Compartment-specific Control of Reactive Oxygen Species Scavenging by Antioxidant Pathway Enzymes*

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Oxidative stress arises from an imbalance in the production and scavenging rates of reactive oxygen species (ROS) and is a key factor in the pathophysiology of cardiovascular disease and aging. The presence of parallel pathways and multiple intracellular compartments, each having its own ROS sources and antioxidant enzymes, complicates the determination of the most important regulatory nodes of the redox network. Here we quantified ROS dynamics within specific intracellular compartments in the cytosol and mitochondria and determined which scavenging enzymes exert the most control over antioxidant fluxes in H9c2 cardiac myoblasts. We used novel targeted viral gene transfer vectors expressing redox-sensitive GFP fused to sensor domains to measure H2O2 or oxidized glutathione. Using genetic manipulation in heart-derived H9c2 cells, we explored the contribution of specific antioxidant enzymes to ROS scavenging and glutathione redox potential within each intracellular compartment. Our findings reveal that antioxidant flux is strongly dependent on mitochondrial substrate catabolism, with availability of NADPH as a major rate-controlling step. Moreover, ROS scavenging by mitochondria significantly contributes to cytoplasmic ROS handling. The findings provide fundamental information about the control of ROS scavenging by the redox network and suggest novel interventions for circumventing oxidative stress in cardiac cells.

Reactive oxygen species (ROS) serve as both signaling molecules and destructive agents, requiring cells to finely regulate their levels. Antioxidant enzymes catalyze ROS conversion directly via an active-site metal ion (e.g. superoxide dismutase or catalase) or through pathways involving the donation of an electron from the moiety-conserved redox couples thioredoxin and glutathione, which require continuous regeneration of the reduced species. Uncontrolled or uncontained ROS accumulation can affect numerous cell functions, including gene/protein expression, calcium handling, myofilament activation, bioenergetics, and substrate metabolism (1–4). Different ROS-generating and scavenging systems are present in distinct cellular compartments, and these may interact in complex ways that have not been well characterized.

In cardiac myocytes, ROS are a byproduct of mitochondrial electron transport (5) and are also produced by extramitochondrial sources, including NADPH oxidase (1), uncoupled nitric oxide synthase (6), xanthine oxidase (7), and monoamine oxidase (8, 9). Both mitochondrial and extramitochondrial sources have been implicated in cardiac disorders including heart failure, myocardial ischemia, and arrhythmias. The dynamics and steady-state levels of ROS in local intracellular domains are determined by the rates of free radical generation and scavenging. Failure to scavenge free radicals via antioxidant fluxes can trigger a vicious cycle of dysfunction, leading to death (10). Recent studies have demonstrated that antioxidant enzymes targeted to the mitochondria, but not the cytoplasm, protect against cardiomyopathies associated with angiotensin-induced heart failure (11) or aging (12).

In this study, we examine the relative contributions of the parallel thioredoxin- and glutathione-driven antioxidant pathways to H2O2 dynamics in specific cellular compartments. We overexpress or knock down key enzymes in the H2O2 scavenger pathways, including those involved in generating NADPH, the master electron donor supporting ROS removal. We describe how antioxidant enzymes respond to external oxidative stress and identify which components are crucial to the ROS scavenging network. Our results support the concept that distributed control of the reactive oxygen gene network moderates steady-state H2O2 levels in distinct cellular compartments for signaling purposes and for protection against oxidative damage.

**Experimental Procedures**

**H2O2 and GSSG Redox Sensors**—Measurement of the cellular redox state is complicated by distinct sources of ROS and antioxidant systems in each intracellular compartment. Redox-active fluorescent compounds are widely used for cell assays, but the interpretation of signals from available sensors is subject to numerous caveats and limitations (13, 14). For example, many fluorescent dyes are irreversibly oxidized, not specific for a given ROS species, cannot be targeted to specific compartments, or are difficult to calibrate for quantitative comparisons.

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2 The abbreviations used are: ROS, reactive oxygen species; GSSG, glutathione disulfide; roGFP, redox-sensitive GFP; NNT, nicotinamide nucleotide transhydrogenase; OE, overexpression; DsRNA, Dicer-substrate RNA; Glc-6-PD, glucose-6-phosphate dehydrogenase; GRX, glutaredoxin; F_vox, fractional oxidation; mitoORP, mito-roGFP-ORP1; cytoORP, Cyto-roGFP-ORP1; mitoGRX, mito-GRX1-roGFP; cytoGRX Cyto-GRX1-roGFP; E_mito, glutathione redox potential; Trx, thioredoxin; TXNRD, thioredoxin reductase; IDH, isocitrate dehydrogenase; KD, knockdown; GSR, glutathione reductase; ME, malic enzyme; GPX, glutathione peroxidase; R_max, maximum florescence; R_min, minimum florescence.
Control of Antioxidant Flux

To overcome these limitations, we generated novel viral gene transfer vectors to express redox-sensitive GFP (roGFP) fused to specific sensor domains to measure either H2O2 or GSSG in intact cells. Because of intramolecular redox group transfer between the specific binding site and roGFP, these probes, developed and characterized previously by Meyer and Dick (15), rapidly and reversibly equilibrate the local redox state of the desired redox couple to a change in the fluorescence spectrum of the probe. The intensity of roGFP emission (510 nm) changes inversely with oxidation at two different excitation wavelengths (405 nm versus 488 nm). Oxidation increases the signal at 405 nm excitation and decreases the signal at 488 nm excitation, whereas reduction causes the inverse response (15, 16).

