 located in the active site, whereas Trx-2 does not. The additional cysteine residues in Trx-1, particularly Cys-72, which is located in a loop in proximity to the active site, can be oxidized leading to dimer formation and a subsequent loss of catalytic activity (Ren, 1993). The reportedly higher resistance to oxidation in Trx-2 than in Trx-1 may be a result of the absence of these additional cysteine residues (Damdimopouilos *et al.*, 2002), although this greater resistance to inactivation may not be true for all oxidants (D. P. Jones, unpublished data). This difference in resistance to oxidative inactivation may also explain the high expression of Trx-2 in tissues with high metabolic activities, and may confer important regulatory and/or protective functions.

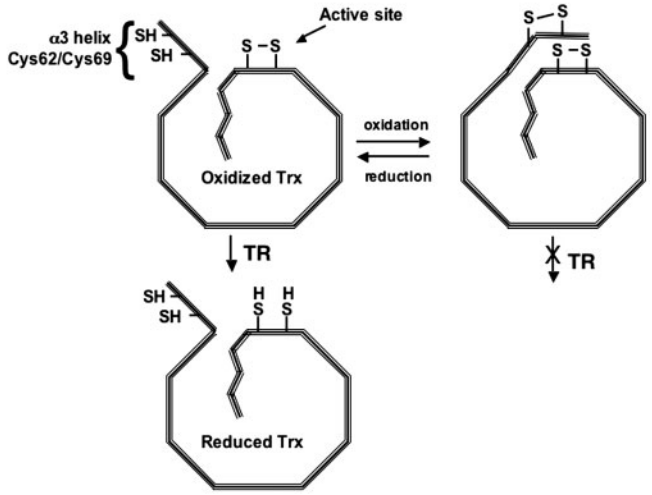
Holmgren and Fagerstedt (1982) showed that dithiol and disulfide forms of bacterial Trx could be separated following treatment with iodoacetic acid. This treatment resulted in (C32)-S-carboxymethyl-Trxand(C32,C35)-bis-carboxymethyl-Trx forms that have additional negative charges associated with the carboxyl groups. These derivatives can be separated from Trx-(C32,C35)-disulfide by native gel electrophoresis. Using this approach, Holmgren and Fagerstedt (1982) showed that Trx is about 60% reduced in *E. coli* in the logarithmic growth phase. Fernando *et al.* (1992) used this separation with a western blot to measure Trx-1 oxidation in endothelial cells. They concluded that essentially all of the Trx-1 was reduced under basal conditions, and that, even following treatment with hydrogen peroxide, 70–85% of the total Trx-1 remained fully reduced. Das *et al.* (1997) used this approach to assess the redox status of oxidized *E. coli* Trx added to A549 cells. They found that approximately 45% of internalized *E. coli* Trx was fully or partially reduced, and that this could be increased to over 80% by the addition of TR and NADPH to the extracellular medium. These authors also found that hyperoxia resulted in substantial oxidation of Trx in premature baboon lung (Das *et al.*, 1999).

This "Redox western" blot approach, using antibodies to Trx-1, was used to determine the standard redox potential (E_o) of Trx-1 (-230 mV; Watson *et al.*, 2003) and to quantify the redox state of Trx-1 in the cytoplasm (Nkabyo *et al.*, 2002, 2003) and nuclei (Watson and Jones, 2003). Trx-1 was 95% reduced in both compartments, corresponding to a redox potential (E_h) of −280 mV.

The redox characteristics of *E. coli* Trx are more favorable for function in reduction of protein disulfides than are the properties of either GSH/glutathione disulfide (GSSG) or glutaredoxin. The $E_{o'}$ value for *E. coli* Trx is about 30 mV more negative than that for GSH/GSSG, and 70 mV more negative than that for glutaredoxin (Aslund *et al.*, 1997; Lundstrom and Holmgren, 1993). The $E_{o'}$ value for human Trx-1 (-230 mV) is very similar to that for GSH/GSSG (Watson *et al.*, 2003), but the redox characteristics of Trx-2 are not known. In cells, Trx-1 is largely reduced under normal conditions (Nkabyo *et al.*, 2002; Watson *et al.*, 2003; Watson and Jones, 2003) so that the redox characteristics are consistent with the conclusion that these proteins normally function as reductants.

