Interplay between cytosolic disulfide reductase systems and the Nrf2/Keap1 pathway

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Abstract
NADPH transfers reducing power from bioenergetic pathways to thioredoxin reductase-1 (TrxR1) and glutathione reductase (GR) to support essential reductive systems. Surprisingly, it was recently shown that mouse livers lacking both TrxR1 and GR ('TR/GR-null') can sustain redox (reduction-oxidation) homeostasis using a previously unrecognized NADPH-independent source of reducing power fuelled by dietary methionine. The NADPH-dependent systems are robustly redundant in liver, such that disruption of either TrxR1 or GR alone does not cause oxidative stress. However, disruption of TrxR1 induces transcription factor Nrf2 (nuclear factor erythroid-derived 2-like-2) whereas disruption of GR does not. This suggests the Nrf2 pathway responds directly to the status of the thioredoxin-1 (Trx1) system. The proximal regulator of Nrf2 is Keap1 (Kelch-like ECH-associated protein-1), a cysteine (Cys)-rich protein that normally interacts transiently with Nrf2, targeting it for degradation. During oxidative stress, this interaction is stabilized, preventing degradation of newly synthesized Nrf2, thereby allowing Nrf2 accumulation. Within the Trx1 system, TrxR1 and peroxiredoxins (Prxs) contain some of the most reactive nucleophilic residues in the cell, making them likely targets for oxidants or electrophiles. We propose that Keap1 activity and therefore Nrf2 is regulated by interactions of Trx1 system enzymes with oxidants. In TR/GR-null livers, Nrf2 activity is further induced, revealing that TrxR-independent systems also repress Nrf2 and these might be induced by more extreme challenges.

Introduction
Reduction-oxidation (redox) reactions involve the transfer of electrons (reducing power) from more reduced (nucleophilic) to more oxidized (electrophilic) molecules. Thus, the terms ‘oxidant’ and ‘electrophile’ are synonymous, although frequently the former is used for small oxidized molecules such as hydrogen peroxide or superoxide radical, whereas the latter is used for larger oxidized compounds, such as drugs or xenobiotics. For the purposes of this mini-review, the terms ‘oxidant’ and ‘electrophile’ will be used interchangeably.

Cytosolic reduction reactions are critical for cell homeostasis in all living systems. For example, DNA is synthesized from deoxyribonucleotides, which universally are produced by cytosolic reduction of ribonucleotides via the enzyme ribonucleotide reductase (RNR) [1]. Also, protein translation and glutathione (GSH) synthesis each require the reduced amino acid cysteine (Cys). Cys is generally acquired as oxidized disulfide forms such as cystine, which must be reduced intracellularly to yield Cys [2]. Once incorporated into proteins or GSH, the sulfur residue of the Cys side chain usually must be in a reduced (thiol) state for the molecules to be functional. These processes would be disfavoured in oxidizing conditions, so cells maintain a reducing cytosol. In cells using aerobic metabolism, incidental production of intracellular reactive oxygen species (ROS) further increases oxidative challenges.

In addition to tolerating constitutive extracellular or metabolic ROS exposures, cells need to resist acute oxidative challenges, for example from inflammation-induced oxidative bursts, nutritional challenges, exposure to electrophilic xenobiotics or transient pathologic perturbations of mitochondrial electron transport [3,4]. In these situations, cellular recognition of the challenge results in activation of the nuclear factor erythroid-derived 2-like-2 (Nrf2) pathway, which regulates a broad array of cytoprotective, antioxidant, drug metabolism and bioenergetic systems [5–7]. The realigned metabolic activities caused by Nrf2 activation promote cytosolic reductase activities, neutralization and export of hazardous molecules and repair of damage. A critical albeit incompletely understood component of this response is the mechanism by which the Nrf2 system senses the challenges so it can respond appropriately to diverse insults [8–12].