To specifically detect H2O2, the redox-sensitive roGFP is fused with the yeast peroxidase ORP1 (17). Upon binding of physiological concentrations of H2O2, ORP1 forms a cysteine sulfenic acid (Cys-SOH) in the active site, and protein disulfide bonds rapidly form in roGFP, thus shifting the 405/488-nm excitation ratio (17). RoGFP-ORP1 has high sensitivity and specificity for H2O2 and can thus report relative differences in H2O2 levels under different conditions. Similarly, GRX1-roGFP utilizes human glutaredoxin to sense GSSG (15, 16). To improve the utility of these probes for expression in both cultured cells and cardiomyocytes, we incorporated the targeted constructs into Gateway vectors (Invitrogen) and developed high-efficiency, high-titer adenoviruses for gene transfer.

Compartment-specific ORP1/GRX1-roGFP Ratiometric Probes—
The construction, cloning, and expression of roGFP-ORP1 is described elsewhere (17). Briefly, the ORP1 sequence is fused downstream of the roGFP cassette by a 30-amino acid linker (GGSGG). These probes were incorporated into adenoviral expression cassettes for gene transfer in multiple cell systems. The Mito-roGFP-ORP1 sequence was synthesized (Bioneer, Inc.) and cloned into a promoterless destination vector, pAd/PL-DEST, using two-fragment multisite Gateway Pro system technology from Invitrogen. Mito-roGFP-ORP1 was cloned into a donor vector, pDONR221P5-P2, resulting in an entry clone, pENTR(5–2)-Mito-roGFP-ORP1. Expression of roGFP-ORP1 was driven by an EF1-α promoter and cloned into another donor vector, pDONR221P1-P5r, resulting in an entry clone, pENTR(1–5r)-EF1α. In a second step, both entry clones and a destination vector, pAd/PL-DEST, were combined, resulting in the expression clone pAd/PL-EF1α-Mito-roGFP-ORP1. Replication-deficient adenovirus particles were produced from pAd/PL-EF1α-Mito-roGFP-ORP1 using the ViraPower adenoviral expression system (Invitrogen).

Construction of cDNA Expression Clones—The cDNA sequence was obtained from Dharmacon, Inc. (Prdx1 cDNA clone ID-7324630, Gpx1 cDNA clone ID-6918849, Txnrd1 cDNA clone ID-6886988, Glc-6-P cDNA clone ID-7108556, GSR1 cDNA clone ID-358965, Prdx3 cDNA clone ID-3963113, Gpx4 cDNA clone ID-4936866, Txnrd2 cDNA clone ID-7191992, ME3 cDNA clone ID-30097608, NNT cDNA clone ID-7381610, and IDH2 cDNA clone ID-7110062). Vector information, antibiotic selection, the sequencing primer, and the accession number are provided on the web sites of the sellers. The cDNA was premade with restriction site 3’NotI and 5’EcoRV for direct insertion into the expression vector. The cDNA was ligated into EcoRV and NotI sites of pcDNA3.1 (Thermo Fisher). Selection was made by Geneticin resistance. The resulting constructs were named pcDNA-OE, where OE is the name of the protein of interest. All constructs were confirmed by sequence analysis.

Cell Culture, Reagents, and Gene Transduction into H9c2 Myoblasts—H9c2 rat cardiac myoblast cell lines were purchased from the ATCC (Manassas, VA). Cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified air (5% CO2) at 37 °C.

Cells were seeded onto 35-mm glass-bottom plates (MatTek, Inc., 1 × 105 cells/plate) 24 h prior to redox sensor viral gene transduction. 24 h after viral transduction, constructs for knockdown or overexpression of antioxidant pathway enzymes were transfected (jetPrime reagent, Polyplus, Inc.). Dicer-substrate RNAs (DsiRNAs) or cDNA plasmids (Integrated DNA Technologies, Inc.) were used for knockdown or overexpression, respectively. DsiRNAs are 27-mer duplex RNAs that are designed to mimic dicer products, thus bypassing the dicer enzyme to increase potency (18, 19). A fluorescent dye-labeled duplex (Tye 3 DS transfection control) was used to make sure that transfection was optimal and indicated that >90% of the cells were transfected.

The major antioxidant enzyme targets (Fig. 1A) included the mitochondrial enzyme isoforms GPX4 (glutathione peroxidase), GSR1 (glutathione reductase), PRX3 (peroxidase), TXNRD2 (thioredoxin reductase), IDH2 (isocitrate dehydrogenase 2), ME3 (malic enzyme 3), and NNT and the cytoplasmic antioxidant enzyme isoforms GPX1, GSR1, PRX1, TXNRD1, and glucose-6-phosphate dehydrogenase (Glc-6-PD). Oligonucleotide was added to the cells in a transfecting mixture consisting of 1:2 siRNA to jetPrime reagent. Medium was changed after 4 h, and cells were analyzed after 48–72 h. Knockdown of proteins was confirmed by Western blot. H9c2 cells transfected with scrambled siRNA were used as controls to compare the level of protein expression. We used GAPDH as an internal standard. Optical densities of the protein bands of interest were divided by the GAPDH band in their respective lanes. Subsequently, the control (scrambled siRNA) was used as a normalizing control, where control values were set to 1, and cells with knockdown and overexpression were fractional values of the control.

The following antibodies were used for Western blots to check the expression levels of the proteins: Prx1 (ab184868), Gpx1 (ab59546), Txnrd1 (ab184868), GSR1 (ab128933), PRX3 (ab73349), Gpx4 (ab125066), NNT (ab10352), and IDH2 (ab131263) from Abcam and Glc-6-PD (12263), Trx2 (3F2-E12-F10), and ME3 (123995) from Cell Signaling Technology, Inc.