This latter conclusion is important because a similar dithiol/disulfide active site is conserved among a broader family of

Scheme II



Scheme 2.

proteins that vary in redox characteristics. Some of the Trx family members (e.g., the protein disulfide isomerases) function to introduce disulfides into proteins rather than to reduce disulfides to dithiols. Thus, the thiol-disulfide exchange reactions catalyzed by Trx could function to oxidize protein thiols. However, recent data indicate that this postulated protein oxidation activity may not be a significant property of Trx-1, because it contains an auxiliary dithiol motif, C62–C69 (Scheme 2), which, upon oxidation to the corresponding disulfide, changes protein–protein interactions. Although the available data only show that oxidation of the C62–C69 motif inhibits reduction of the active site disulfide by TR1 (Watson *et al.*, 2003), the conformational changes that alter interactions of Trx-1 with TR1 also may inhibit interactions with other proteins because of the proximal position of the C62–C69 motif to the Trx-1 active site. On the other hand, by inhibiting TR1-mediated reduction of the catalytic disulfide, the half-life of the oxidized form of Trx-1 will be prolonged. This will increase the time this oxidized species has to undergo reactions that are otherwise slower than its reduction by TR1, such as thiol-disulfide exchange with other proteins.

Functions of Thioredoxin

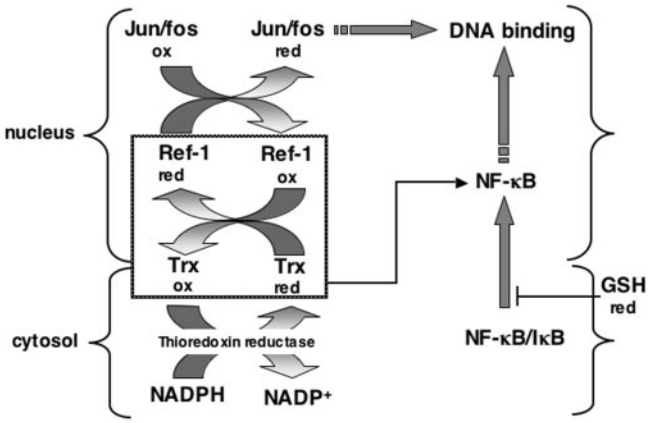
Numerous activities have been identified for Trx-1 (Table 1). These include acting as a growth factor, antioxidant, and enzyme cofactor (particularly for ribonucleotide reductase), and in regulating certain transcription factors. This latter activity is based on the ability of Trx-1 to reduce thiols that are critical for DNA binding on nuclear transcription factors such as AP-1 and NF-κB (Nishiyama *et al.*, 2001) (Scheme 3). Trx-1 also binds to proteins (perhaps modulating folding), plays a role in tumor resistance to anticancer drugs (Sasada *et al.*, 1996), and provides protection against oxidative stress, bleomycin-induced lung damage, and doxorubicin-induced cardiotoxicity (Andoh *et al.*, 2002; Gon *et al.*, 2001; Shioji *et al.*, 2002; Tanaka *et al.*, 2000). Further protective effects will undoubtedly be identified. The underlying mechanisms by which Trx-1 protects cells remain largely unexplored but seem more likely to involve effects on signaling pathways than to be mediated by direct scavenging, because Trx is present at much lower concentrations than are other endogenous antioxidants and nucleophiles such as GSH.

The antioxidant activities of all forms of Trx are clearly related to its thiol content, particularly at the active site. These activities are most likely mediated through its function as a cofactor for peroxidases known as the peroxiredoxins (Rhee *et al.*, 2001), although some direct antioxidant capacity exists. Trx-1 may also function as a cofactor in secondary antioxidant repair systems. For example, methionine sulfoxide reductase reduces methionine sulfoxide residues using electrons from Trx-1 (Lowther *et al.*, 2000).

