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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 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 and thioredoxin reductase, generate H_2O_2 by leaking electrons from their reduced flavoprotein to O_2 when electron flow is impaired by inhibitors or because of limited availability of their natural electron acceptors, GSSG and oxidized thioredoxin. The spillover of H_2O_2 under these conditions depends on H_2O_2 reduction by peroxiredoxin activity, which may regulate redox signaling in response to endogenous or exogenous factors. These findings may explain how ROS production during reductive stress overwhelms ROS scavenging capability, generating the net mitochondrial ROS spillover causing oxidative injury. These enzymes could potentially be targeted to increase cancer cell death or modulate H_2O_2-induced redox signaling to protect the heart against ischemia/reperfusion damage.

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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 H_2O_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 H_2O_2 scavenging systems in the matrix, the glutathione/glutathione peroxidase3 (Gpx)/glutathione reductase (GR) and the peroxiredoxin3 (Prx)/thioredoxin2 (Trx)/thioreductase2 (TrxR2) systems (Fig. 1). Although catalase is also present in the matrix [4,5], it has a much lower affinity for H_2O_2 [6] and therefore is not likely to play an important role at micromolar concentrations of H_2O_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 perioxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; ICDH, isocitrate dehydrogenase; pCMB, p-chloromercuribenzoic acid; pCMPS, p-chloromercuriphenyl-sulphonate; Prx, peroxiredoxin; $O_2^{\bullet-}$, superoxide; TCA, tricarboxylic acid; TrxR1, thioredoxin reductase 1; TrxR2, thioredoxin reductase 2; Trx, thioredoxin 1 and 2

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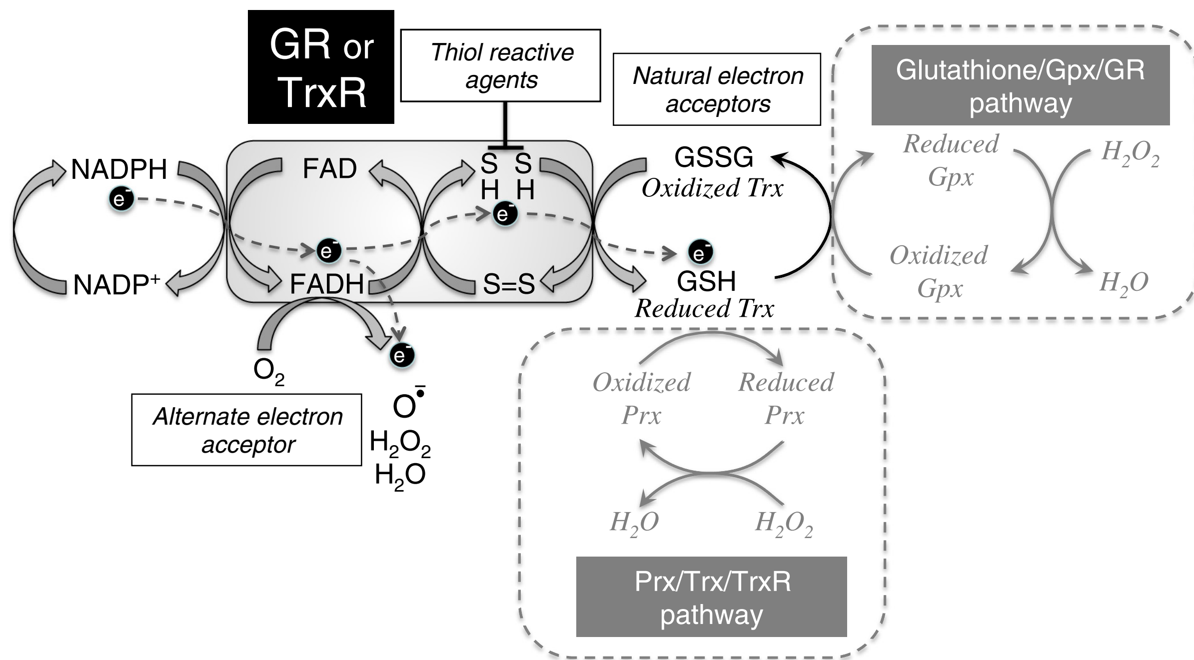

Fig. 1. Proposed mechanism of ROS generation by GR and TrxR2 during reductive stress. Flow of electrons (e^-) as indicated from NADPH to FAD to cysteine-related sulfhydryl groups (S) and then to the natural electron acceptors GSSG for GR, and oxidized Trx for TrxR2. If oxidized GSSG or Trx is in limited supply, the flavin reduction state is increased and electron flow is shunted from $FADH_2$ to O_2 as an alternative electron acceptor, with one-electron reduction producing superoxide, two-electron reduction producing H_2O_2 or four-electron reduction producing H_2O. Similarly, thiol reactive agents which react with SH groups in GR or TrxR2 also prevent electron transfer to the natural electron acceptors, resulting in superoxide, H_2O_2 and H_2O production instead.

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 TrxR's. Indeed, the cytoplasmic isoform 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 O_2, to generate superoxide and H_2O_2 by one or two electron reduction of O_2, 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 H_2O_2 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 Zn^{2+}. 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 O_2 consumption by recombinant GR was measured. Mitochondria

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