H2O2 Challenge and Live Cell Imaging—Redox sensor-transduced cells were imaged using a spinning disk confocal microscope (Olympus/Andor Revolution XD) with continuous perfusion of extracellular solution at 37 °C. Cells were first perfused with a modified Tyrode’s solution (130 mM NaCl, 5 mM L-ketoglutarate, 1 mmol/liter MgCl2, 10 mmol/liter sodium HEPES, 2 mmol/liter CaCl2, 10 mmol/liter glucose, 2
mmol/liter pyruvate, and 0.3 mmol/liter ascorbic acid (pH 7.4)) and then challenged with continuous perfusion of 1 mm tert-butyl hydroperoxide (referred to as exogenous H$_2$O$_2$ in the experiment descriptions and figures), which forms a more stable peroxy radical in solution than hydrogen peroxide. At the end of each experiment, the signals were calibrated using diamide to oxidize the GSH pool and obtain R$_{\text{max}}$ for the roGFp probe and DTT to obtain R$_{\text{min}}$. Mito/cyto-roGFp-ORP1/GRX were excited with the 405- and 488-nm laser lines, and emission was detected at with a 500- to 554-nm band pass filter. Images were collected at a 2-s interval over 30 min. 405/488-nm excitation ratio images were created and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) as 16-bit TIFF files. After normalization of the ratiometric signals to R$_{\text{max}}$ (1) and R$_{\text{min}}$ (0) to obtain the fractional oxidation, F$_{\text{Fox}}$ of the sensor, the change in GRX1-roGFp or roGFp-ORP1 signal from baseline (ΔF$_{\text{ox}}$) was determined during exposure to H$_2$O$_2$ for statistical analysis unless otherwise indicated. 50% duration of recovery of the GSH pool was determined as the time from the peak GRX1-roGFp oxidation to half recovery to the baseline ratio.

**Summary Data and Statistical Analysis**—To examine the regulatory impact of individual antioxidant enzymes, the mean ROS response (ΔF$_{\text{ox}}$) in knockdown or overexpression cells was compared with the relative decrease or increase in protein levels determined by Western blot (normalized ΔE). All data are expressed as mean ± S.E. of separate experiments (n ≥ 5) and compared by Student’s t test. Differences between two treatment groups were considered significant at $p < 0.05$.

**Results**

**Compartmentalized Expression of Redox Sensors**—Fig. 1A provides a schematic of the antioxidant pathways and enzymes in mitochondria and cytoplasm examined in this study. Live cell imaging of transfected H9c2 cells showed optimal expression and specific subcellular localization of the roGFp probes (Fig. 1B). The mito-roGFp-ORP1 (mitoORP)-transfected cells loaded with tetramethylrhodamine methyl ester demonstrated that this probe localized specifically to the mitochondria (Fig. 1B, top panels). In contrast, the Cyto-roGFp-ORP1 (cytoORP) localized to the cytoplasm. Similar results were obtained with the GRX1-roGFp probes (mitoGRX and cytoGRX, Fig. 1B, bottom panels). Compared with fluorescent dye-labeled control RNAs, transfection of DsiRNAs resulted in knockdown of antioxidant enzymes by 50–90% at 48–72 h after transfection, respectively (Fig. 1C). Expression levels were subsequently tested by Western blots. Protein bands and overall expression levels are reported in Figs. 2, 3, and 4.

**The Role of Mitochondrial Antioxidant Enzymes**—We first examined the contribution of the mitochondrial antioxidant enzymes to local and global H$_2$O$_2$ dynamics by knocking down or overexpressing key enzymes in the glutathione or thioredoxin scavenging pathways (shaded enzymes in Fig. 2A). Mitochondrial and cytosolic H$_2$O$_2$ levels were measured during exposure of the cells to exogenous H$_2$O$_2$, followed by calibration with DTT and diamide. Fig. 2B shows example records of the H$_2$O$_2$ signal (mitoORP ratio) in control cells or with knockdown of mitochondrial PRX3. The increase in the mitoORP ratio upon H$_2$O$_2$ exposure reports the local increase of H$_2$O$_2$ in the mitochondrial matrix, which was accentuated with PRX3 knockdown (Fig. 2B, gray line). Subsequent exposure to the strong reducing agent DTT directly reduces the disulfide of the roGFp, causing a decrease in the fluorescence ratio to a minimum (R$_{\text{min}}$), whereas the thiol oxidant diamide increased the ratio to obtain a maximum (R$_{\text{max}}$). These limits were then used to normalize the response to between 0 and 1, enabling the calculation of a calibrated ΔF$_{\text{ox}}$ for the H$_2$O$_2$ response in each group of cells. Cells transfected with scrambled DsiRNA were used as a control (Fig. 2B, black line).

Decreased H$_2$O$_2$ scavenging (i.e. larger ΔF$_{\text{ox}}$ upon H$_2$O$_2$ exposure) was observed for knockdown of PRX3, TXNRD2, GPX4, and GSR1 compared with the control (TXNRD2 > GPX4 ≈ PRX3 ≈ GSR1 > control, $p < 0.05$). Overexpression of GPX4 and GSR1 significantly improved H$_2$O$_2$ scavenging (Fig. 2C). However, overexpression of PRX3 or TXNRD2 had little effect on the response. Thus, although it is critical to prevent depletion of either the thioredoxin or glutathione enzymes, there appears to be adequate capacity of the PRX3 and TXNRD2 but not the GPX4 and GSR1 enzyme reserves. Indeed, overexpression of GSR1 improved H$_2$O$_2$ scavenging by ~10-fold over the controls ($p < 0.05$), implicating it as a rate-controlling enzyme.

**Measuring compartment-specific H$_2$O$_2$ responses permitted us, for the first time, to examine the impact of the mitochondrial H$_2$O$_2$ scavenging machinery on cytosolic H$_2$O$_2$. Notably, knockdown of the mitochondrial antioxidant pathway enzymes (PRX3, TXNRD2, and GSR1) significantly impaired H$_2$O$_2$ scavenging in the cytoplasm, as reported by cytoORP (Fig. 2D). The GSR1 knockdown effect on cytoplasmic scavenging was particularly severe. However, this may due to the fact that GSR1 is present in both mitochondria and the cytoplasm, with its localization dependent on alternative splicing (20). The overexpression of the mitochondrial enzymes PRX3, TXNRD2, or GPX4 did not significantly decrease the cytosolic H$_2$O$_2$ levels. However GSR1 expression did improve cytosolic H$_2$O$_2$ scavenging (Fig. 2D).

