Increased reactive oxygen species production during reductive stress: The roles of mitochondrial glutathione and thioredoxin reductases

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Abstract

Both extremes of redox balance are known to cause cardiac injury, with mounting evidence revealing that the injury induced by both oxidative and reductive stress is oxidative in nature. During reductive stress, when electron acceptors are expected to be mostly reduced, some redox proteins can donate electrons to \( \text{O}_2 \) instead, which increases reactive oxygen species (ROS) production. However, the high level of reducing equivalents also concomitantly enhances ROS scavenging systems involving redox couples such as NADPH/NADP\(^+\) and GSH/GSSG. Here our objective was to explore how reductive stress paradoxically increases net mitochondrial ROS production despite the concomitant enhancement of ROS scavenging systems. Using recombinant enzymes and isolated permeabilized cardiac mitochondria, we show that two normally antioxidant matrix NADPH reductases, glutathione reductase (GR), the peroxiredoxin3 (Prx)/thioredoxin2 (TrxR2) systems (Fig. 1). Although catalase is also present in the matrix [45], it has a much lower affinity for \( \text{H}_2\text{O}_2 \) [6] and therefore is not likely to play an important role at micromolar concentrations of \( \text{H}_2\text{O}_2 \). Thus, matrix antioxidant power is ultimately dependent on

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1. Introduction

Both the production and removal of reactive oxygen species (ROS) in the mitochondrial matrix play critical roles regulating mitochondrial function. ROS-induced redox modifications of mitochondrial proteins are required for redox signaling to adjust metabolism to changing conditions and to regulate cell death and survival pathways. Conventionally, an excess of ROS and reactive nitrogen species (RNS) relative to reducing equivalents is defined as oxidative stress. Conversely, a relative shortage of ROS compared with reducing equivalents in the form of redox couples (GSH/GSSG, NADPH/NADP\(^+\), NADH/NAD\(^+\), etc.) is defined as reductive stress [1]. However, the latter definition is somewhat confusing because in isolated mitochondria, reductive stress, in the form of high matrix NADH/NAD\(^+\) ratios, is known to promote excessive ROS production to a level exceeding ROS scavenging capability, resulting in net \( \text{H}_2\text{O}_2 \) spillover from mitochondria [2]. Specifically, experiments have demonstrated that ROS production by alamethicin-permeabilized mitochondria increases steeply at high NADH/NAD ratios [3], which is traditionally attributed to complex I of the respiratory chain, but can also involve certain tricarboxylic acid cycle (TCA) enzymes, specifically those containing lipoamide dehydrogenase, the ROS-producing E3 component of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase [3].

When the NADH/NAD\(^+\) pool becomes highly reduced, so does the NADPH/NADP\(^+\) pool, which increases matrix antioxidant power to compensate for increase in ROS production and limit ROS spillover. NADPH is a key reducing equivalent supplying the major \( \text{H}_2\text{O}_2 \) scavenging system, the glutathione/glutathione peroxidase3 (Gpx3)/glutathione reductase (GR) and the peroxiredoxin3 (Prx3/thioredoxin2 (TrxR2) systems (Fig. 1). Although catalase is also present in the matrix [45], it has a much lower affinity for \( \text{H}_2\text{O}_2 \) [6] and therefore is not likely to play an important role at micromolar concentrations of \( \text{H}_2\text{O}_2 \). Thus, matrix antioxidant power is ultimately dependent on

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; FCCP, trifluorocarbonylcyanide phenylhydrazone; GDH, glutamate dehydrogenase; GR, glutathione reductase; Gpx1, glutathione peroxidase1; GSSG, oxidized glutathione; H\(_2\)O\(_2\), hydrogen peroxide; iCDH, isocitrate dehydrogenase; pCMR, p-chloromercuribenzoic acid; pCMPS, p-chloromercuriphenyl-sulphonate; Prx, peroxiredoxin; O\(_2\)••, superoxide; TCA, tricarboxylic acid; TrxR1, thioredoxin reductase1; TrxR2, thioredoxin reductase2; Trx, thioredoxin 1 and 2

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an adequate supply of NADPH generated by transhydrogenase, isocitrate dehydrogenase, malic enzyme and glutamate dehydrogenase to keep the matrix NADPH/NADP⁺ pool reduced.

However, experimental evidence indicates that the enhancement of antioxidant power by the reduced NADPH/NADP⁺ pool during reductive stress is insufficient to counterbalance the increased ROS production resulting from the reduced NADH/NAD⁺ pool, leading to net ROS spillover, matrix oxidation, cytotoxicity and protein aggregation causing cardiomyopathy [7–10]. These observations and others support the “redox-optimized ROS balance” hypothesis recently put forth by O'Rourke and colleagues [11,12]. According to this hypothesis, redox balance is lost and ROS production increases at both extremes of oxidation and reduction of redox couples involved in respiratory chain activity (NADH/NAD⁺) or ROS scavenging (NADPH/NADP⁺, GSH/GSSG). In other words, the extent of ROS production is defined by the overall change of matrix redox environment in either direction. In a highly reduced environment, ROS production is accelerated to the point that it overwhelms ROS scavenging capacity, even though the latter should be maximally potentiated [11,12].

To better understand the factors that lead to net ROS production and oxidative cytotoxicity during reductive stress, we explored the possibility that two of the NADPH-dependent reductases that normally enhance antioxidant function, namely GR and TrxR2, begin to directly generate significant ROS when the NADPH/NADP⁺ pool becomes highly reduced. This hypothesis is based on the premise that these enzymes belong to the same family of disulfide reductase flavoenzymes as lipoamide dehydrogenase, the E3 component of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase. Lipoamide dehydrogenase has been shown to produce ROS with a rate equal to or even higher than complex I when the NADH/NAD⁺ pool is highly reduced [3,13]. It is structurally similar and catalyzes the transfer of electrons between pyridine nucleotides and disulfides with very similar reaction chemistry as GR and TrxR1. Indeed, the cytoplasmic isomerase of thioredoxin reductase (TrxR1) has been previously shown to generate ROS robustly in the presence of NADPH when its preferred endogenous substrate, oxidized thioredoxin 1 (Trx), is in limited supply [14]. Under these conditions, it is thought that the reduced flavoprotein redox site of TrxR1 directly donates electrons obtained from NADPH to molecular O₂, to generate superoxide and H₂O₂ by one or two electron reduction of O₂, respectively.