Many human cancer cells have increased Trx-1 levels, and cellular resistance to chemotherapy seems to be related to the growth factor activities of Trx-1. However, Trx-1 is an atypical growth factor, as it does not appear to bind to any specific receptor. Thus, Trx-1 should probably not be considered a growth factor, but rather an important growth co-factor. The mechanisms underlying the pro-growth activities of Trx-1 may be through an ability to prevent the inactivation, or to enhance more directly the activities of other endogenous growth factors.

The large number of activities identified for Trx-1 clearly suggested that this molecule would be critical for cell viability. This expectation was realized when Matsui *et al.* (1996) found that Trx-1 null mice were embryonic lethal at a very early stage of development. Nonn *et al.* (2003) recently showed that, like Trx-1, deleting Trx-2 is also embryonic lethal to mice. Fibroblasts cultured from Trx-2 null embryos are not viable and the null cells showed extensive apoptosis. Interestingly, lethality appeared at the mid-point of development when oxidative phosphorylation began. This contrasts with lethality in Trx-1

Scheme III



Scheme 3.

null mice which occurs shortly after implantation (Matsui *et al.*, 1996). The authors concluded that the generation of reactive oxygen species (ROS) was the key event in cell death in Trx-2 null mice, and thus that Trx-2 is a critical component in an important antioxidant defense system. Overall, while the basic redox biochemistry of Trx-1 and Trx-2 is similar, and both are essential for fetal viability, the differences in staging of fetal deaths indicate distinct essential functions for the respective proteins.

While Trx-1 knockouts are lethal, possibly more subtle effects of Trx-1 underexpression have not been studied. Trx-2 heterozygous animals showed no obvious defects (Nonn *et al.*, 2003), although they were not tested for sensitivity to oxidants or other xenobiotics. A study *in vitro* (Tanaka *et al.*, 2002) examining the importance of Trx-2 made use of chicken B cells expressing a *tet*-repressible Trx-2 transgene. These researchers showed that, following suppression of Trx-2 for 5 days, there was a 2- to 3-fold increase in intracellular dichlorofluorescein oxidation (an index of ROS). In addition, apoptosis increased, reaching 45% of total cells by day 7 (Tanaka *et al.*, 2002). The Trx-2 suppressed cells were also more susceptible to apoptosis induced by serum-withdrawal than were control cells. The effect of Trx-2 suppression on xenobiotic-induced apoptosis was not tested. Interestingly, consistent with data in whole animals showing Trx-2 knockout is embryonic lethal (Nonn *et al.*, 2003), these authors were unable to create any homozygous Trx-$2^{-/-}$ clones. Similar studies have not yet been done in mammalian cells.

A number of functions have been found for Trx-2. Trx-2 isolated from pig brain mitochondria catalyzes the regeneration of native 4-aminobutyrate aminotransferase from the oxidized enzyme (Park and Churchich, 1992). Pig Trx-2 is catalytically active as a reductant for *E. coli* ribonucleotide reductase, but no mitochondrial substrates were identified (Bodenstein and Follman, 1991). Studies in yeast showed that Trx-2 acts as a reductant to a peroxiredoxin that functions in peroxide reduction (Pedrajas *et al.*, 2000). Mutant yeast cells that were deficient in Trx-2 reductase were more sensitive to peroxide-induced toxicity (Pedrajas *et al.*, 1999). Reddy *et al.* (1999) found that in Emory mouse lens, a model for age-related cataract formation, there was an increased amount of Trx-1 mRNA and protein after three weeks, but a decreased amount of Trx-2 mRNA, relative to time zero beginning 6 weeks after photochemical treatment, suggesting that the failure to maintain or increase Trx-2 could contribute to injury. In cultured rat retinal pigment epithelial cells transfected with human Trx-2, immunoelectron microscopy revealed increased staining associated with mitochondria following hydrogen peroxide treatment (Gauntt *et al.*, 1994). Finally, Trx-2, like Trx-1, can reduce insulin in the presence of NADPH and thioredoxin reductase, a reaction that forms the basis for the most widely used activity assay (Holmgren and Bjornstedt, 1995).

