The in vivo gene expression signature of oxidative stress

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1Department of Biological Science, University of Tulsa, Tulsa, Oklahoma; 2Department of Cellular and Structural Biology, 3Barshop Institute for Longevity and Aging Studies, and 4Center for Epidemiology and Biostatistics, University of Texas Health Science Center at San Antonio; and 7Geriatric Research Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, Texas; 6Department of Pediatrics, University of California, San Francisco, California; and 7Departments of Pharmacology and Medicine, Vanderbilt University, Nashville, Tennessee

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Han E-S, Muller FL, Pérez VI, Qi W, Liang H, Xi L, Fu C, Doyle E, Hickey M, Cornell J, Epstein CJ, Roberts LJ, Van Remmen H, Richardson A. The in vivo gene expression signature of oxidative stress. Physiol Genomics 34: 112–126, 2008. First published April 29, 2008; doi:10.1152/physiolgenomics.00239.2007.—How higher organisms respond to elevated oxidative stress in vivo is poorly understood. Therefore, we measured oxidative stress parameters and gene expression alterations (Affymetrix arrays) in the liver caused by elevated reactive oxygen species induced in vivo by diquat or by genetic ablation of the major antioxidant enzymes CuZn-superoxide dismutase (Sod1) and glutathione peroxidase-1 (Gpx1). Diquat (50 mg/kg) treatment resulted in a significant increase in oxidative damage within 3–6 h in wild-type mice without any lethality. In contrast, treatment of Sod1−/− or Gpx1−/− mice with a similar concentration of diquat resulted in a significant increase in oxidative damage within an hour of treatment and was lethal, i.e., these mice are extremely sensitive to the oxidative stress generated by diquat. The expression response to elevated oxidative stress in vivo does not involve an upregulation of classic antioxidant genes, although long-term oxidative stress in Sod1−/− mice leads to a significant upregulation of thiol antioxidants (e.g., Mt1, Srxn1, Gclc, Tmnr1), which appears to be mediated by the redox-sensitive transcription factor Nrf2. The main finding of our study is that the common response to elevated oxidative stress with diquat treatment in wild-type, Gpx1−/−, and Sod1−/− mice and in untreated Sod1−/− mice is an upregulation of p53 target genes (p21, Gdf15, Plk3, Atf3, Trp53inp1, Ddit4, Gadd45a, Btg2, Ndrg1). A retrospective comparison with previous studies shows that induction of these p53 target genes is a conserved expression response to oxidative stress, in vivo and in vitro, in different species and different cells/organisms.

REACTIVE OXYGEN SPECIES (ROS) such as superoxide (O2−/HO2•), hydrogen peroxide (H2O2), and the hydroxyl radical (OH•) are deleterious by-products of aerobic metabolism (14, 61). ROS can be pathogenic (6, 62, 82) and, if left unchecked, are incompatible with life in higher organisms (20, 22, 49, 54, 63). Oxidative damage to cellular components by ROS can compromise the structure and function of a variety of macromolecules, e.g., DNA, lipids, and proteins (6, 77). This in turn can lead to impaired physiological function and has been suggested as a cause of a variety of diseases and aging (6, 32, 82).

An elaborate network, which is not completely understood, of nonenzymatic and enzymatic antioxidant defense mechanisms has evolved to cope with ROS. The best-characterized nonenzymatic ROS scavengers include the antioxidant vitamins E and C, as well as glutathione and thioredoxin. However, this picture is likely far from complete, e.g., the biliverdin/bilirubin system has recently been discovered to function as an antioxidant (4). The main enzymatic branches of the antioxidant network include the superoxide dismutases (SODs), the glutathione peroxidases (GPXs), the peroxiredoxins, and catalase (28, 88). The SODs catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (58). The latter is then converted to water by catalase, the glutathione peroxidases, or peroxiredoxin families [which of these is most relevant and under what circumstances is not yet fully understood (88)]. There are three mammalian SODs: cytosolic CuZn-SOD (Sod1), the most abundant (58); mitochondrial matrix Mn-SOD (Sod2) (85); and extracellular SOD (EC-SOD, Sod3) (57). There are at least four glutathione peroxidases (Gpx1 through 4) and six peroxiredoxins (Prdx1 through 6) in mammalian cells. The cytosolic/mitochondrial selenoprotein Gpx1 is traditionally thought to be the main scavenger of cellular H2O2 (28), but the importance of peroxiredoxins is increasingly recognized (50, 63, 83).