**NADPH-producing Enzymes Control Antioxidant Pathway Flux**—NADPH, which has the most negative redox potential of the redox couples involved, is absolutely required to drive the thioredoxin and glutathione antioxidant pathways in the mitochondrial matrix and in the cytosol. In mitochondria, NADPH supply is both directly and indirectly coupled to the TCA cycle. The NNT is a transmembrane protein in the inner membrane that can couple NADH oxidation to NADP$^+$ reduction at the expense of the proton gradient or, conversely, can couple NADPH oxidation to NAD$^+$ reduction while extruding a proton in the thermodynamically unfavorable reverse reaction (21). IDH2 and ME3 couple the oxidation of TCA cycle intermediates to the reduction of NADP$^+$, generating NADPH in the following reactions:

- IDH2:NADP$^+$-dependent isocitrate dehydrogenase
- Isocitrate + NADP$^+$ + H$^+$ → α-ketoglutarate + NADPH
- ME3:malic enzyme
- Malate + NADP$^+$ + H$^+$ → pyruvate + NADPH

Hence, changes in flux through the TCA cycle or oxidative phosphorylation (by altering the rate of NADH oxidation) can
impact H$_2$O$_2$ scavenging in the matrix. We next assessed the impact of varying NADPH-supplying enzymes on the response to exogenous H$_2$O$_2$. Knockdown of either IDH2, NNT, or ME3 marked impaired H$_2$O$_2$ scavenging (Fig. 3B), with NNT depletion having the largest effect of any intervention. Remarkably, the antioxidant system appears to be operating at a suboptimal level in terms of NADPH supply. Overexpression of any one of these enzymes substantially improved H$_2$O$_2$ scavenging, almost completely eliminating the increase in the mitoORP $\Delta F_{\text{ox}}$ during H$_2$O$_2$ exposure (Fig. 3B). Knockdown of these enzymes did not, however, significantly alter basal H$_2$O$_2$ levels in the absence of external oxidative stress (Fig. 3C).

FIGURE 1. Schematic of the antioxidant network and targeting of redox sensors. A, major components of the antioxidant network in the mitochondria and cytoplasm. Electron leak from the respiratory chain (complexes I–IV) leads to the formation of superoxide anion (O$_2^\cdot$), which is dismutated to H$_2$O$_2$ by mitochondrial superoxide dismutase (SOD2). Similarly, SOD1 catalyzes O$_2^\cdot$ conversion to H$_2$O$_2$ in the cytoplasm. H$_2$O$_2$ is reduced to water by several antioxidant enzymes, including catalase (not shown) or GPX1–4 and PRX1–3, which depend on the maintained availability of reduced GSH and Trx(SH)$_2$, respectively. Regeneration of GSH/Trx(SH)$_2$ by glutathione reductase or Trx reductase requires NADPH, which is oxidized to NADP$^+$ in the reaction. NADPH is generated in the mitochondria by NNT, IDH2, or ME3. Cytosolic NADPH is predominantly provided by Glc-6-PD or by the cytosolic isoforms IDH1 and ME1. H$_2$O$_2$, but not O$_2^\cdot$ can diffuse through membranes. NADPH may be indirectly shuttled between compartments through coupled enzyme reactions and mitochondrial metabolite transporters. B, confocal fluorescence images of the mitochondrially targeted H$_2$O$_2$ (mitoORP) or GSSG:GSH (mitoGRX) redox sensors with the mitochondrial membrane potential probe tetramethylrhodamine methyl ester (TMRM) in H9c2 cells. The degree of colocalization of the probes with the mitochondria is evident in the merged overlays. CytoORP and CytoGRX images show the cytoplasmic location of the respective redox probes. C, example of H9c2 cells transfected with fluorescent dye-labeled DsiRNA duplex in transmitted light (top panel), 48 h after transfection (center panel), and 72 h after transfection (bottom panel), showing high transfection efficiency by 72 h. Protein expression levels were subsequently tested by Western blots (see Figs. 2, 3, and 4).
To determine the extent of control exerted by individual pathway components on H$_2$O$_2$ scavenging rates, the ROS response after knockdown or overexpression of each enzyme was plotted as a function of the concentration of enzyme (normalized to control cells) (Fig. 3D), determined from Western blots performed in parallel experiments. Notably, the relationship between antioxidant flux and protein level was steepest for the enzymes responsible for NADPH supply, as summarized by calculating the H$_2$O$_2$ control coefficients ($\frac{\Delta F_{\text{ox}}}{\Delta E}$, Fig. 3E).

Cross-talk between Mitochondrial and Cytosolic Antioxidant Responses—Next we investigated whether changes in antioxidant pathway enzymes in one compartment impact ROS scavenging in another. We measured the effect of knockdown of the cytoplasmic antioxidant enzyme isoforms TXNRD1, PRX1, GPX1, GSR1, and Glc-6-PD (shaded in Fig. 4A) on cytoplasmic and mitochondrial redox state using cytoORP (Fig. 4B) or mitoORP (Fig. 4C). Knockdown of any one of these enzymes significantly decreased the cytoplasmic H$_2$O$_2$ scavenging rate during exposure to H$_2$O$_2$ (Fig. 4B), with Glc-6-PD having the largest effect (>3-fold increase in steady-state H$_2$O$_2$ compared with the control). Because Glc-6-PD is the primary NADPH-producing enzyme in the cytosol, this result aligns with our findings from the mitochondrial antioxidant pathways, where the NADPH-producing enzymes were the key regulators driving the scavenging of H$_2$O$_2$. Overexpression of the cytosolic enzymes did not substantially improve cytosolic H$_2$O$_2$ scavenging, indicating that endogenous enzyme concentration was not rate-limiting in the cytosol (Fig. 4B).