As noted above, Trx-1 overexpression also provides protection against oxidative stress and some xenobiotic-induced toxicities (Andoh *et al.*, 2002; Gon *et al.*, 2001; Shioji *et al.*, 2002; Tanaka *et al.*, 2000). Other data have shown that overexpression of Trx-2 makes human osteosarcoma cells resistant to oxidant-induced apoptosis (Chen *et al.*, 2002) and human embryo kidney cells resistant to etoposide (Damdimopoulos *et al.*, 2002). This last finding is of interest since etoposide is not considered to be toxic via an oxidative mechanism. However, etoposide does have some interesting dose-dependent effects on mitochondrial apoptosis-signaling pathways (Ott *et al.*, 2002) that Trx-2 may be able to alter.

Finally, Trx-2 may interact with components of the mitochondrial respiratory chain, thereby regulating mitochondrial potential ($\Delta\psi_m$) and perhaps functioning as an anti-apoptotic protein (Ly *et al.*, 2003). Trx-2 and cytochrome *c* co-immunoprecipitate (Tanaka *et al.*, 2002), and overexpression of Trx-2 increases $\Delta\psi_m$ (Damdimopoulos *et al.*, 2002), which supports these concepts. Furthermore, Trx-2 overexpressing cells are more sensitive to rotenone, and Trx-2 may interfere with the activity of ATP synthase (Damdimopoulos *et al.*, 2002). Although the characterization of Trx-2 is limited, taken together with known properties of Trx-1 and *E. coli* Trx, available results strongly support the interpretation that Trx-2 functions in protection against oxidative stress, maintenance of protein thiols, and prevention of mitochondria-mediated apoptosis.

Thioredoxin and Apoptosis

As described above, it is apparent that Trx-2 has the potential to exert significant control over apoptosis. A similar or perhaps complementary role for Trx-1 has been suggested by several studies (reviewed in Powis and Montfort, 2001). For example, exogenous addition of Trx-1 can prevent oxidant-induced apoptosis in neuroblastoma cells (Andoh *et al.*, 2002) while WEHI7.1 cells transfected to overexpress Trx-1 are resistant to apoptosis induced by a variety of agents (Baker *et al.*, 1997). Similarly, increased Trx-1 levels are related to decreased apoptosis in gastric carcinomas (Grogan *et al.*, 2000). Conversely, lymphocytic cells transfected to express a redox-inactive Trx-1 exhibit enhanced susceptibilities to apoptosis induced by various xenobiotics (Freemerman and Powis, 2000).

The mechanisms by which Trx affects apoptosis are not known. As noted above, direct interactions with chemically reactive species may occur, but effects on signaling pathways are likely to be more important in Trx functions. Trx-1 binds with ASK1 (Saitoh *et al.*, 1998), creating an inactive complex. Certain apoptosis-inducing stresses (particularly oxidative) can break this complex, activating ASK1 and leading to the activation of *c*-Jun amino-terminal kinase (JNK)/p38 mitogen-activated protein (MAP) kinases and apoptosis (Tobiume *et al.*, 2001).

The JNK and p38 kinase pathways have been characterized in some detail. These pathways belong to the MAP kinase-signaling cascade, which typically consists of three layers of

protein kinases including MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK) (Davis, 1994; Waskiewicz and Cooper, 1995; Widmann *et al.*, 1999). Through hierarchical phosphorylation, MAPK is activated and then, in turn, regulates the activities of downstream transcription factors or other kinases to control gene expression.

ASK1 was identified as a MAPKKK by showing that it activated SEK1-JNK and MKK3/MKK6-p38 signaling cascades (Ichijo *et al.*, 1997). Several studies suggested ASK1 to be a key element in the mechanism of cytokine- and stress-induced apoptosis. For example, overexpression of ASK1 induced apoptotic cell death, while one kinase-inactive mutant of ASK1 (ASK1-K709R) reduced TNFα-induced apoptosis (Ichijo *et al.*, 1997; Tobiume *et al.*, 1997). Recent data from ASK1-deficient mice also suggests that sustained activation of JNK/p38 and apoptosis induced by TNFα and ROS required ASK1 (Tobiume *et al.*, 2001).