The inner workings and interrelationships within the antioxidant system in vivo are at present poorly understood. While it is clear that ROS can play a role in modulating cell signaling (reviewed in Ref. 24), how the antioxidant system and indeed the cell as a whole respond to elevated oxidative stress in vivo is not well understood. Studies in this area have largely been conducted with cells in culture (18, 19). With regard to oxidative stress, this presents a strongly confounding factor because culturing cells under atmospheric oxygen tension (21% vs. physiological oxygen tension of ~5%) is now well established to be a potent oxidative stress in itself (13, 38, 67). Indeed, even though Sod1−/− mice are viable, cells from Sod1−/− mice do not grow under standard culture conditions (11, 41). Another potential problem with cell culture is whether the type and degree of oxidative stress applied is physiologically relevant (for example, is the application of a bolus dose of 100 μM H2O2 representative of a possible in vivo situation?).

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In this study, we measured global changes in gene expression induced by oxidative stress in vivo in liver with Affymetrix arrays. We employed a dual strategy to induce oxidative stress (which we quantified by measuring oxidative damage to DNA and lipids), namely, the injection of mice with the redox cycler diquat (78) and/or the ablation of the major antioxidant enzymes Sod1 and Gpx1 (34, 41). Oxidative stress is thus induced via the unchecked production of ROS from endogenous physiological sources as well as the overproduction of ROS by the exogenously added diquat. We observed no increased upregulation of classic antioxidant enzymes in the livers of wild-type (WT), Gpx1−/−, or Sod1−/− mice treated with diquat, even though diquat induced a dramatic increase in oxidative stress in vivo in the livers of these mice. However, untreated Sod1−/− mice, which exhibited significantly increased oxidative stress compared with untreated WT and Gpx1−/− mice, showed a significant increase in thiol-based antioxidant defense genes.

We identified a panel of genes that showed a common pattern of gene expression in response to both endogenous and exogenous oxidative stress in vivo: an upregulation of p53 target, checkpoint genes. To our knowledge this is the first study to identify p53 target genes as a common response of cells/tissues to oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Animals**

C57BL/6J mice used in this study were obtained from the aging colonies of mice maintained by the San Antonio Nathan Shock Aging Center (originally purchased from The Jackson Laboratory, Bar Harbor, ME), Sod1−/− and Gpx1−/− mice were generated in the laboratories of C. J. Epstein and Y. S. Ho (Wayne State Univ., Detroit, MI), respectively (34, 41). These mice were maintained in the heterozygous state on a C57BL/6J background (backcrossed for >10 generations) under specific pathogen-free conditions. All the mice were male, 3–6 mo of age, group housed at four animals per cage, and fed Harlan Teklad LM-485 mouse/rat sterilizable diet 7912 (Madison, WI) ad libitum. Mice were maintained on a 12:12-h light-dark cycle (lights on at 6:00 AM). Diquat was delivered intraperitoneally at 50 mg/kg body mass, a dose chosen because it is not lethal to WT mice. All animals were killed between 9:00 and 11:00 AM to minimize potential variation due to circadian rhythms. The rodents were humanely euthanized at 0, 1, 3, 6, and 12 h after diquat treatment, and tissues were collected immediately, frozen in liquid nitrogen, stored at −80°C, and analyzed within 30 days. All procedures for handling the mice were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the Subcommittee for Animal Studies at the Audie L. Murphy Memorial Veterans Hospital.

**Oxidative Damage and Liver Necrosis**

**Plasma alanine aminotransferase activity.** Plasma alanine aminotransferase (ALT) activity was measured with a commercially available kit (transaminases ALT/glutamic-pyruvic transaminase (GPT) and aspartate aminotransferase (AST)/glutamic-oxalacetic transaminase (GOT), Sigma) according to the manufacturer’s instructions. ALT activity was calculated from the calibration curve and expressed as Sigma-Frankel (SF) units per milliliter (69).

**Lipid peroxidation.** Lipid peroxidation was measured in mouse plasma and liver by the levels of F2-isoprostanes. Mouse blood was collected under deep anesthesia, and plasma was isolated by centrifugation. Because of the small volume, it was necessary to pool sera from three mice. Mouse livers (~200 mg) were homogenized, and whole lipid was extracted with chloroform-heptane. The levels of F2-isoprostanes from blood (free) and liver (esterified) were determined with gas chromatography-mass spectrometry (GC-MS) as initially described by Morrow et al. (59) and currently used in this laboratory (84).