Inducible cytoprotective activities: the Keap1/Nrf2 pathway
Nrf2 is an evolutionarily conserved member of the ‘cap-‘n-collar’ subfamily of BZIP (basic leucine zipper) proteins. Nrf2 is a basic leucine zipper (BZIP) protein that is transcriptionally activated by small electrophiles that form covalent adducts with its Nrf2-binding domain (Keap1). Activation of Nrf2 by electrophiles leads to the induction of cytoprotective gene expression, including those encoding thioredoxin reductase (TrxR1), glutathione reductase (GR), and peroxiredoxins (Prxs). These proteins function in the detoxification of reactive oxygen species and the maintenance of cellular redox balance.

Key words: cytoprotective response, glutathione, oxidative stress, peroxiredoxin, thioredoxin.

Abbreviations: BZIP, basic leucine zipper; Cul3, cullin-3; Cys, cysteine; ECH, enoyl-CoA-hydratase; EpRE, electrophile-response element; Gpx, glutathione peroxidase; GR, glutathione reductase; Gs, glutathione reductase gene; Keap1, Kelch-like ECH-associated protein-1; Nrf2, nuclear factor erythroid-derived 2-like-2; Prx, peroxiredoxin; RNR, ribonucleotide reductase; ROS, reactive oxygen species; Sec, selenocysteine; Trx1, thioredoxin-1; TrxR1, thioredoxin reductase-1; Txnrd1, thioredoxin reductase-1 gene.

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transcription factors, a family that can function as either hetero- or homo-dimers [13,14]. Nrf2 is continually and ubiquitously translated. In unstressed cells, newly translated Nrf2 interacts transiently with Kelch-like ECH-associated protein-1 (Keap1). Keap1 transfers Nrf2 to cullin-3 (Cul3), an ubiquitin E3 ligase, thereby targeting Nrf2 for proteosomal degradation [10,15]. Keap1 contains 25 (mouse) or 27 (human) Cys residues; the redox states of several of these modulate activity [8,11,16]. Thus, in unstressed conditions, the Cys residues are in a thiol state and Nrf2 is degraded. Under conditions of electrophilic stress, several Cys residues are oxidized to disulfides or conjugated to electrophiles. This causes Nrf2 to become stably associated with Keap1, preventing transfer of Nrf2 to Cul3 and thereby encumbering Keap1 [17]. As a result, newly synthesized Nrf2 bypasses degradation, accumulates in the nucleus, heterodimerizes with small Maf (avian musculoaponeurotic fibrosarcoma homolog) proteins or other BZIP family members and activates genes containing an electrophile-response element [EpRE, also called an antioxidant-response element (ARE); 10].

Studies on cells and tissues with genetic disruptions of Keap1 have identified the genes that respond to Nrf2 [18,19]. Among these, genes with EpRE elements are thought to be direct Nrf2 targets. These include genes encoding enzymes on the pentose phosphate and GSH biosynthesis pathways, cytosolic thioredoxin reductase-1 (TrxR1), glutathione reductase (GR) and drug metabolism enzymes. Genes repressed by Nrf2 include several associated with lipogenic processes [19,20], although it is less clear whether these are direct targets. In concert, Nrf2 induction causes cells to exhibit increased capacity to generate intracellular NADPH and GSH, increased ability to reduce Trx1, increased capacity to conjugate GSH or saccharides to electrophiles and increased ability to export the conjugated toxins out of the cells [10,21].

The NADPH-dependent disulfide reductase systems: a sulfur-based redox economy

To maintain intracellular redox homoeostasis, mammalian cells have two major disulfide reductase systems: the GSH system and the thioredoxin (Trx) system [22,23]. Together, these drive a ‘dithiol/disulfide-based economy’, which supports many cytosolic reduction reactions. NADPH is the universal ‘currency’ that is able to traffic reducing power into this economy. The two NADPH-dependent disulfide reductases, TrxR1 and GR, are the ‘cashiers’ that can accept this currency. Upstream of this, glucose generally is the ‘natural resource’ that is obtained from extracellular sources and drives this sulfur-based economy and the pentose phosphate pathway is the most familiar ‘industry’ for converting natural resources into this currency (Figure 1A).