Trx-1 is a key regulator of ASK1 functions. The reduced form of Trx-1 is bound directly to the N-terminal part of ASK1, thereby inhibiting ASK1 activity as well as ASK1-dependent apoptosis. Downregulation of Trx-1 levels caused activation of endogenous ASK1 (Saitoh *et al.*, 1998). It has also been reported that HIV Nef protein inhibited ASK1 activity by preventing Trx-1 release from the Trx-1-ASK1 complex (Geleziunas *et al.*, 2001). Recent data suggested that Trx-1 could promote ASK1 ubiquitination and degradation in endothelial cells (Liu and Min, 2002). As described above, the inhibition of ASK1 by Trx-1 is dependent on the oxidation state of Trx-1 (Saitoh *et al.*, 1998). Both single mutants, but not the double mutant at the redox-active site of Trx (C32S, C35S) retain binding activity for ASK1 and an ability to induce ASK1 ubiquitination/degradation. This suggests that Trx-1 may form a stable complex with ASK1 through either of its Cys residues (Liu and Min, 2002). This type of complex has been shown between the single Cys-containing Trx-1 and Trx-1 reductase (through C32) or NF-κB (through C35) (Qin *et al.*, 1995; Wang *et al.*, 1996).

The co-immunoprecipitation of Trx-2 with cytochrome *c* (Tanaka *et al.*, 2002), and the importance of cytochrome *c* in apoptosis (Wang, 2001), are suggestive that this thiol may be released in response to apoptotic stimuli and play a role in critical regulatory pathways associated with this form of cell death, perhaps through effects on the formation of the apoptosome and the activation of caspases (which require reduced cysteine to be active). This latter possibility is supported by one study showing that the maintenance of the cellular reducing environment by thioredoxin (Trx-1 vs. Trx-2 was not examined), as well as by GSH, was required for caspase-3 to become activated and induce apoptosis (Ueda *et al.*, 1998). Clarifying the importance of Trx-1 and Trx-2 and the mechanisms through which the two thioredoxins modulate apoptosis and protect cells from oxidants and electrophiles, requires additional research.

Thioredoxin and Transcription Factors

Many genes have effects on cell division and are modulated in response to stressors. Redox-regulated genes and transcription factors are particularly prevalent. At least 64 redox-regulated transcription factors have been identified (Allen, 1998; Gabbita *et al.*, 2000). A number of these have critical thiol moieties and are known to be regulated, at least in part, by the Trx system. This role has been highlighted in some recent reviews (Forman *et al.*, 2002; Haddad, 2002). Of particular note are p53, NF-κB, AP-1, Nrf2, GR, and ER, each of which is thiol-dependent and has been implicated in cell proliferation and apoptosis (Aggarwal, 2000; Grippo *et al.*, 1985; Hayashi *et al.*, 1997; Kim *et al.*, 2003; Shaulian and Karin, 2002; Sheikh and Fornace, 2000).

More so than most other transcription factors, there is an extensive body of information regarding the activation of NF-κB. This activation requires phosphorylation of the inhibitory IκB subunit, which results in its dissociation from the inactive complex and its degradation. A role for ROS in NF-κB activation is based on the observations that oxidizing conditions activate NF-κB in several cell types, antioxidants can block this activation, and ROS production is enhanced by NF-κB inducers such as tumor necrosis factor α. However, at the nuclear level, NF-κB must be reduced in order to bind to DNA. Thus, the redox status of specific subcellular sites is crucial for determining the activation state of NF-κB (Flohé *et al.*, 1997). Although GSH was considered necessary for NF-κB reduction (Rupec and Baeurle, 1995), it is now evident that Trx-1 is the proximate factor (Hirota *et al.*, 1999). Given that numerous xenobiotics alter transcription factors such as NF-κB and AP-1, and given the myriad effects of these transcription factors on cellular growth and death (Gius *et al.*, 1999), Trx-1 is likely to exert at least some of its effects through actions on redox-regulated transcription factors.