**DNA oxidative damage.** Oxidative damage to nuclear DNA was determined by measuring the levels of 8-oxo-2-deoxyguanosine (8-oxo-dG). Mouse livers were homogenized in Dounce homogenizers in ice-cold lysis solution provided with a DNA Extractor WB kit (Wako Chemicals, Richmond, VA), and nDNA was isolated according to the instructions of the DNA Extractor WB and hydrolyzed as described by Hamilton et al. (29). The 8-oxo-dG and 2-deoxyguanosine in the hydrolysates were resolved by high-pressure liquid chromatography and quantified by electrochemical detection. The data were expressed as the ratio of nanomoles of 8-oxo-dG to 10^6 mmol of 2-deoxyguanosine.

**Statistical analysis of oxidative damage data.** Both the t-test and ANOVA Tukey-Cramer methods were used to analyze the statistical significance of the results. Unless indicated, the P value represents the statistical result from the Tukey-Cramer method. P < 0.05 is considered as statistically significant.

**Gene Expression**

**RNA isolation.** Total RNA was extracted from liver tissues of control and diquat-treated WT, Sod1−/−, and Gpx1−/− mice (RNA was obtained for 7 mice for each group) as previously described (72). The RNA yield of each sample was determined spectrophotometrically, assuming that 1 optical density at 260 nm (OD260) unit = 40 mg/l. The quality of total RNA extracted from each sample was monitored by absorbance (A)260-to-A280 ratio and 1.0% agarose formaldehyde gel electrophoresis. All samples had A260-to-A280 ratios of ~2 and exhibited discrete 28S and 18S rRNA bands. Several samples were randomly chosen and subjected to Northern blot analysis for further mRNA quality control with glyceraldehyde-3-phosphate dehydrogenase as a probe to ensure the quality of the RNA samples.

**Measurements of mRNA transcripts by Affymetrix GeneChip arrays.** Mouse Expression Array 430A (MOE430A) GeneChips were purchased from Affymetrix (Santa Clara, CA). The MOE430A GeneChip contains ~22,000 genes, ~14,500 of which are well-annotated genes with known full-length sequences and the remainder being unknown genes. For the 9 treatment groups, 63 GeneChip arrays were hybridized (1 GeneChip/mouse, 7 mice/treatment group). Before the labeling reaction, RNA samples were subjected to a cleanup process using columns from the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). We followed the vendor’s protocols for GeneChip hybridization and scanning (31).

**Statistical analysis of microarray data.** Affymetrix GeneChip Operating Software (GCOS version 1.1.1) was used to quantify each GeneChip. The summary intensities for each probe (as contained in the CEL files) were loaded into DNA-Chip Analyzer (dChip) (53) version 1.3 for normalization and standardization. To normalize the arrays, i.e., placing the arrays on a common measurement scale by adjusting for differing “brightness” among arrays that might arise because of the amount of starting RNA or labeling efficiency, we used a nonlinear approach that is the method of normalization implemented in the dChip software package. The same software was used to combine probe level data, comprising 11 pairs of 25-mer probes for each gene, into a single gene-specific summary estimate of expression. On visual inspection, no array had any obvious contamination or single outliers are solitary probes of unusual expression for further mRNA quality control with glyceraldehyde-3-phosphate dehydrogenase as a probe to ensure the quality of the RNA samples.

**Statistical analysis of oxidative damage data.** Both the t-test and ANOVA Tukey-Cramer methods were used to analyze the statistical significance of the results. Unless indicated, the P value represents the statistical result from the Tukey-Cramer method. P < 0.05 is considered as statistically significant.
ranged from 0.02% to 0.36%. Single outliers were treated as missing values in subsequent analyses. The percentage of genes called “present” by the GCOS software ranged from 53.42% to 64.69%.

To determine which genes showed a statistically significant change in expression between the comparison groups, we ran unpaired t-tests, a commonly used method to evaluate the differences in means between two groups. The t-test comparison assumes that the data are approximately normally distributed and the variances of the separate groups are approximately equal. For this reason, we standardized and log transformed the data before analysis. To correct for multiple testing, we calculated the Hochberg and Benjamini (35) false discovery rate (FDR) and set the FDR-adjusted P value (α) for the unpaired t-test results at <0.005. The results were further restricted by deleting those probe sets with “absent” GCOS detection calls across all chips in both comparison groups. Considering the GenBank accession numbers to represent unique genes, we deleted repeated accession numbers except in cases when the probe set name designation indicated that the probe sets recognized alternative transcripts from the same gene. Otherwise, we discarded the repeated accession number results for those probe sets that were not unique to a single gene (see Appendix B in Affymetrix’s Data Analysis Fundamentals Manual).