The Trx1 and GSH systems are both based on the transfer of electrons along voltage-gradients from more reduced molecules or residues to more oxidized ones. In general, these transfers occur via reversible thiol/disulfide exchanges (Figure 1B), although in some cases, selenothiol/selenosulfide exchange reactions involving the twenty-first amino acid selenocysteine (Sec) participate [24–26]. TrxR1 or GR are homologous NADPH-dependent flavoproteins [26]. Two electrons of reducing power are extracted from NADPH and transferred via a flavin cofactor to an N-terminal active site where they reduce the disulfide of a Cys pair into a dithiol [26]. In the case of GR, the electrons are then used to reduce glutathione disulfide (GSSG) into two molecules of GSH. In the case of TrxR1, the N-terminal active site dithiol is used to reduce the selenosulfide between a Cys–Sec pair in the G-terminal active site of the reciprocal subunit of the homodimeric TrxR1 enzyme. The selenothiol pair then reduces the disulfide in the active site Cys pair of oxidized Trx1 to a dithiol pair, yielding reduced Trx1 [25,26]. GSH or reduced Trx1 can then distribute this reducing power throughout the cytosol and nucleus, reducing disulfide bonds in other proteins. This sustains the reduced state of most surface exposed Cys residues and supplies reducing power to reductive enzymes including peroxiredoxins (Prx), glutathione peroxidases (Gpx), RNR and others. Usually it is only the final enzyme in the pathway that might transfer the electrons to an acceptor that is neither sulfur- nor selenium-based, for example in the reduction of ribonucleoside diphosphates by RNR or the reduction of peroxides by Prxs or Gpxs.

The tripeptide GSH (L-γ-glutamyl-L-cysteinylglycine) carries a transferrable electron on the sulfur of the Cys side chain [2]. Typically, two GSH molecules each donate their electron to an oxidized acceptor, reducing the acceptor and forming GSSG. GR-null mice cannot directly reduce GSSG into 2GSH, yet they are healthy [27]. Also, cells unable to synthesize GSH are viable in culture [28,29] and adult mice tolerate systemic depletion of GSH [30]. It is generally thought that redundancies between the GSH and Trx systems preserve redox homoeostasis following disruption of the GSH system.

Trx1 is a 105-amino acid protein that carries reducing power as two electrons in a dithiol motif on an active site Cys-pair [31]. Following transfer of these electrons to an acceptor, the oxidized disulfide form of the enzyme is recycled to the dithiol form by TrxR1, thereby oxidizing one molecule of NADPH [26]. Trx1- or TrxR1-deficient embryos grow to contain several thousand cells, but the embryos are disorganized and fail to gastrulate [32,33]. TrxR inhibitors are not harmful to mice or humans [26] and conditional disruption of the gene encoding TrxR1 has revealed that TrxR1-null cells and tissues are healthy [32,34,35]. TrxR1-deficient mouse livers require continuous GSH synthesis to replicate DNA suggesting that, in the absence of TrxR1, the GSH system provides electrons to RNR for synthesis of DNA precursors [36]. Thus, redundancies between the Trx1 and GSH systems sustain redox homoeostasis when either one of the systems is compromised.
**Figure 1** | The cellular disulfide reductase economy  
(A) Flow of electrons in cells is analogous to the flow of ‘value’ in an economy. (B) Activity and partitioning of thiol/disulfide couples. Inside cells, the cytosol is reducing (red colour, ‘red’) whereas the extracellular milieu is oxidizing (yellow colour, ‘ox’). Most redox-active sulfurs within cells are in a thiol (R-SH) state and carry a transferable electron (e\(^{-}\)), although intracellular dithiol/disulfide exchange reactions, as discussed in the text, will generate transient disulfide species. In the oxidizing environment outside of cells, the sulfurs are generally present as disulfides (R-S-S-R, right).

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**An NADPH-independent disulfide reductase system in liver**

Although concurrent disruption of both TrxR1 and GSH in hepatocytes prevents DNA replication (see above), it remained untested whether the disulfide reducing power for RNR needed to be transferred from NADPH to the cytosolic disulfide reductase systems. Based on the evolutionary conservation of these systems across phyla, it seemed likely that their role would be essential [37,38]. Nevertheless, it was also known that mutations compromising NADPH production in humans or mice are often benign, yet the compensatory pathways remained obscure [39,40]. To test whether having at least one of the two major cytosolic NADPH-dependent disulfide reductases was essential for hepatocyte homeostasis in vivo, our group ablated the *Txndl* gene, encoding TrxR1 [32], in the hepatocytes of mice homozygous for a germline disruption of the *Gsr* gene, encoding GR [27]. Remarkably, we found that mice lacking both TrxR1 and GR in all hepatocytes (‘TR/GR-null’ livers) were able to maintain hepatic redox homoeostasis and long-term organismal survival [41].