Recent data have suggested that Trx-1 may also activate NF-κB by affecting the degradation of IκB, which is mediated through the JNK-signaling pathway. In response to the overexpression of redox active Trx-1 in A549 cells, NF-κB was activated while IκB was degraded (Das, 2001). In MCF-7 cells stably expressing Trx-1, a NF-κB-dependent reporter was also activated (Freemerman *et al.*, 1999). A link between NF-κB and JNK signaling was suggested, based on the evidence that overexpression of MEKK1 (one MAPKKK upstream of JNK) activated NF-κB (Hirano *et al.*, 1996; Meyer *et al.*, 1996). Though it has been shown that MEKK1 may be the initiating kinase of the JNK pathway that mediates the NF-κB activation by Trx-1, the mechanism requires further investigation.

AP-1 is a ubiquitous collection of protein complexes known to regulate transcription in response to environmental stimuli. It is composed of gene products from the *fos* and *jun* proto-oncogene families. The products of these genes form homodimeric (Jun-Jun) and heterodimeric (Fos-Jun) complexes that bind to DNA (Rupec and Baeurle, 1995). In addition to being

stimulated by a wide range of xenobiotics or factors that promote cell proliferation, several studies demonstrate that cellular thiol redox state plays an important role in the activation of AP-1 (Pinkus *et al.*, 1993; Rupec and Baeurle, 1995). In particular, the transcriptional activity of AP-1 is regulated by a direct association between Trx-1 and Ref-1 (Hirota *et al.*, 1997).

Several genes involved in protection against oxidative stress and xenobiotics contain within their promoter an antioxidant response element (ARE). Transcriptional regulation through the ARE involves binding by Nrf2, which forms a heterodimer with small MafK proteins (Nguyen *et al.*, 2000). Nrf2 is normally retained in the cytoplasm through its association with Keap1. In response to oxidative stress and certain dietary inducers, cysteine residues within Keap1 are oxidized, and Nrf2 is released from Keap1 and enters the nucleus binding to ARE-containing gene promoters (Dinkova-Kostova *et al.*, 2002). Although oxidizing conditions in the cytoplasm promote the activation of Nrf2, it should be noted that oxidizing conditions in the nucleus inhibit Nrf2 binding to the ARE (Kim *et al.*, 2003).

The Trx system can affect p53, a well-studied redox-sensitive tumor suppressor protein with many roles in cell signaling and apoptosis (Stewart and Pietenpol, 2001). Because activation of p53 by genotoxic agents and oxidative stress results in cell-cycle arrest, upregulation of repair pathways, and the initiation of apoptosis if repair is not possible, p53 has been called the guardian of the genome (Lane, 1992). In addition, p53 contains several critical cysteines in its DNA-binding domain. Some of these cysteines are required for the coordination of zinc to form a zinc finger domain, whereas others are not involved in zinc binding but come into contact with the DNA (Hainaut and Mann, 2001). Both zinc binding and DNA binding require that these cysteine residues be in the reduced form, and Trx-1, both directly and through Ref1, enhances the DNA binding activity of p53 in the nucleus (Ueno *et al.*, 1999).

Several transcription factors have critical cysteine residues in their DNA binding domains. In principle, all such transcription factors are susceptible to oxidation and, therefore, Trx-1 has the capability for maintaining them in their reduced and functional forms. Different effects may occur in the cytoplasm, where signal initiation occurs, and in the nucleus, where DNA binding occurs. Most evidence indicates that redox regulation by Trx-1 is more likely to occur in the cytoplasm where the key signaling and regulatory machinery reside, with the nuclear function limited to maintenance of DNA binding activity. However, Trx-1 may provide a further level of transcriptional regulation in the nucleus. For example, overexpression of a nuclear-targeted Trx-1 construct was associated with increased NF-κB-mediated gene expression (Hirota *et al.*, 1999). More research will be required to define the distinct functions of nuclear Trx-1 and cytoplasmic Trx-1 in the regulation of gene expression.