In this study, we tested the hypothesis that, analogous to lipoamide dehydrogenase and TrxR1, GR and TrxR2 generate ROS when their natural electron acceptors (GSSG and oxidized Trx, respectively) are in limited supply, as illustrated schematically in Fig. 1. Using recombinant GR and TrxR1, we show that both enzymes generate robust amounts of H₂O₂ when provided with NADPH in the absence of their preferred electron acceptors, or in the presence of inhibitors which interfere with electron transfer to their preferred electron acceptors (Fig. 1). Moreover, we present evidence that these same reactions occur with GR and TrxR2 in cardiac mitochondria exposed to reductive stress, and that the amounts of ROS generated under these conditions are quantitatively comparable to those generated by respiratory complexes and TCA cycle dehydrogenases during reductive stress. Finally, we show that ROS spillover during reductive stress is very sensitive to Prx inhibition by Zn²⁺. These findings help to explain how respiratory complexes and NAD⁺–related matrix dehydrogenases, together with GR and TrxR2 directly generating ROS, combine to overwhelm NADPH-dependent antioxidant mechanisms, resulting in ROS efflux from the matrix during reductive stress.

2. Material and methods

This study was approved by the UCLA Chancellor’s Animal Research Committee (ARC 2003-063–23B) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with UCLA Policy 990 on the Use of Laboratory Animal Subjects in Research (revised 2010). All measurements were carried out using customized Fiber Optic Spectrofluorometer (Ocean Optics) in a continuously stirred cuvette at room temperature (22 to 24 °C). The cuvette was partially open to air during isolated mitochondria experiments, but was tightly closed when O₂ consumption by recombinant GR was measured. Mitochondria
were isolated from rabbit and mouse hearts as described previously [15]. Recombinant proteins (5–400 nM) were added to incubation buffer containing 110 mM KCl, 10 mM Hepes, pH 7.4 with Tris. Mitochondria (0.5 to 1.0 mg/ml) were permeabilized with addition of alamethicin (20 μg/ml) to sucrose buffer (250 mM sucrose, 10 mM HEPEs, pH 7.4 with Tris). Alamethicin creates pores which allow equilibration of low-molecular weight components across the inner membrane, while high-molecular weight proteins are retained in the matrix and intermembrane space [16]. Rat recombinant TrxR1 and Trx from Escherichia coli (oxidized form) were obtained from Cayman Chemical. Nox2 inhibitor gr91ds-tat was from Eurogentec. Other reagents, including recombinant human GR, recombinant human Prx1, oxidized glutathione (GSSG), 1-chloro-2,4-dinitrobenzene (CDNB), p-chloromercuribenzoic acid (pCMB), auransofin, mersalyl, p-chloromercuriphenyl-sulphonate (pCMPS), trifluorocarbonylcyanide phenylhydrazone (FCCP), rotenone and antimycin, were obtained from Sigma. For experiments with isocitrate dehydrogenase, we used commercially available enzymes purified by Sigma.

Mitochondrial O$_2$ consumption was measured continuously by monitoring buffer O$_2$ content using a fiber optic oxygen sensor FOXY-AL300 (Ocean Optics) [17].

H$_2$O$_2$ release from mitochondria or production by recombinant GR or TrxR was measured using 10 μM Amplex UltraRed and 0.2 U Horse Radish Peroxidase (HRP) in the buffer (excitation/emission, 540/590 nm).

NAPDH oxidation by GR or TrxR1 was measured by the rate of decrease in fluorescence (366/460 nm, excitation/emission) after adding NAPDH (50 μM) to the cuvette.

2.1. Statistical analysis

For each data set, the median and 95% confidence intervals (CI) are reported. The conventional percentile bootstrap-resampling approach with 10,000 replications was used for estimating 95% confidence intervals (CI) as well as examining the significant difference between groups (effect size statistics) [18–20]. A P value < 0.05 was considered statistically significant.

3. Results

3.1. NADPH-dependent ROS production by purified recombinant GR

To determine whether GR is capable of generating ROS when provided with NAPDH in the absence of its natural electron acceptor, as hypothesized in Fig. 1, we added purified recombinant GR at different concentrations (50 to 400 nM) to KCl buffer in the absence of GSSG (Fig. 2A). Addition of 100 μM NAPDH to GR rapidly initiated H$_2$O$_2$ production that was dependent on the GR concentration. Subsequent addition of catalase (2 μM) rapidly inhibited further H$_2$O$_2$ increase. NADPH addition in the absence of GR increased fluorescence very slowly, which rapidly increased after GR addition (Fig. 2A, lower red trace). Inset shows the dependency of H$_2$O$_2$ production (in nM/min) on GR concentration (n = 2). In contrast, when GSSG was provided as the natural electron acceptor for GR, NADPH addition did not stimulate significant H$_2$O$_2$ production (Fig. 2B, blue trace b versus red trace a). The GSSG concentration required for 50% inhibition of NADPH-induced production of ROS by GR (IC50) was 106 μM when fit to a Hill equation (Fig. 2C).