In contrast to the effect on cytoplasmic scavenging, knockdown of the cytosolic antioxidant enzymes had no statistically significant effect on mitochondrial matrix H$_2$O$_2$ (Fig. 4C). This finding supports the robust ability of mitochondria to couple substrate oxidation to the production of reducing equivalents for ROS scavenging. Scavenger reserve capacity was sufficient

FIGURE 2. Effects of knockdown or overexpression of mitochondrial antioxidant pathway enzymes on mitochondrial and cytoplasmic H$_2$O$_2$ responses. A, enzymes targeted are shaded in gray. H$_2$O$_2$ levels were measured in either the mitochondrial or cytoplasmic compartments. B, representative recordings of the mitoORP 405/488-nm excitation ratio (530-nm emission) in control H9c2 cells (black line) or those transduced with DsiRNA against PRX3 (gray line). Cells were continuously perfused at 37 °C on the stage of the confocal microscope, and exogenous H$_2$O$_2$ (1 mM) was applied as indicated, followed by DTT and diamide to obtain the R$_{\text{min}}$ and R$_{\text{max}}$ ratios, respectively, used to calibrate the ratio in terms of F$_{\text{ox}}$, C, the mean mitochondrial ROS response, i.e. the maximal change in F$_{\text{ox}}$ ($\Delta F_{\text{ox}}$) of mitoORP upon exposure to H$_2$O$_2$ in control cells (white column), or those with overexpression (gray columns), or knockdown (striped columns) of the mitochondrial enzymes PRX3, TXNRD2, GPX4, or GSR1. D, the mean cytosolic ROS response to H$_2$O$_2$ exposure, $\Delta F_{\text{cytoORP}}$, in control (white column), overexpression (gray columns) and knockdown (striped columns) of the mitochondrial enzymes PRX3, TXNRD2, GPX4, or GSR1. n = 5 replicates for all experiments. Error bars show S.E. *, $p < 0.05$ versus control group; †, $p < 0.05$ overexpression versus knockdown. E, Western immunoblots showing the protein OE, control, and KD of respective isoforms of enzymes in H9c2 cells. GAPDH was used as the internal standard. Bar graphs show protein levels in milligram per milliliter normalized to control (control = 1). GAPDH bands were spliced from the gel for presentation purposes (n = 5 replicates).
to buffer the exogenous H$_2$O$_2$ diffusing into the matrix even when the cytoplasmic system was impaired. Similarly, overexpression of cytosolic isoforms did not affect the H$_2$O$_2$ response. The only enzyme that had an effect on the mitochondrial H$_2$O$_2$ response when overexpressed was GSR1, which, as mentioned above, is the common isoform in mitochondria and the cytoplasm. Hence, mitochondria are not only a major source of ROS but are effective whole-cell H$_2$O$_2$ scavenging organelles operating more or less independently of cytoplasmic antioxidant pathways.

Antioxidant Pathways and Redox Potential of the Glutathione Redox Couple (E$_{GSH}$)—GSSG is reduced to two molecules of GSH by GSR1 using reducing equivalents provided by NADPH. In response to changes in the levels of H$_2$O$_2$, there is a transient increase in GSSG:GSH because of activation of GPX, followed by activation of GSR1 to restore the glutathione redox potential (16). Because the GSH/GSSG redox couple is the most abundant source of reducing equivalents, it represents the first line of defense for maintaining thiol redox balance.

We measured GSSG:GSH in the mitochondria or cytoplasm using the mitoGRX (Fig. 5) or cytoGRX (Fig. 6) probes in cells with antioxidant enzymes knocked down. Fig. 5B shows a representative recording of the mitoGRX response under oxidative stress. In the control cell, exogenous perfusion of H$_2$O$_2$ caused a transient increase in mitoGRX ratio because the antioxidant enzymes respond to eliminate H$_2$O$_2$. When the GSSG/GSH signal reached its peak, the GSR1 rate increased, and GSSG reduction via NADPH was initiated (decay in the GSSG/GSH plot in Fig. 5B). This process recovers EGSH, thereby maintaining cellular redox homeostasis. Knockdown of GSR1 (Fig. 5B) accentuated the peak GSSG/GSH increase and prolonged the recovery time of E$_{GSH}$.

Knockdown of PRX3, TXNRD2, GPX4, or GSR1 in the mitochondrial compartment all had the same effect, increasing peak ΔF$_{ox}$ (Fig. 5C) and slowing recovery of the GSSG/GSH ratio (Fig. 5D). Similar effects were observed after knockdown of the mitochondrial NADPH-producing enzymes IDH2, NNT, or ME3 (Fig. 5, E and F). In the case of
GSR1 knockdown, some cells were so sensitive that there was no recovery of the GSH pool for the duration of the experiment (these were not included in the average time to recovery). The average time to 50% recovery of the GSH pool in the control cells was \( T_{50} = 85 \text{ s} \), which was significantly prolonged in knockdown cells (GPX4 > PRX3 > GSR1 > TXNRD2; \( T_{50} > 200 \text{ s} \)).

Correspondingly, knockdown of the cytosolic antioxidant enzymes PRX1, TXNRD1, GPX1, GSR1, or Glc-6-PD led to higher peak \( \Delta F_{\text{ox}} \) of cytosolic GSSG/GSH upon \( \text{H}_2\text{O}_2 \) exposure compared with the control cells. In this case, GPX1 or GSR1 knockdown had the most pronounced effects on the GSSG/GSH ratio (Fig. 6B). The \( T_{50} \) of the control cells (~105 s) was significantly shorter than in knockdown cells (PRX1 > TXNRD1 > GPX1 > Glc-6-PD > GSR1 > control, ~180–300 s; \( p < 0.05 \), Fig. 6C).