Electrophiles, Thioredoxin, and Transcription-Factor Activation

Thiol moieties represent major nucleophilic sites within cells. Electrophilic species, through their formation via xenobiotic metabolism, their widespread environmental presence, and their ability to react with cellular macromolecules are significant toxicants. In order to adapt to electrophilic stresses, cells have evolved DNA response elements that respond to electrophiles and oxidants. The oxidant *t*-butylhydroquinone activates the Trx-1 gene, apparently through binding of the Nrf2/small Maf complex to the antioxidant response element (ARE); binding that is itself enhanced by Trx-1 (Kim *et al.*, 2003). The authors suggested that this induction might contribute to protection against chemical carcinogenesis.

One of the most electrophilic aldehydes to which humans are exposed is acrolein (Srivastava *et al.*, 1999). Acrolein is present in our food, is generated endogenously through lipid peroxidation (Uchida *et al.*, 1998) and oxidation of hydroxyamino acids by myeloperoxidase (Anderson *et al.*, 1997), and is a metabolic product of cyclophosphamide, spermine, spermidine, allyl alcohol and allylamine (Ghilarducci and Tjeerdema, 1995). At sublethal doses, acrolein exhibits subtle broad-based effects reflected in decreased proliferation (Horton *et al.*, 1997; Ramu *et al.*, 1996), while at higher doses, massive cell and tissue injury ensues.

Acrolein induces many genes regulated by the ARE (Kehrer and Biswal, 2000). Acrolein reacts rapidly with nucleophiles, especially cellular thiols such as GSH (Horton *et al.*, 1997, 1999), and similar interactions occur with the sulfhydryl moieties of Trx and TR (J. P. Kehrer, unpublished data). Although Trx activity was virtually eliminated in A549 cells immediately after treatment with acrolein, western blot analyses of Trx-1 protein levels revealed no changes in levels of immunoreactive protein. By 4 h after 25 and 50 μM, acrolein immunoreactive protein levels were greater in acrolein-treated than in control cells (JPK unpublished data). These data indicate a rapid synthesis of bioactive Trx-1 in acrolein-treated cells, and is consistent with the report that electrophiles induce Trx expression (Kim *et al.*, 2003).

The depletion of cellular thiols such as GSH and Trx by electrophiles likely affects transcription factor activation. For example, NF-κB activity after exposure to acrolein is inhibited (Horton *et al.*, 1999; Li *et al.*, 1999). This inhibition appears to involve a loss of regulatory thiols as well as a direct binding of acrolein to the critical nucleophilic cysteine (Kumar *et al.*, 1992; Toledano *et al.*, 1993) on the p50 and/or p65 subunits (Horton *et al.*, 1999) of NF-κB. The effects of acrolein on AP-1 and p53 are similar to those seen with NF-κB. Specifically, there is adduct-formation and an inhibition of activity (Horton *et al.*, 1999; Biswal *et al.*, 2002, 2003). However, the specific role of Trx-1 in affecting xenobiotic-induced changes in the activation of these transcription factors has not yet been determined.

TABLE 2
Comparison of GSH and Thioredoxin-1 Systems

	GSH	Trx-1
Molecular mass (Da)	307	11,606
Concentration in cells	>1 mM	≤1 μM
Electrons donated/molecule during reduction	1	2
Biological reducing system	NADPH+ Flavoprotein	NADPH+ Flavoselenoprotein
E_o	−240 mV	−230 mV
E_h, Proliferating cells	−260 to −230	−280 mV
Differentiated cells	−230 to −190	−280 mV
Apoptotic cells	−170 to −150	−270 mV
Biosynthetic functions	Yes	Yes
Detoxification of peroxides	Yes	Yes
Detoxification of electrophiles	Yes	Maybe
Repair functions	Minimal	Multiple

Other data exist indicating that electrophiles can form adducts with Trx-1. For example, the metabolism of 1,2-dichlorothane involves conjugation with GSH, yielding S-(2-chloroethyl)-glutathione. This product is capable of alkylating Trx-1 (Erve *et al.*, 1995). In addition, endogenously formed cyclopentenone prostaglandins such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 are electrophilic and can bind to Trx-1 (Shibata *et al.*, 2003). It has also been reported that Trx-1 is sensitive to *S*-nitrosylation and that this can cause dissociation from ASK1 leading to its activation (Sumbayev, 2003).