Organic mercurial compounds such as pCMB have a high affinity for thiols and at least one functionally important thiol in GR is known to react with soluble thiol reactive agents in vitro [21]. Binding of thiol reactive agents to reduced SH-groups interferes with electron flow from reduced pyridine nucleotides via FAD and thiol groups to GSSG (Fig. 1). Consistent with this mechanism, pCMB (50 μM) accelerated NADPH-induced H$_2$O$_2$ production by GR by 3.2-fold (95% CI [3.1, 3.3], n = 3), even when GSSG was present (2.2-fold increase, 95% CI [2.1, 2.4], n = 4) (Fig. 2B, green and purple traces c and d, respectively). Like pCMB, other organic mercural thiol reactive agents such as mersaryl and pCMPS also increased NADPH-dependent H$_2$O$_2$ production (Fig. 2D) (2.7-fold increase, 95% CI [2.5, 2.8], and 8.3-fold increase, 95% CI [7.9, 8.6] for mersaryl (n = 4) and pCMPS (n = 4), respectively).

Fig. 2E shows NADPH consumption by GR under the same conditions as in Fig. 2B. With no GSSG present, the rate of NADPH consumption increased in parallel as the rate of H$_2$O$_2$ production was accelerated by adding mersaryl (blue trace b) or pCMPS (green trace c). Note that in the absence of mersaryl or pCMPS, addition of GR’s natural electron acceptor GSSG abruptly increased NADPH consumption by many orders of magnitude (red trace a), indicating that the rate of electron transfer to GSSG is extremely fast compared to its maximal rate of electron leak to O$_2$ when no GSSG is present. In the presence of mersaryl (blue trace b) or pCMPS (green trace c), GSSG had no effect on the rate of NADPH consumption, as expected if electron transport from NADPH to GSSG is blocked. However, addition of the reducing agent DTT (1 mM) rapidly reversed the effects of mersaryl and pCMPS, causing NADPH consumption to accelerate rapidly, again by orders of magnitude. Further additions of NADPH were also rapidly consumed.

In Fig. 3, we quantitatively analyzed the relationship between NADPH consumption, O$_2$ consumption and H$_2$O$_2$ production rates by GR, which averaged 76.9, 42.7 and 6.5 nmol/min/mg enzyme respectively in the presence of 5 μM mersaryl (Fig. 3A), and 269.1, 143.0 and 18.8 nmol/min/mg enzyme respectively in the presence of 5 μM pCMPS (Fig. 3B). In both cases, the rates of NADPH and O$_2$ consumption are much greater than can be accounted for by H$_2$O$_2$ production via two-electron reduction of O$_2$. Specifically, assuming that one NADPH and one O$_2$ molecule are consumed for each H$_2$O$_2$ molecule produced, the amounts of NADPH and O$_2$ consumption unaccounted for by H$_2$O$_2$ production are (76.9–6.5) = 70.4 and (42.7–6.5) = 36.2 nmol/min/mg enzyme with mersaryl present, and 250.3 and 124.2 nmol/min/mg enzyme with pCMPS present. The ratio of the NADPH oxidized to O$_2$ consumed that is not accounted for by H$_2$O$_2$ production is thus similar and near 2.0 in both cases (1.94 for mersaryl and 2.02 for pCMPS). To demonstrate that the NADPH consumption under these conditions was directly linked to O$_2$ consumption, we performed the same experiment in the absence of O$_2$ (Fig. 3B, inset). Under anaerobic conditions, pCMPS did not induce NADPH oxidation by GR, and NADPH fluorescence only decreased after O$_2$ was readmitted. Besides two-electron reduction of O$_2$ to generate H$_2$O$_2$, other possible reactions that could consume NADPH include one-electron reduction of O$_2$ to form O$_2^-$, four-electron reduction of O$_2$ to form H$_2$O [22] or uptake of electrons by the added mercurial compounds. The first possibility was excluded by including superoxide dismutase (15 U/ml) in the cuvette, which did not enhance NADPH-induced H$_2$O$_2$ production by GR (data not shown). In principle, the second possibility could account for all of the remaining NADPH consumption, assuming that two NAPDH molecules are reduced for each O$_2$ molecule to generate two H$_2$O molecules, assuming that GR is capable of this reaction. However, we cannot exclude that the third possibility is also important. Despite this limitation, the findings collectively indicate that ROS production by reduced GR depends on the rate of electron transport from NADPH to GSSG: leak of electrons to O$_2$ to form H$_2$O$_2$ is insignificant when GSSG reduction is fast, but becomes appreciable when oxidized substrate is absent or electron transport is inhibited at the level of functionally important thiols by pCMB, mersaryl or pCMPS.

3.2. NADPH-dependent ROS production by purified recombinant TrxR1

Since recombinant TrxR2 with intact enzymatic activity was not commercially available, we used recombinant TrxR1 (the cytoplasmic isoform) as a proxy, under the assumption that the matrix isoform TrxR2 is likely to have similar properties. Fig. 4A demonstrates that recombinant TrxR1 generates H$_2$O$_2$ in the presence of NADPH when its natural electron acceptor, oxidized Trx, is absent. When 100 μM NADPH was added to recombinant TrxR1 in KCl buffer, H$_2$O$_2$ generation increased rapidly in proportion to the added TrxR1 concentration. The
control case with no TrxR1 (red trace labeled “0”) showed only a very small nonspecific increase in resorufin fluorescence upon NADPH addition. The inset to Fig. 4A summarizes H₂O₂ production in nmoles/min for different TrxR1 concentrations. When expressed per mg protein, H₂O₂ production rate by TrxR1 averaged 8.3 nmoles/mg/min (95% CI [1.0, 1.1], n = 7) in the absence of inhibitors.

Fig. 4B shows that the rate of NADPH-induced H₂O₂ production by TrxR1 (50 nM) saturated between 10 and 25 μM NADPH. The TrxR1 inhibitor CDNB (50 μM) increased H₂O₂ production at each NADPH concentration. For 50 μM NADPH, the increase was 3-fold, from 8.3 nmoles/mg/min to 25.1 nmoles/mg/min (95% CI [21.9, 27.9], n = 6), as shown in Fig. 4C, which compares the average values of NADPH-dependent H₂O₂ production by TrxR1 and GR in the absence and presence of inhibitors, respectively.