Discussion

The high energy demand of the heart necessitates a high rate of oxidative phosphorylation while maintaining ROS below a destructive level. This study quantifies the relative contribution of specific enzymes in the antioxidant network of the mitochondrial matrix and the cytosol on \( \text{H}_2\text{O}_2 \) scavenging rates within each intracellular compartment. The major findings are that thioredoxin- and glutathione-coupled reactions in the mitochondrial matrix contribute equally to optimal \( \text{H}_2\text{O}_2 \) scavenging; knockdown of mitochondrial thioredoxin reductase had a disproportionally large effect on \( \text{H}_2\text{O}_2 \) scavenging; NADPH-producing enzymes are the major rate-controlling steps in \( \text{H}_2\text{O}_2 \) scavenger flux; cytosolic \( \text{H}_2\text{O}_2 \) scavenging is dependent on mitochondrial \( \text{H}_2\text{O}_2 \) scavenging, but mitochondrial \( \text{H}_2\text{O}_2 \) scavenging is less dependent on cytosolic \( \text{H}_2\text{O}_2 \) scavenging; and the kinetics of glutathione oxidation and
reduction are significantly altered by impairment of either the Trx- or GSH-dependent reactions.

Parallel Pathways for Detoxifying $H_2O_2$—In its simplest interpretation, the antioxidant system can be viewed as a network with parallel redox circuits (22) downstream of superoxide dismutase, utilizing glutathione or thioredoxin redox couples, whose reduction is driven by the common electron donor NADPH. Although both branches of the pathway are capable of eliminating $H_2O_2$, qualitative and quantitative differences exist that need to be considered from the perspective of ROS regulation. The kinetic contribution to scavenging for each component depends on the concentration of the electron donors, redox potential of the couple, enzyme concentration, and rate of the reaction with hydrogen peroxide. Taking these factors into account, Cox et al. (23) proposed that the relative abundance and kinetic properties of PRX3 favor it as the predominant mitochondrial scavenger of $H_2O_2$ compared with other peroxidases. On the other hand, GSH is present in millimolar amounts within the cell (24) and represents the largest-capacity redox reserve (25). With respect to cardiac mitochondrial $H_2O_2$ scavenging, the relative contributions of GSH- versus thioredoxin-driven $H_2O_2$ removal were recently assessed by Aon et al. (26), using inhibitors of each pathway. It was found that GSH depletion resulted in a larger increase in mitochondrial $H_2O_2$ emission ($\sim$19-fold) compared with thioredoxin reductase inhibition (7-fold), although species-dependent differences were observed (e.g. the impact of the two branches was roughly equal in guinea pig heart mitochondria). In contrast, the thioredoxin system accounted for 60% and GSH for only 20% of the increase in $H_2O_2$ emission in rat brain mitochondria (27), with catalase making a minimal contribution.

In this work, knockdown of PRX3 or GPX4, the mitochondrial peroxidases directly scavenging $H_2O_2$, decreased the ROS response to a similar degree, whereas knockdown of the upstream reductase TXNRD2, which might be expected to affect only the thioredoxin arm, had a larger impact on $H_2O_2$.
scavenging. Consistent with this enhanced role of TXNRD2, a dominant negative TXNRD2 mutation was shown to markedly increase oxidative stress in cultured cells (22). In addition, Zhang et al. (28), showed that early embryonic death in TXNRD2 knockout Zebrafish was associated with increased hepatic cell death and defective liver development. This was accompanied by increased ROS levels and an imbalance of members of the Bcl-2 family. Although increased ROS may contribute to this strong knockout phenotype, it should be recognized that thioredoxins can directly interact with protein complexes and transcription factors (29) and mediate alternative redox reactions such as trans-nitrosylation (30), demonstrating that their role extends beyond H\textsubscript{2}O\textsubscript{2} scavenging alone. This pleiotropic role of mitochondrial TXNRD2 may explain why early homozygous knockout results in embryonic lethality in mice (31). Nevertheless, TXNRD2 plays a critical role in the removal of toxic ROS and maintenance of mitochondrial integrity in mice (32), and heart-specific deletion of the TXNRD2 gene leads to dilated cardiomyopathy and death shortly after birth. A less dramatic effect was observed for overexpression of a dominant negative cytosolic TXNRD1, which increased oxidative stress markers and induced cardiac hypertrophy (33). These effects were mitigated by antioxidant treatment or expression of wild-type TXNRD1. These findings highlight thioredoxin reduction as a potential therapeutic target in hypertrophic and dilated cardiomyopathies.

The amplified mitochondrial ROS response in TXNRD2 knockout cells illustrates that the GSH system alone is insufficient to fully compensate for oxidative stress. One possible explanation is that loss of the ability to reduce thioredoxin may have a broad impact on all thioredoxin-dependent targets, including mitochondrial peroxiredoxins other than PRX3, or Trx-dependent protein complexes that might impact ROS production or scavenging indirectly (34). Second, increased oxidative stress upon TXNRD2 knockdown could be further enhanced by hyperoxidation of PRX3, which is known to occur after treatment with auranofin, a thioredoxin reductase inhibitor, or other stressors (35–38). This hyperoxidation results in inactivation of PRX3 (37). Thus, negative synergistic effects could exacerbate the

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**FIGURE 6. Effects of knockdown of cytoplasmic antioxidant pathway enzymes on cytoplasmic GSSG:GSH response during H\textsubscript{2}O\textsubscript{2} exposure.** A, enzymes targeted are shaded in gray. The redox state of the GSSG/GSH couple was measured in the cytoplasmic compartment using cytogrX. B, the mean cytosolic GSSG/GSH response, \( \Delta F_{\text{cmax}} \), cytogrX, upon exposure to H\textsubscript{2}O\textsubscript{2} in control cells (white column), or those with knockdown (gray or patterned columns) of the cytosolic enzymes PRX1, TXNRD1, GPX1, GSR1, or Glc-6-PD. C, time to 50% recovery of the cytosolic GSH redox state during H\textsubscript{2}O\textsubscript{2} exposure for cells in which PRX1, TXNRD1, GPX1, GSR1, or Glc-6-PD was knocked down. \( n \leq 5 \) replicates for all experiments. Error bars show S.E. *, \( p < 0.05 \) versus the control group.
Control of Antioxidant Flux

Impairment of H$_2$O$_2$ scavenging even though some redundancy is present in the system.