How redox homoeostasis was maintained in TR/GR-null livers posed a compelling conundrum: what was the alternative source of electrons? An important clue in our search came when it was revealed that the TR/GR-null livers, although unable to use NADPH to reduce GSSG into 2GSH, had roughly normal levels of total glutathione and most of this was in the GSH form [41]. Therefore, either GSSG was being reductively recycled by an NADPH-independent process or *de novo* GSH biosynthesis coupled with non-reductive GSSG elimination was involved. Buthionine sulfoximine, an inhibitor of GSH biosynthesis, induced rapid global hepatocyte necrosis and precipitous deterioration of animals with TR/GR-null livers [41].
indicated that the TR/GR-null livers required uninterrupted GSH biosynthesis, pointing to this as the pipeline of reducing power for redox homeostasis. Subsequent analyses showed that, although the TR/GR-null livers could not use extracellular cystine as a source of Cys, they required *trans*-sulfuration of homocysteine for survival. Metabolic labelling showed that hepatic Cys was being supplied by converting dietary methionine to homocysteine in the methionine cycle and then using this in the *trans*-sulfuration pathway to generate Cys [41].

**TrxR1-specific repression of the Nrf2 pathway**

Transcriptome analyses on mice having liver-specific disruption of a conditional-null allele of *Txnrd1* revealed two important observations: First, of the mRNAs expressed in wild-type liver, only ~0.3% show substantial differences in TrxR1-null livers. This indicates that, despite not having TrxR1, most activities in the liver are unperturbed. Second, many of these differentially expressed mRNAs are encoded by Nrf2 target genes [34]. By contrast, livers of mice lacking GR do not exhibit a significant Nrf2-response (Justin R. Prigge, Emily A. Talago, Michael McLaughlin and Edward E. Schmidt, manuscript in preparation), indicating that the Trx1 and the GSH pathways are not equivalent in terms of Nrf2 induction. Western blotting and immunostaining verified that TrxR1-deficient livers over-accumulate nuclear Nrf2 protein and ChIP assays verified that EpREs in the regulatory regions of these genes have increased occupancy by Nrf2 protein [34]. Importantly, these livers do not have hallmarks of oxidative stress, such as accumulation of lipid hydroperoxides, protein carbonyls or a shift in the GSH:GSSG redox couple [34]. Thus, disruption of TrxR1 was revealed to induce Nrf2 in the absence of oxidative or electrophilic stress, suggesting that the activity of the Trx1 pathway might be a critical part of the mechanism that keeps the Nrf2 pathway inactive in unstressed cells. Studies in mouse lung show that pharmacologic disruption of TrxR1 and Trx1 system, but not the GSH system, is an integral part of the mechanism that triggers Nrf2 induction (Figure 2A).

**Why would the Trx1 pathway be involved in triggering Nrf2?**

The thiol in Keap1 that function in induction of Nrf2 are orders of magnitude less reactive than the active-site thiol selenols in reductase enzymes [48,49]. Recent articles have suggested the need for a more reactive ‘sensor’ to catalyse the oxidation of Cys residues in Keap1 for activation of Nrf2 and compelling arguments have been made for Gpxs serving this function [8,9]. Gpxs contain an active site Sec residue that, as one of the most nucleophilic entities in cells [50,51], will be extremely sensitive to oxidants or nucleophiles [8,9]. However, we would expect that, if Gpxs served as the sensor for Keap1, then Nrf2 induction should be responsive to disruptions in the GSH pathway that feeds electrons to the Gpxs, not to the Trx1 pathway. Importantly, TrxR1, like Gpxs, contains a highly reactive Sec residue [25,26,50] and the active site thiols within Trx1-dependent Prxs are also orders of magnitude more reactive than are structural thiols, including the Nrf2-triggering Cys residues of Keap1 [52–55]. Therefore, we reason that the reactive residues in TrxR1 or Prxs are likely electrophile sensors that then induce catalytic oxidation of the less reactive Cys residues on Keap1.