We used Expression Analysis Systematic Explorer (EASE) to statistically test for significant overrepresentation of the Gene Ontology (GO) Consortium category Biological Process in our results. Instead of ranking functional clusters by the number of selected genes per category, this software ranked functional clusters by statistical overrepresentation of individual genes in specific categories relative to all genes in the same category. The EASE score is a modification of Fisher’s exact test that attenuates the significance of categories overrepresentation of individual genes in specific categories relative to all genes in the same category. The EASE score is a modification of Fisher’s exact test that attenuates the significance of categories overrepresentation of individual genes in specific categories relative to all genes in the same category. The EASE score is a modification of Fisher’s exact test that attenuates the significance of categories overrepresentation of individual genes in specific categories relative to all genes in the same category.

Real-time quantitative reverse transcription-polymerase chain reaction assay. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was used to independently verify the changes in mRNA levels identified by Affymetrix arrays. Primers were designed with the OligoPerfect Designer (Invitrogen, Carlsbad, CA) and purchased from Invitrogen. RNA used for the GeneChip array experiments were used for the real-time QRT-PCR. Primers were designed with the OligoPerfect Designer (Invitrogen, Carlsbad, CA) and purchased from Invitrogen. The data in Supplemental Table S3 give the primer sequences used and their annealing temperatures.1 18S rRNA was used as an internal control for PCR quantification. PCR reactions were carried out as previously described (25). Relative quantification of gene expression was performed as described previously (16, 25). Briefly, logarithmic transformations of raw fluorescence data from the log-linear portion of real-time PCR growth curves for both target and reference genes (18S rRNA in our experiment) were analyzed with a SAS/STAT Mixed Procedure program, which is specifically designed to give a point estimate of the relative expression ratio of the target gene with the associated 95% confidence interval.

Tissue fractionations. Liver tissues were homogenized in 50 mM Tris pH 7.4 supplemented with protease inhibitor cocktail (Calbiochem, La Jolla, CA). The homogenates were centrifuged at 600 g for 10 min at 4°C; the pellet was used for nuclei isolation, and the supernatant was then centrifuged at 10,000 g for 10 min at 4°C to obtain the mitochondrial pellet. The supernatant was further centrifuged at 100,000 g for 60 min at 4°C, yielding the cytosolic fraction.

Nuclei isolation. Nuclei were obtained by ultracentrifugation of the crude pellet obtained after the first slow centrifugation (600 g for 10 min) through 2.2 M sucrose containing 1 mM MgCl2. The nuclei were further purified by two washings at 10,000 g for 10 min with a buffer containing 0.32 M sucrose, 1 mM MgCl2, 2 mM CaCl2, 10 mM Tris buffer pH 7.4, and 0.5% Triton X-100. The pellets were resuspended in 10 mM Tris buffer pH 8.0 containing 0.14 M NaCl and 1 mM MgCl2 and centrifuged at 10,000 g for 10 min. The final pellet was homogenized in 0.1 M Tris buffer pH 7.5 containing 2 mM MgCl2 and 2 mM CaCl2 supplemented with protease inhibitor mixture and sonicated (2 × 10 s). The samples were centrifuged at 10,000 g for 15 min, and the supernatant was used for Western blot analysis (8, 15).

Mitochondrial extract. The mitochondrial pellet was washed twice with 50 mM Tris buffer pH 7.4 and resuspended in 50 mM Tris buffer pH 7.4 containing 0.5% Triton X-100 and protease inhibitor cocktail. The samples were incubated for 45 min at 4°C and centrifuged at 100,000 g for 15 min, the pellet was discarded, and the supernatants (mitochondrial extracts) were used for Western blot analysis. The protein concentration was determined with the Bradford protein assay reagent (Bio-Rad, Richmond, CA).