We also examined the effects of oxidized Trx on NADH oxidation and H₂O₂ production by recombinant TrxR1 (Fig. 4D and E). NADPH is consumed by TrxR to reduce oxidized Trx, which is thought to be available at low micromolar levels in the matrix [23]. In the absence of both TrxR1 and Trx, NADPH fluorescence was stable in KCl buffer (Fig. 4D, red trace). Subsequent addition of TrxR1 caused NADPH oxidation at a low rate, which was further accelerated by 3.4-fold (95% CI [3.2, 3.5], n = 3) upon addition of CDNB (50 μM). Under the same conditions, the blue trace shows that addition of oxidized Trx (10 μM) transiently stimulated rapid NADPH oxidation by TrxR1 until all of the Trx was reduced, which was repeated again with 2.5 μM oxidized Trx. Fig. 4E shows the corresponding rates of H₂O₂ production. The oxidized Trx decreased NADPH-induced H₂O₂ production by TrxR1 in a concentration-dependent manner, with 10 μM oxidized Trx causing a 2.4-fold decrease (95% CI [2.2, 2.6], n = 3) that was relieved by CDNB (red trace).

Matrix NADPH concentration is expected to be in large excess relative to Trx. In addition, reduced Trx reacts with H₂O₂ rather slowly (rate constant 1.05 M⁻¹ s⁻¹) [24]. Therefore, H₂O₂ detoxification in the matrix depends on reduced Prx, whose cysteine residues react directly and rapidly with H₂O₂ (rate constant 10² M⁻¹ s⁻¹) [24]. Once oxidized, Prx becomes inactive and requires donation of electrons from reduced Trx to scavenge H₂O₂ again. This is illustrated with purified TrxR1 in Fig. 5A. When TrxR1 was added to KCl buffer containing NADPH (green trace), H₂O₂ production was initiated. Addition of 1 μM oxidized Trx had no immediate effect on H₂O₂ production, but addition of Prx (100 nM) rapidly inhibited further increases in resorufin fluorescence, which remained stable even after CDNB (50 μM) was added. If CDNB was added first to
enhance NADPH-induced H2O2 production by TrxR1, Prx also prevented further increases in resorufluorescence (Fig. 5A, red and blue traces), indicating that continued H2O2 production by TrxR1 was masked by reduced Prx competing with HRP for H2O2. The masking effect of Prx on H2O2 production could be revealed by adding redox inert Zn2+ (blue trace), which is known to bind to accessible thiol groups [25]. Neither ZnCl2 (20 μM), CDNB (50 μM) nor pCMPS (5 μM) had no significant effect on the Amplex UltraRed/HRP (resorufluorescence) response to H2O2 in the buffer (Fig. 5B). Because Zn2+ had no significant effect on the ability of TrxR1 to generate H2O2 in the presence of NADPH and CDNB, Zn2+’s effect at unmasking H2O2 production could be attributed to Prx inhibition. Removing Zn2+ from functionally important thiol groups by adding EDTA prevented further H2O2 spillover (Fig. 5A).

To mimic conditions in the mitochondrial matrix in which NADPH is generated endogenously from NADP+ by matrix enzymes such as isocitrate dehydrogenase, we also incubated recombinant TrxR with purified isocitrate dehydrogenase, together with isocitrate and NADP+ in KCl buffer containing EDTA to chelate free Mg2+ (Fig. 6A). When excess Mg2+ was added to activate NADPH generation by isocitrate dehydrogenase, H2O2 production increased gradually as NAPDH increased, similar to the effects of thiol reactive agents on NADPH-induced H2O2 production by GR (Fig. 2). In contrast, in the absence of TrxR1, isocitrate dehydrogenase activation by Mg2+ generated NADPH (Fig. 6B, top trace) but no significant H2O2 (bottom trace). Note that in these experiments we used a relatively high CDNB concentration (50–100 μM), because 100 μM is required for complete inhibition of isolated TrxR1 [26], although lower concentrations are able to induce H2O2 production to a lesser extent.

3.3. NADPH-dependent ROS production in isolated mitochondria

To determine whether NADPH-dependent ROS production by GR and TrxR2 occurs in isolated cardiac mitochondria subjected to reductive stress, we studied alamethicin-permeabilized mitochondria in which free (unbound) low molecular weight compounds like NADH/NAD+, NADPH/NADP+, GSH/GSSG, substrates, etc., equilibrate through alamethicin-induced membrane pores, while proteins and protein-bound metabolites are retained in the matrix [16]. In Fig. 7, mitochondria in sucrose buffer were permeabilized with alamethicine and then left unperturbed to allow endogenous GSH/GSSG and other small molecules to diffuse out of the matrix. After 5 min, addition of NADPH resulted in a marked increase in H2O2 production (red trace a). In contrast, addition of NADPH immediately after permeabilization had no effect on H2O2 production initially (blue trace b). The lack of H2O2 production immediately after permeabilization is likely due to an oxidized matrix environment characterized by high GSSG levels and low GSH [27]. Under these conditions, NADPH is consumed by GR for GSSG reduction rather than H2O2 production, until either the GSSG has all been converted to GSH and/or has diffused out of the matrix through alamethicine pores. After 5 min, however, addition of a second bolus of NADPH increased ROS production comparably to that in trace a.