The Role of NADPH Supply in ROS Scavenging—The major source of reducing power for biosynthesis and detoxification of oxidative species in the cell is NADPH, which drives the reduction of both GSSG and TrxSS, representing the link between substrate catabolism and mitochondrial ROS scavenging (39). Our results emphasize that disruption of any of the NADPH sources, i.e. NNT, IDH2, or ME3, strongly impacts the H$_2$O$_2$ scavenging capacity of the mitochondria. Moreover, overexpression of any of the NADPH-producing enzymes was highly effective at suppressing oxidative stress responses, suggesting a novel way to potentially improve mitochondrial ROS scavenging kinetics. With regard to NADPH availability, it has been known for some time that a spontaneous NNT mutation in C57BL/6J (B6)-NntMUT) mice eliminates trans-hydrogenation activity. The NNT mutant is associated with redox abnormalities, including higher rates of H$_2$O$_2$ release, spontaneous oxidation of NADPH, a poor ability to metabolize organic peroxide, and a higher susceptibility to trigger a Ca$^{2+}$-induced mitochondrial permeability transition. The oxidized/reduced glutathione ratio is also increased compared with wild-type mice (40). Importantly, the NNT knockout has been linked to increased susceptibility to metabolic diseases, including diet-induced weight gain and diabetes (40, 41).

Myocardial tissue is typically thought to have low catalase-specific activity (42) and it is usually considered to be a minor contributor to mitochondrial H$_2$O$_2$ scavenging (perhaps with the exception of rat heart mitochondria (43)). However, it should be noted that catalase has been shown to be an effective mitochondrial ROS suppressor when artificially targeted and overexpressed in the mitochondria, decreasing oxidative damage, inhibiting cardiac pathology, and extending the life span of mice under stress (44–46). Importantly, catalase also requires NADPH, but only as a co-factor, not a primary substrate. Hence, one might expect less of an impact of low NADPH supply (or impaired metabolism) when using catalase overexpression as an antioxidant strategy. This remains to be tested experimentally.

Dependence of Cytosolic H$_2$O$_2$ Scavenging on Mitochondria—Our findings support the idea that mitochondria should not only be viewed as important sources of ROS but also as potent ROS-scavenging organelles. Impairment of mitochondrial scavenging markedly increased H$_2$O$_2$ accumulation in the cytosolic compartment during the exogenous H$_2$O$_2$ challenge. This is presumably because of H$_2$O$_2$ diffusion into the matrix, where metabolism-driven reduction drives antioxidant fluxes to act as a ROS sink. The increase in cytosolic H$_2$O$_2$ with inhibition of mitochondrial matrix scavenging was, in fact, roughly equivalent to the effect of knockdown of cytosolic antioxidant enzymes. As expected, the cytoplasmic H$_2$O$_2$ responses also strongly depended on NADPH, derived from the primary cytoplasmic source, Glc-6-PDH. In this study, we could not determine whether mitochondrial NADPH sources contributed to cytoplasmic reductase activity via metabolite shuttles in the inner membrane (e.g. malic enzyme reaction coupled to pyruvate/malate transport or IDH reaction coupled to isocitrate/α-ketoglutarate transport (47)). Theoretically, the transfer of NADPH equivalents from the matrix to the cytoplasm, or vice versa, could also contribute to cross-talk between the compartments.

Although mitochondria appear to be effective ROS sinks, the reverse is not true. There was no statistically significant impact of knockdown of the cytosolic antioxidant enzymes on the mitochondrial ROS response under our conditions.

Compartmental GSSG/GSH Responses to Oxidative Stress—The catalytic ability of antioxidant pathways depend on recycling of Trx and GSH. Two molecules of GSH are oxidized by H$_2$O$_2$ through GPX, producing GSSG (48, 49). The resulting GSSG is then reduced by GSR1 and NADPH, regenerating GSH. Thus, E$_\text{GSH}$ (detected as changes in E$_\text{roGFP}$) follows transient kinetics, rapidly normalizing during maintained oxidative stress. As anticipated, GSR1 activity is rate-controlling in this compensatory response. Moreover, overexpression of GSR1 improves the ROS-scavenging activity profoundly, indicating that the cell has a limiting enzyme concentration of GSR1. The finding that there is not an overabundance of GSH-driven scavenger capacity may reflect the need for precise control of redox balance in the cell. Some level of oxidation of GSSG/GSH redox potential or a minimal level of signaling ROS may be required for normal cell function in the absence of oxidative stress.

Because mitochondria cannot synthesize GSH and oxidized glutathione cannot be transported to the cytosol through the membrane, GSSG reduction strictly depends on compartmentalized mitochondrial NADPH production. When the NADPH-producing enzymes were knocked down, we not only observed H$_2$O$_2$ accumulation but also GSH depletion and a change in E$_\text{GSH}$. This will have serious repercussions because accumulation of mitochondrial GSSG above a threshold level impairs ROS scavenging and triggers mitochondrial membrane potential instability or oscillation (50). Early GSH depletion can result in accumulation of hydrogen peroxide and lipid hydroperoxides, which can initiate necrotic or apoptotic cell death (51, 52).