GSH and Trx

The GSH and Trx systems have many similarities and differences (Table 2). GSH is present at millimolar concentrations in cells and functions along with several peroxidases and GSH S-transferases to provide a primary protection against ROS and electrophiles (Sies, 1999). GSH also protects against free radical damage by maintaining vitamins C and E in their reduced, radical-scavenging forms (Smith *et al.*, 1996). These functions allow GSH to protect protein thiols from oxidation. In addition, GSH can reduce protein disulfides and sulfenic acids by nonenzymatic and enzymatic reactions (Cotgreave and Gerdes, 1998).

A large number of factors that have, or lead to, oxidant/free-radical activity, or exhibit antioxidant effects, have been identified as having effects on cell division and apoptosis. As the major cellular thiol, GSH has been extensively studied in this context. Some investigators have observed a positive correlation between GSH levels and cell proliferation (Frischer *et al.*, 1993; Horton *et al.*, 1997; Spyrou and Holmgren, 1996), while others have noted either no correlation or even a suppression of proliferation following the administration of exogenous GSH (Cantin *et al.*, 1990). Cell-specific differences may account for some of these variations, but despite the substantial circumstantial evidence for a role of GSH in redox control and signaling, there are also reasons to doubt that GSH directly controls the function of signaling and regulatory proteins. Studies of GSH reactions with protein disulfides indicate that the rates are slow, relative to that needed for efficient regulation (Gilbert, 1990, 1995). Even though there are enzymes (the protein disulfide isomerase family) that catalyze thiol-disulfide exchange reactions with GSH, their redox characteristics indicate that they function to introduce disulfide bonds, not to reduce disulfides to thiols (Ferrari and Soling, 1999).

GSH and Trx redox appear to be independently controlled. Trx-1 is maintained in a reduced state, even under conditions resulting in GSH depletion and oxidation (Nkabyo *et al.*, 2002). Other work in yeast confirms that the redox state of the Trx system is maintained independently of the GSH system (Trotter and Grant, 2003). Recently, Casagrande *et al.* (2002) showed that GSH can form a mixed disulfide with Trx-1 at one of the nonactive site cysteines and that this glutathionylation inhibited the activity of Trx-1, suggesting a mechanism by which the GSH system could regulate the Trx system. Thus, there appears to be a potential for cross talk between GSH and Trx-1, but the two systems certainly operate independently under some, or even most, conditions. Overall, additional research is needed on the toxicologic role of Trx to complement the extensive database available on GSH.

Summary, Significance, and Future Studies

Over the years, many studies have examined the roles of GSH/GSSG in cellular responses to toxicants. The Trx system is a relatively recent addition to the recognized cellular defense armamentarium. As a result, significant work remains to be done to understand its roles. Because of the nucleophilic sites found on Trx, it clearly has the ability to interact with oxidants and electrophiles. While Trx is present at substantially lower levels than GSH, its roles in regulating cellular events appear to be more direct, making it a potentially critical target for toxicants. Moreover, the presence of two thiols in the active site makes Trx well suited for 2-electron reductions of protein disulfides and sulfenic acids.

Many different oxidants and electrophiles have the potential to interact with Trx. Surprisingly, the toxicities of xenobiotics that are believed to act through nonoxidant/electrophile mechanisms are also affected by Trx. For example, as noted earlier, etoposide toxicity is inhibited by the overexpression of Trx-2 (Damdimopoulos *et al.*, 2002). Clearly, additional research is needed to investigate how the different forms of Trx can modulate cellular toxicity induced by agents that act through purportedly nonoxidant mechanisms.

The protective effects of Trx-1 versus Trx-2 are not yet clear. Recent data indicate that both have complementary, but not overlapping functions. Further studies examining the independent roles of each thiol are needed to fully understand their toxicologic significance, particularly in terms of electrophiles, oxidants, and signal transduction pathways. In addition, the

mechanisms underlying the effects of Trx-1 and Trx-2 need to be better defined. Such data will improve our understanding of the molecular mechanisms by which cells defend themselves against endogenous and exogenous reactive molecules and may assist in developing therapeutic strategies to protect normal tissues from oxidizing and electrophilic toxicants.

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