Recently completed transcriptome analyses on the TR/GR-null livers indicates these have an even more dramatic Nrf2 response, with most Nrf2-response mRNAs induced rough a different order of magnitude higher than in TrxR1-deficient livers (Justin R. Prigge, Emily A. Talago, Michael McLaughlin and Edward E. Schmidt, manuscript in preparation). Importantly, TR/GR-null livers, although viable and able to support long-term survival of the mice, are not normal. Mice with TR/GR-null livers exhibit a substantially higher rate of spontaneous acute liver failure and all TR/GR-null livers show hepatomegaly, a high incidence of necrotic hepatocytes, low-grade chronic inflammation, elevated serum markers of hepatic damage and biliary hyperplasia, suggesting that, unlike either GR-null or TrxR1-null livers, the TR/GR-null livers represent a severely pathological condition ([41] and Justin R. Prigge, Emily A. Talago, Sofi Eriksson and Edward E. Schmidt, unpublished data). It is tempting to speculate that the augmented activity of the Nrf2 pathway in TR/GR-null livers reflects the combined loss of the Trx1 pathway in the Nrf2 triggering mechanism with an increased level of cytosolic oxidative stress. Additional studies will be required to test this. Regardless, it suggests that the Nrf2 pathway responds to at least two triggering mechanisms: one that requires TrxR1 activity and one that can further amplify this response in severe situations. How the two mechanisms suggested by these genetic studies relate to the previously reported Keap1-dependent and -independent pathways of Nrf2 activation [10,56] remains to be determined.
Figure 2 | Interplay between disulfide reductase systems and Nrf2

(A) Model for Trx1-dependence of Nrf2 activity. At left, under unstressed conditions both the GSH and the Trx1 systems are constitutively active in cells and reduce oxidants; however the Trx1 system, in addition to providing reducing power to combat oxidative stress, maintains the active state of Keap1 and thereby the inactive state of Nrf2. At right, following an oxidative siege on the cells, highly nucleophilic Sec and Cys residues in the active sites of Trx1 system enzymes sensitively respond to the electrophilic challenge and catalyse oxidative inactivation of Keap1. This activates Nrf2, inducing a cytoprotective response. Whereas Nrf2 will further activate the Trx1 system, the flow of reducing power might preferentially go to the reactive electrophiles constituting the challenge and only reduce the relatively less reactive oxidized Cys residues on Keap1 after the challenge subsides. (B) Nrf2 induces disulfide reductase systems in liver. Oxidative stress is proposed to induce Nrf2 via a mechanism that involves the Trx1 system and Keap1 (red). In addition to inducing drug metabolism pathways and realigning energy metabolism pathways, Nrf2 induces production of NADPH (e.g., by inducing the pentose phosphate pathway and malic enzyme), the Trx1 system (e.g., by inducing the genes encoding Trx1 and TrxR1) and the GSH system (e.g., by inducing the genes encoding GR and the GSH biosynthesis pathway). It remains unclear whether Nrf2 participates in regulating the methionine-dependent disulfide reductase system (e.g., by regulating genes involved in amino acid transport and metabolism, dashed line). See text for details.

Conclusions

In hepatocytes, the mechanisms of sustaining cytosolic redox homoeostasis are enormously robust as a result of having three pathways, two NADPH-dependent and one methionine-dependent, to reduce disulfides, any one of which can alone maintain adequate hepatic function for long-term survival. The disulfide reductase systems are integrated with the cytoprotective Nrf2 pathway, thereby allowing cells to match reductive activities with varying stress conditions (Figure 2B). Thus, genes encoding components of each of the disulfide reductase systems are induced by Nrf2 activation and the Trx1 pathway plays a key role in regulating Nrf2 activation. The details of these interactions are continually coming to light and each new revelation has the potential
to uncover novel approaches for regulating oxidative stress-responses, detoxification and energy metabolism. The broad scope of physiologic activities affected by Nrf2 promises to make this pathway an increasingly important target for therapeutic strategies related to a vast range of diseases and conditions.

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