It could be argued that when the GSH/GSSG pool was depleted by permeabilizing mitochondria in Fig. 7, the robust H2O2 production

![Fig. 3. NADPH consumption, O2 consumption and H2O2 production rates by GR. A. Recombinant GR (200 nM) was added to KCl buffer in the absence of GSSG. Upon addition of NADPH, NADPH fluorescence (F_NADPH, upper trace), O2 (middle trace) and H2O2 fluorescence (F_H2O2, lower trace) were recorded, before and after adding mersalyl (5 μM). B. Same, but with pCMPS (5 μM) in place of mersalyl. Shaded inset shows that during anoxia (N2, lighter gray shaded area), GR (200 nM) and pCMPS (5 μM) did not induce any decrease in NADPH fluorescence until O2 was readmitted (darker gray shaded area).]
Fig. 4. Recombinant TrxR1 generates ROS when provided with exogenous NADPH. A. Rat recombinant TrxR1 was added to KCl buffer in the concentrations indicated (5–75 nM) at the end of traces. NADPH addition (100 μM) resulted in H₂O₂ production that was dependent on [TrxR1]. In the absence of TrxR1, the NADPH-induced non-specific increase in resorufin fluorescence was minimal (red trace 0). Inset to Fig. 4A shows H₂O₂ production in pmol/min for different TrxR1 concentrations (n = 2). B. H₂O₂ production by 50 nM TrxR1 was activated by NADPH added at the indicated concentrations (10, 25, 50 and 100 μM). Addition of 50 μM CDNB significantly accelerated ROS production at all NADPH concentrations. C. Average H₂O₂ production by TrxR1 or GR expressed per mg of protein in the absence or presence of inhibitors (50 μM CDNB, 5 μM pCMPS). D. NADPH (50 μM) fluorescence in KCl buffer was stable until TrxR1 (50 nM) was added, initiating NADPH oxidation that was further accelerated by CDNB (50 μM) (red trace). Addition of oxidized Trx (10 followed by 2.5 μM) rapidly increased NADPH oxidation, which returned to the former rate after Trx was reduced. CDNB addition accelerated NADPH oxidation (red and blue trace). E. H₂O₂ production by 50 nM TrxR1 was initiated by 25 μM NADPH in the absence of Trx (green trace) or presence of 2.5 (blue trace) or 10 μM (red trace) oxidized Trx. Addition of 50 μM CDNB significantly accelerated depressed H₂O₂ production in the presence of Trx (red trace). (Additions to both traces are indicated by black arrows; additions to one trace only are indicated by the same color arrows).

Fig. 5. Prx inhibition by Zn²⁺ unmasks NADPH-induced H₂O₂ production by TrxR1. A. NADPH (50 μM) and TrxR1 (50 nM) addition to KCl buffer initiated H₂O₂ production that was not significantly affected by 1 μM Trx, but was rapidly inhibited by adding Prx (100 nM), even after CDNB (50 μM) (green trace). Prx addition also stopped NADPH-induced H₂O₂ production by TrxR1 enhanced by CDNB (30 μM) (red and blue traces). Addition of ZnCl₂ (20 μM) to prevent Prx from reducing H₂O₂ partially reactivated H₂O₂ production and was reversed by adding EDTA (250 μM) to chelate Zn²⁺ (blue trace). Similar results were obtained in 4 preparations. (Additions to both traces are indicated by black arrows; additions to one trace only are indicated by the same color arrows). B. CDNB (50 μM, green trace), ZnCl₂ (20 μM, lilac trace) or pCMPS (5 μM, red trace), present in the buffer from the start, had no significant effect on Amplex UltraRed/HRP response to H₂O₂ additions (control, blue trace).
induced by NADPH was due to the loss of antioxidant power, rather than direct stimulation of ROS generation by GR and TR2. In this case, an alternative mechanism for NADPH-induced ROS generation would be required. NADH-related ROS production by electron chain complexes or NAD-related dehydrogenases is unlikely, because no exogenous substrates or NADH was provided after permeabilization, and most of the endogenous substrates and pyridine nucleotides would be expected to diffuse out of the matrix following membrane permeabilization, although some fraction may remain bound [28]. Even then, this fraction is likely to be oxidized in the absence of substrates [27]. This was confirmed in Fig. 8A (red trace a), which shows that addition of rotenone to inhibit complex I in permeabilized mitochondria did not result in significant H2O2 production, unless NADH was also added. Antimycin to inhibit complex III did not further potentiate H2O2 production under these conditions. NADH added before or after CDNB also did not result in significant H2O2 production, indicating that CDNB does not inhibit respiratory complexes to the extent required for ROS production (blue trace b). NADPH was specifically required to initiate H2O2 production by CDNB (green trace c). Note that the rate of NADPH-induced H2O2 production in the presence of CDNB in trace c was comparable to that of NADH-dependent H2O2 production when respiratory complexes were maximally inhibited by rotenone and antimycin in trace a, with rates averaging 0.35 nmoles/min/mg, 95% CI [0.34, 0.38], and 0.33 nmoles/min/mg, 95% CI [0.31, 0.38], respectively, in 4 paired mitochondria preparations (Fig. 8A, inset). Fig. 8B provides further evidence that CDNB did not inhibit respiratory chain activity in permeabilized mitochondria, since the rate of NADH oxidation was similar before or after CDNB was added (red trace a). In contrast, rotenone completely inhibited NADH oxidation (blue trace b). Similar findings were obtained using the TrxR inhibitor auranoxin in place of CDNB (Fig. 8C&D). These findings were confirmed in 4 preparations, and together show that neither CDNB nor auranoxin causes NADH-related ROS production by directly inhibiting respiratory complexes or stimulating ROS production by NADH-related TCA cycle dehydrogenases.

There are some reports that Nox4, a constitutively active NAPDH oxidase, may be present in the mitochondrial inner membrane, and could be a source of NADPH-dependent ROS production. To test this possibility, we obtained Nox4−/− mice [29], a generous gift of Dr. Junichi Sadoshima, from which we isolated cardiac mitochondria. In response to CDNB or auranoxin, H2O2 efflux from Nox4−/− cardiac mitochondria was similar compared to wild-type murine cardiac mitochondria (Fig. 9), indicating the NADPH-induced ROS production under these conditions could not be attributed to Nox4 activity. In addition, incubation of permeabilized mitochondria from rabbit heart with 5 μM gr91ds-tat, considered to be a specific inhibitor of Nox2 [30], did not suppress NADPH-dependent H2O2 production induced by CDNB (results not shown).