Redox Environment and ROS Balance—In developing a model of control of the redox status of the cell, Schafer and Buettner (25) proposed that the entire "redox environment" should be considered when assessing the ability to withstand redox stress. They defined the redox environment by a formula taking into account both the amounts and redox potentials of the NADP$^+$/NADPH, GSSG/GSH, and TrxSS/Trx(SH)$_2$ redox couples. Building upon this notion, we previously proposed the "redox-optimized ROS balance" hypothesis to emphasize the fact that ROS overload can occur with both high and low mitochondrial redox potentials (53). When the redox environment is most reduced, ROS production by the electron transport chain is enhanced, possibly to a point that exceeds the antioxidant scavenger capacity, whereas, in a more oxidized redox environment, depletion of the antioxidant-reducing capacity evokes a ROS increase as basal ROS production exceeds scavenging. Our results are consistent with this hypothesis, emphasizing, at a molecular level, the degree of control exerted by key enzymes involved in determining the redox environment specifically in the mitochondrial matrix and the cytoplasm.

Limitations—A potential limitation of this study is that manipulation of antioxidant gene levels may induce adaptive
changes in the transcriptome/proteome. ROS-induced hore- 

misis (54) could occur, whereby changes in other antioxidant 
genes could be evoked by acute rox stress after single gene 
knockdown. For example, knockout of TXNRD1 in hepatocytes 
leads to activation of Nrf2-mediated antioxidant gene tran-
scription (55). Chronic adaptive remodeling is highly likely in 
cells or organisms with stable gene knockout and is a major 
complicating factor in the interpretation of results. We cannot 
rule out that adaptive processes may be present in our study. 
However, this should be minimized by the subacute nature of 
the transient transfection protocol we used. A hormetic 
response would likely depend on whether the gene knockdown 
was severe enough to chronically alter the rox state of the cell 
under baseline conditions. In our study, antioxidant gene 
knockdown typically did not have a large effect on baseline ROS 
or GSSG signals (see individual records in Figs. 2B and 5B and 
summary data in Fig. 3C), but it did affect the response to H2O2 
challenge. The redundant parallel nature of the antioxidant 
pathway may be responsible for resistance to alterations in the 
basal rox state by single gene expression modulation under 
non-stressed conditions, but additional proteomic analysis of 
possible adaptive changes in antioxidant pathways bears fur-
ther investigation in the future.

Another limitation is that, although the ratiometric probe 
signal reports local “levels” of the selective species, which are 
the product of the rate of production and removal of each, exact 
quantification is challenging. For example, the concentration 
range over which the sensor responds can shift based on con-
ditions. This is especially true for the Grx1-roGFP probe, where 
the total glutathione pool size impacts the sensitivity range for 
GSSG detection (16). As such, without knowing the exact glut-
athione concentration in a given cell or in a given compart-
ment, one cannot easily calculate GSSG concentration from the 
fluorescence ratio, although it is theoretically possible. Simi-
larly, precise H2O2 concentrations cannot be determined, but 
reporting changes in relative H2O2 levels is appropriate (16).

Conclusions and Implications for Antioxidant Therapy—

Despite a number of randomized trials of antioxidants (e.g. vita-
mins C and E, probucol, coenzyme Q10, allopurinol, and alli-
 cin) (56–60), the clinical effectiveness of antioxidant therapy in 
cardiovascular disease remains unproven. By analyzing the 
control and compartment specificity of ROS scavenging, this 
work sheds light on this important problem. The strong depen-
dence of antioxidant flux on metabolism for regeneration of the 
pyridine nucleotide pool sets strict limitations on how fast the 
main electron donors for detoxifying ROS can be recycled to 
their reduced forms. We have reported that this failure to main-
tain the NAD(P)H supply during increased work is a key con-
tributor to heart failure and sudden cardiac death, in part 
because of impaired mitochondrial Ca2+ signaling to the TCA 
cycle, leading to ROS overload (61). Hence, the key to maintain-
ning ROS balance lies in preventing metabolic (especially mito-
chondrial) dysfunction, which often becomes progressively 
impaired during the progression of disease. Without a mecha-
nism to reduce the oxidized forms of exogenous antioxidants, 
they will be rendered inactive, or possibly become pro-oxi-
dants, during long-term treatment, even if they can be targeted 
to the right compartment. For example, the coenzyme Q deriv-
atives MitoQ (62) or SkQ (63) accumulate in the mitochondria 
and can be “recharged” by the electron transport chain. How-
ever, recharging depends on NADH supply and pro-oxidant/
prodeath effects have been reported (63, 64). It is perhaps 
unlikely that enough exogenous antioxidants could be loaded 
and specifically localized near key ROS-sensitive targets to 
mimic the finely regulated parallel antioxidant pathways of the 
cell. Nevertheless, an intriguing recent finding is that the small 
peptide “antioxidant” SS-31 (65) (commercially developed as 
Bendavia (66)) may work by specifically interacting with cardi-
olipin to suppress oxidation (67) rather than functioning as an 
antioxidant, as originally assumed.

Our findings suggest that alternative approaches to modulate 
cellular responses to oxidative stress may be possible, which 
could include gene therapies to alter NADPH availability or to 
decrease the NADPH requirement of ROS scavenging (e.g. by 
expressing mitochondrial catalase). Additional work will be 
required to determine whether such strategies can be imple-
mented at the organ or organism level, especially in the context 
of existing metabolic or cardiovascular disease.

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ments, analyzed the data, interpreted the results, and wrote the manu-
script. A.S. contributed to the experiments. B.O. designed the 
experiments, interpreted the results, and edited the manuscript.

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