Western Blot Analysis

Samples were lysed in Laemmli buffer containing 100 mM β-mercaptoethanol and 0.4% SDS for 10 min at 95°C. The amount of sample loaded varied for each antibody and is indicated in RESULTS. Samples were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The antibodies used were rabbit polyclonal antibody anti-Nrf2 (sc-722), goat polyclonal antibody anti-peroxiredoxin 1 (sc-73831), and rabbit polyclonal antibody anti-sulfiredoxin (sc-51211) from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-mouse anti-p53 antibody (no. 2524) and polyclonal anti-rabbit phospho-p53 (no. 9284) were from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-heme oxygenase-1 (clone no. HO-1-1) and mouse monoclonal anti-metallothionein (clone no. UC1MT) antibodies were from StressGene Bioreagents (Ann Arbor, MI). Rabbit polyclonal anti-Txn-2 (LF-PA002) and mouse monoclonal anti-Txn-2 (MA-0079) antibodies were from Lab-Frontier (Seoul, Korea). Mouse monoclonal anti-CD36 (MAB1258) antibody was from Chemicon International (Temecula, CA), and rabbit polyclonal antibody anti-Gpx4 was generated as described elsewhere (91) with a 17-amino acid peptide corresponding to the COOH terminal of Gpx4 protein as antigen. β-Actin, ATPase β-subunit, and histone H1 were used as the loading controls for cytosolic, mitochondrial, and nuclear fractions, respectively.

Thioredoxin Reductase Activity

Thioredoxin reductase activity was measured in the cytosolic fraction with the 5,5′-dithiobis(2-nitrobenzoic acid)-reduction aurothioglucoside inhibition method (27). Briefly, reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) by NADPH was followed at absorbance of 412 nm. A second assay was performed as described above, with the addition of 20 μM aurothioglucose, a specific inhibitor of thioredoxin reductase. Thioredoxin reductase activity was calculated as the differences between the activities measured in the absence and presence of aurothioglucoside and expressed as relative units per milligram of protein extract. The assay was performed on the same cytosolic fractions used for Western blot.

RESULTS

Diquat Treatment Causes Oxidative Stress and Hepatotoxicity, Which Is Enhanced in Mice Lacking Sod1 or Gpx1

Before screening the genome for changes in gene expression after an oxidative stress, we first measured the levels of oxidative stress in vivo in liver tissue from Gpx1−/− and Sod1−/− mice before and after injection of diquat, which generates superoxide anions in the liver in vivo (45, 68, 78). Hepatotoxicity induced by diquat can be followed by the extent of liver necrosis, which can be quantified by measuring the levels of ALT in plasma (69). As can be seen in Fig. 1A, intraperitoneal injection of 50 mg/kg diquat induced significant
liver injury 3–6 h after diquat treatment in WT mice; there was no significant increase in ALT 1 h after diquat treatment. Because diquat undergoes redox cycling (78) leading to the generation of superoxide (which will dismutate to H$_2$O$_2$), it stands to reason that animals with a deficiency in an antioxidant enzyme would suffer more severe toxicity when exposed to diquat. While all WT control mice survived 50 mg/kg diquat (data not shown), the two knockout models were unable to survive more than a few hours after this dose of diquat. 

$\text{Sod1}^{-/-}$ mice died 1–3 h (mean ± SE: 1.45 ± 0.26 h; n = 4) after diquat administration, and $\text{Gpx1}^{-/-}$ mice died after 4–6 h (mean ± SE: 3.84 ± 0.30 h; n = 6). The data in Fig. 1B show that $\text{Gpx1}^{-/-}$ and $\text{Sod1}^{-/-}$ mice demonstrated increased hepatotoxicity to diquat in 1 h after treatment as measured by ALT levels. When $\text{Gpx1}^{-/-}$ mice were treated with diquat, they showed a 120% increase in plasma ALT activity 1 h after diquat injection that was statistically significant compared with the diquat-treated WT and untreated $\text{Gpx1}^{-/-}$ mice. $\text{Sod1}^{-/-}$ mice showed an even greater increase (200%) in ALT activity, which was significantly greater than either the diquat-treated WT or untreated $\text{Sod1}^{-/-}$ mice. There were no differences in ALT levels between untreated knockout and WT mice.