These findings indicate that during reductive stress in permeabilized cardiac mitochondria, GR and/or TrxR2 can generate ROS spillover directly, at a rate comparable to NADH-dependent ROS production by inhibited respiratory chain complexes and/or NAD-related dehydrogenases. The
Fig. 8. Unlike NADPH, NADH does not increase H$_2$O$_2$ production in permeabilized mitochondria, either before or after adding CDNB or auranothin. A. After permeabilization with alamethicine (ala), mitochondria in sucrose buffer were exposed to either: 10 $\mu$M rotenone followed by 50 $\mu$M NADH followed by 5 $\mu$M antimycin (red trace a); 50 $\mu$M NADH followed by 100 $\mu$M CDNB followed by additional NADH (blue trace b); or 50 $\mu$M NADPH followed by 100 $\mu$M CDNB (green trace c). The rate of H$_2$O$_2$ production was quantitatively similar for NADPH + CDNB as for rotenone + NADH + antimycin (inset), but markedly less for NADH + CDNB. B. Mitochondria in sucrose buffer were permeabilized with alamethicine (ala). NADH (50 $\mu$M) was oxidized at a high rate before or after 100 $\mu$M CDNB was added (red trace a). In contrast, 10 $\mu$M rotenone completely prevented NADH oxidation (blue trace b). C. Mitochondria in sucrose buffer were permeabilized with alamethicine (ala). NADH added before or after 1 $\mu$M auranothin failed to generate significant H$_2$O$_2$, which required addition of NADPH. D. Mitochondria in sucrose buffer were permeabilized with alamethicine (ala). NADH (50 $\mu$M) was rapidly oxidized at a similar rate before or after 1 $\mu$M auranothin was added. (Additions to both traces are indicated by black arrows; additions to one trace only are indicated by the same color arrows).

Fig. 9. CDNB- and auranothin-induced H$_2$O$_2$ production is similar in alamethicine-permeabilized cardiac mitochondria isolated from wild-type and Nox4 $-/-$ mice. A. Cardiac mitochondria isolated from wild-type mice incubated in EDTA-sucrose buffer were permeabilized with alamethicine (ala). NADP$^+$ (0.5 $\mu$M), malate and isocitrate (2.5 mM each) were added, after which NADPH production was initiated by activating malate- and isocitrate dehydrogenase with excess Mg$^{2+}$. H$_2$O$_2$ production rapidly increased when CDNB (50 $\mu$M, trace a) or auranothin (1 $\mu$M, trace b) was added, compared to control conditions without inhibitors (trace c). Note that additions to all superimposed traces are indicated by black type, whereas additions specific to each colored trace are indicated by the same color type. B. Same protocol with cardiac mitochondria isolated from Nox4 $-/-$ mice, showing similar levels of H$_2$O$_2$ production as wild-type mice in A. (Additions to both traces are indicated by black arrows; additions to one trace only are indicated by the same color arrows). C. Comparison of the maximum rates of H$_2$O$_2$ production (nmol/min/mg protein) in wild-type versus Nox4 $-/-$ mice.
quantitative contributions of GR versus TrxR2 to total ROS production in permeabilized mitochondria, however, are difficult to assess. Even with GSSG/GSH depleted by permeabilization, Prx remained effective at masking H2O2 production. This is demonstrated in Fig. 10A, in which alamethicin-treated mitochondria were incubated with CDNB in the absence (blue trace) or presence of Zn2+ at concentrations of 5 (red trace), 10 (green trace) or 20 (dashed line) μM. Zn2+ significantly enhanced NADPH-induced H2O2 production, which saturated at 10 μM (Fig. 10B).

4. Discussion

4.1. Major findings of this study

Our major novel finding is the demonstration that both reduced GR and TrxR’s, like other members of the disulfide reductase flavoenzyme family including lipoamide dehydrogenase (the E3 component of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase), can leak electrons to O2 and generate significant amounts of ROS spillover when the supply of their natural electron acceptors is limited or electron transport to acceptors is inhibited (Fig. 1). Similar to these enzymes, reduced flavoprotein in isolated complex I has also been demonstrated to generate significant ROS [31] in the absence of electron acceptors other than O2 or when intrinsic electron transport to electron acceptors is prevented by inhibitors. Therefore, thermodynamically, in the absence of electron transport or acceptors, ROS production by all these proteins is directly demonstrated. The lack of an effect of SOD increases in ROS production, relative the rate of H2O2 production with no Zn2+ (100%), in 2 preparations.

Using the Amplex UltraRed/HRP system to detect H2O2 from resorufin fluorescence, we have shown that both GR and TrxR’s produce H2O2 in the presence of NADPH and O2. Reduced flavoproteins are known to be oxidized by molecular O2 with formation of O2•− and H2O2 [32], and H2O2 formation by lipoamide dehydrogenase [33] and Nox4 [34] has been directly demonstrated. The lack of an effect of SOD on H2O2 production by these proteins suggests that spontaneous dismutation of O2•− to H2O2 is not rate-limiting [35]. Acceleration of non-enzymatic dismutation has been shown with Nox4 [34], and may be the reason why many oxidases (glucose, xanthine oxidase) are known to produce mainly H2O2. Whereas lipoamide dehydrogenase uses NADH as its preferred electron donor, GR and TrxR’s use NADPH exclusively for this purpose. These results provide new insights into how reductive stress generates net ROS production, by converting enzymes that normally serve antioxidant functions into ROS producers.

The link between reductive stress and oxidative injury has been substantiated at multiple levels, including the in vivo setting. For example, mutations in crystallin, a small heat shock protein, result in protein aggregation causing cardiomyopathy in mice. The mechanism underlying protein aggregation disease has been attributed to reductive stress associated with increased activity of glucose-6-phosphate dehydrogenase (G6PD) raising NADPH levels [10]. In studies of experimental heart failure, G6PD activity was found to be significantly elevated resulting in a two-fold increase in NADPH concentration and increased ROS production [9]. In cultured cells, reductive stress associated with increased GSH levels has shown to trigger mitochondrial oxidation and cytotoxicity, although no increase in ROS production was reported based on DCF fluorescence [7]. Long-term treatment of mice with dioxin resulted in increased GSH levels in liver mitochondria that significantly enhanced O2•− and H2O2 production by mitochondria [8]. These findings are consistent with the “reduced-optimized ROS balance” hypothesis put forward by O’Rourke’s group [11,12], which posits that reduct balance is lost and ROS production increases at both extremes of oxidation and reduction of reduct couples involved in respiratory chain activity (NADH/NAD+) or ROS scavenging (NADPH/NADP+, GSSG/GSH). In addition, their recent work [12] shows that mitochondrial bioenergetics and ROS production are closely linked, such that reductive stress may also compromise cellular function by impairing energy production as well as by promoting oxidative injury.

Our findings provide novel insights into the “reduced-optimized ROS balance” hypothesis showing that the previous assumption that NAD+−related ROS production from respiratory complexes and/or NADH-related dehydrogenases simply overwhelms the capacity of NADPH-dependent ROS scavenging during reductive stress is not the full story. The novel information provided here is that ROS-scavenging capacity by both the glutathione/Gpx/GR and Prx/Trx/TrxR2 systems...
may become severely impaired during reductive stress due to the conversion of GR and TrxR2 into ROS producers, as a result of NADPH-dependent electron leak to O2 when their usual electron acceptors are in limited supply.

The evidence can be summarized as follows: 1) purified recombinant GR and TrxR1 both generated substantial amounts of H2O2 when supplied with NADPH in the absence of their usual electron acceptors GSSG and oxidized Trx, or if electron flow to their natural electron acceptors was impaired by thiol reactive agents such as pCMPS, mesaryl or pCMB for GR and CDNB for TrxR2 (Figs. 2 & 4); 2) in permeabilized mitochondria in which no NADH was provided for ROS generation by respiratory complexes or NAD+-related matrix dehydrogenases, NADPH stimulated robust H2O2 production when GR and TrxR2 were inhibited by lack of GSSG and/or CDNB (Fig. 8). Under these conditions, addition of NADH did not induce H2O2 production, indicating that these thiol reactive agents did not inhibit respiratory chain complexes directly at the concentrations used (Fig. 8); 3) in permeabilized mitochondria, the rate of NADPH-dependent H2O2 production by inhibited GR and TrxR2 during reductive stress was quantitatively comparable to the rate of NADH-dependent H2O2 production when respiratory complexes were maximally inhibited by rotenone and antimycin; 4) we also excluded the possibility that endogenous matrix Nox4 or Nox2 are responsible for NADPH-dependent ROS generation (Fig. 9). However, these observations notwithstanding, it still remains to be proved whether conditions sufficient to activate ROS production by GR and TrxR2 occur in intact mitochondria during reductive stress in vivo. Although not applicable to permeabilized mitochondria, it should be mentioned that in intact mitochondria transhydrogenase is a major mechanism responsible for the transfer of reducing equivalents between NAD(H) and NADP(H), coupled to the translocation of protons across inner membrane. Under physiological conditions about half of NADPH production is abolished by uncouplers, consistent with transhydrogenase-generated NADPH, with similar estimates from flux measurements [36].

4.2. Mechanisms of ROS generation by GR and TrxR’s

Although ROS production by GR has not, to our knowledge, been previously reported, TrxR1 was reported to be irreversibly inhibited by CDNB resulting in NADPH oxidation in the presence of NADPH and O2 almost 20 years ago [26]. Later studies showed that one of the ROS species generated by CDNB-inhibited TrxR1 was superoxide, since O2− detection (by the adrenochrome method) was eliminated by SOD [37]. In the absence of thioredoxin, O2− production by TrxR1 was also demonstrated using the EPR spin trap DEPMPO method [14]. However, as we have shown above in the presence of NADPH, TrxR1 also generates H2O2 (Fig. 4) that is likely due to accelerated spontaneous dismutation of O2− as described above. TrxR1’s proposed inherent NADPH oxidase activity [14] can also be induced by inhibiting electron flow to Trx with low molecular weight compounds like auranofin, curcumin, juglone, motexan gadolinium and other dinitrohalobenzenes [14,37–40], resulting in ROS production. As shown in Fig. 3, the rate of NADPH oxidation by GR was fully accounted for by the rate of O2 consumption, but both NADPH and O2 consumption were considerably greater than the rate of H2O2 production. The quantitative analysis indicated that only about 15% the electron leak from GR produces H2O2 directly by two-electron reduction. An important point to note is that the maximal rate of electron leak by GR to form H2O2 and reduce other possible ROS is orders of magnitude lower than its capacity to transfer electrons to GSSG. This is obvious in Fig. 2D, in which the rate of NADPH consumption associated with H2O2 production by GR in the absence of GSSG is orders of magnitude slower than when GSSG is added to the cuvette. Thus, O2 becomes viable as an electron acceptor from the flavoprotein redox site of GR only when the rate of electron transfer to GSSG is markedly impaired. Nevertheless, this low level of NADPH oxidase activity is still sufficient to generate significant amounts of H2O2, at rates comparable to the inhibited respiratory chain complexes during reductive stress (Fig. 8A).

4.3. Physiological implications, limitations and significance

To protect matrix elements against oxidative damage by ROS, mitochondria have evolved an integrated set of thiol systems. The glutathione/Gpx1/GR and Prx/Tx/TrxR2 systems are the most important, although catalase is also present in the matrix [4,5] and may come into play at micromolar concentrations of H2O2 [6]. Under normal conditions, H2O2 efflux from mitochondria energized with NAD+-related substrates is very low, suggesting that NADPH-dependent H2O2 scavenging is able to balance H2O2 production in the matrix. At either extreme of redox balance, however, this is no longer the case. This is obvious for oxidative stress, which is defined by excess ROS production relative to ROS scavenging. For reductive stress, however, the cause of the imbalance is less obvious, since both NADPH-dependent ROS production and NADPH-dependent ROS scavenging normally increase in parallel. The finding that NADPH-dependent ROS scavenging is directly impaired by the conversion of GR and TrxR2 from their antioxidant functions into ROS producers during reductive stress offers a resolution to this seeming paradox.

In addition to direct ROS production by GR and TrxR2, however, we also found that Prx activity has important influences on the degree of ROS spillover from the matrix during reductive stress. Specifically, we found that although there is an oxidized Trx concentration-dependent depression of H2O2 production (Fig. 4E) by TRxR’s, for normal NADPH and Trx concentrations inside the matrix (about 200 μM and ~10 μM correspondingly), Trx will be rapidly reduced (Fig. 4D) allowing H2O2 production to continue. However, the expected increase in [H2O2] was partly masked by Prx rapidly reducing the generated H2O2, as demonstrated when Zn2+ was used to inhibit Prx in Figs. 5A and 10. Therefore, it seems that H2O2 production by TrxR2/NADPH is not the primary determinant of matrix H2O2 spillover, but rather Prx activity. This could be an important regulatory mechanism for redox signaling or other purposes. Although free Zn2+ concentration is normally very low, metallothionein, the main Zn2+ binding protein in the cytoplasm, releases Zn2+ from its thiols. Metallothionein thiols have very low redox potential and are readily oxidized to release Zn2+ [41] even by relatively mild oxidants. This initial cytoplasmic Zn2+ release, if induced by ROS production from respiratory chain complexes or matrix dehydrogenases, can be taken up into the matrix by the Ca2+-uniporter as well as through independent import mechanism that is still not well defined [41]. In addition mitochondria have been shown to contain an independent store of Zn2+ that can be mobilized [42]. Zn2+ has been repeatedly shown to increase mitochondrial ROS production [41]. Although Prx is thought to be responsible for scavenging most of the H2O2 produced in the matrix [43], this could be an overestimate [23] and the relative contribution of other antioxidant systems requires further clarification. In this regard, one problem is that chemicals used to “selectively and independently” inhibit GSH/Trx systems are not completely specific: GSH depletion with CDNB, which is a good substrate for glutathione-S-transferase, also inhibits TrxR activity with a concomitant increase in ROS production. Significant inhibition of TrxR activity (>50%) has been reported at low concentrations of CDNB (~10 μM) and this effect was almost 10,000-fold faster than alkylation of GSH [26]. Irrespective of these issues, Prx inhibition by Zn2+ could be an important step that increases matrix H2O2 levels, further exacerbating ROS spillover during reductive stress. Ischemia/reperfusion, ROS and peroxynitrite have been shown to cause significant cytoplasmic and mitochondrial Zn2+ release in cardiomyocytes as demonstrated by high-affinity Zn2+ selective probes [44]. In addition, exogenous Zn2+ delivered either at the onset of reoxygenation/reperfusion (mimicking post-conditioning) or before ischemia (mimicking pre-conditioning) is cardioprotective [45–49], attributed to Zn2+-induced activation of the RISK pathway inhibiting mitochondrial permeability transition pore opening upon reperfusion [45,50,51]. Since cardioprotection by pre- and post-conditioning ischemia can be abolished by ROS...
factor exacerbating the massive ROS burst upon reperfusion that has cytokotoxic effects. Further experiments will require to delineate the regulation of $\text{Zn}^{2+}$ release by endogenous and exogenous factors as a cardioprotective versus injurious factor in the setting of ischemia/reperfusion.

An important limitation of our study is that our findings were all obtained using purified recombinant enzymes or isolated permeabilized mitochondria. We cannot exclude the possibility that GR and TrxR2 might behave differently in intact mitochondria in vivo, even though the relationship between reductive stress and oxidative injury in vivo is well-established, as reviewed above. Also, due to the lack of availability of recombinant TrxR2 with intact activity, we used recombinant TrxR1 as a proxy assuming that its properties are similar. In permeabilized mitochondria with both GR and TrxR2 present, we could not quantitatively assess their separate contributions to total H$_2$O$_2$ production. However, assuming that TrxR2 behaves similarly to recombinant TrxR1, then it is highly likely to make an important contribution for two reasons: 1) the capacity of recombinant TrxR1 to generate NADPH-dependent H$_2$O$_2$ is 8-fold higher than recombinant GR (Fig. 4C); and 2) in the matrix GSH/GSSG of recombinant TrxR2 with intact activity, we used recombinant TrxR1 to generate NADPH-dependent H$_2$O$_2$ was 8-fold higher than recombinant GR (Fig. 4C); and 2) in the matrix GSH/GSSG of recombinant TrxR1 to generate NADPH-dependent H$_2$O$_2$ was 8-fold higher than recombinant GR (Fig. 4C); and 2) in the matrix GSH/GSSG.

The collective findings raise the intriguing possibility that Zn$^{2+}$ likely to make an important contribution for two reasons: 1) the capacity of recombinant TrxR1 to generate NADPH-dependent H$_2$O$_2$ was 8-fold higher than recombinant GR (Fig. 4C); and 2) in the matrix GSH/GSSG of recombinant TrxR2 with intact activity, we used recombinant TrxR1 to generate NADPH-dependent H$_2$O$_2$ was 8-fold higher than recombinant GR (Fig. 4C); and 2) in the matrix GSH/GSSG.