Diquat is a superoxide generator, and it is recognized that the hepatotoxicity resulting from diquat treatment arises from oxidative stress/damage (33, 70, 76). We quantified the level of oxidative stress after diquat administration by measuring oxidative damage to lipids and DNA. Lipid peroxidation was assessed by measuring the levels of F$_2$-isoprostanes, which are stable, prostaglandin-like products formed in vivo by free radical-catalyzed nonenzymatic, cyclooxygenase-independent peroxidation of arachidonic acid (60). These are formed during lipid peroxidation reactions in lipid membrane bilayers and are subsequently cleaved and eliminated via plasma (59). As shown in Fig. 2, A and B, both plasma free F$_2$-isoprostanes (that is, those that have been cleaved from lipids and are in the free fatty acid form in the process of being eliminated) and liver total lipid esterified F$_2$-isoprostanes (that is, F$_2$-isoprostanes still in the complex lipids in membranes) were significantly increased 3–6 h after diquat treatment in WT mice. Plasma free F$_2$-isoprostanes, but not esterified liver F$_2$-isoprostanes, significantly increased as early as 1 h after diquat injection in WT mice. Compared with the WT mice, $\text{Gpx1}^{-/-}$ mice had significantly higher levels of plasma free F$_2$-isoprostanes and liver total F$_2$-isoprostanes 1 h after diquat treatment. Again, diquat-treated $\text{Sod1}^{-/-}$ mice showed even higher plasma free and liver esterified F$_2$-isoprostane levels compared with diquat-treated $\text{Gpx1}^{-/-}$ mice and WT mice (Fig. 2, C and D). Importantly, untreated $\text{Sod1}^{-/-}$ mice had significantly higher basal plasma free F$_2$-isoprostane levels than $\text{Gpx1}^{-/-}$ mice, which had the same basal F$_2$-isoprostane levels as WT mice (Fig. 2B). It is worth noting that plasma F$_2$-isoprostanes exhibited a much greater increase after diquat than liver esterified F$_2$-isoprostanes. This effect has been described previously (3), and a similar phenomenon is seen in the untreated $\text{Sod1}^{-/-}$ mice (Fig. 2).

Oxidative stress was also measured in liver by the presence of 8-oxo-dG in nDNA, which is one of the most widely used markers for DNA oxidative damage (30). In WT mice, 8-oxo-dG levels in liver increased after diquat treatment and reached statistical significance at 3–6 h. Mice lacking Gpx1 had significantly higher 8-oxo-dG levels than WT mice 1 h after diquat treatment, while $\text{Sod1}^{-/-}$ mice showed even higher 8-oxo-dG levels than $\text{Gpx1}^{-/-}$ mice 1 h after diquat treatment. Like the basal plasma free F$_2$-isoprostane level, the basal level of 8-oxo-dG in $\text{Sod1}^{-/-}$ but not $\text{Gpx1}^{-/-}$ mice was again higher than that of WT (Fig. 3B).

Diquat Treatment Causes Dramatic Changes in Global Gene Expression

Because diquat induces oxidative stress, which peaked at 3–6 h after injection, gene expression was measured in the liver tissues of male C57BL/6 WT mice at 0 (control, no diquat treatment), 1, 3, 6, and 12 h after diquat injection. Comparisons of the control (untreated) WT mice with each of the four groups treated with diquat (1 h diquat, 3 h diquat, 6 h diquat,
and 12 h diquat) were carried out to identify genes whose expression are altered by diquat. The number of genes whose expression changed significantly (P < 0.005) by diquat treatment at the various time points (as compared with the untreated control) are presented in Supplemental Table S5 (see Supplemental Table S1 for gene names, GenBank accession numbers, fold changes, and P values). Supplemental Table S5 shows that the expression of 245, 1,237, 1,642, and 4,129 genes were altered at 1, 3, 6, and 12 h after diquat, respectively. EASE analysis of gene ontology Biological Process is presented in Supplemental Table S2. Also indicated in Supplemental Table S5 (row 5) are the number of genes altered at any given time point of diquat treatment (4,551 unique genes), as well as those genes found to show significantly different levels of expression at all time periods after diquat injection compared with untreated control samples (153 genes; 60 increased, 93 decreased). To judge the relative magnitude of the gene expression changes, these are further subdivided into the number of genes whose expression was significantly changed more than 1.5-, 2-, and 2.5-fold. The number and magnitude of gene expression alterations increased throughout the time course of diquat treatment as shown in Table 1.

Global Changes in Gene Expression Are More Extensive in Sod1<sup>−/−</sup> Than in Gpx1<sup>−/−</sup> Mice

We next investigated the effect of ablating the two major antioxidant enzymes Sod1 and Gpx1 on gene expression in the liver. In these experiments, Sod1<sup>−/−</sup> and Gpx1<sup>−/−</sup> mice were either untreated or treated with diquat for 1 h, and gene expression was compared with WT mice either untreated or treated with diquat for an hour. The data in Supplemental Table S6 show that 1,404 (638 up- and 766 downregulated) genes were changed in the untreated Sod1<sup>−/−</sup> mice (row 3) and 648 (245 increased, 403 decreased) genes were changed in the untreated Gpx1<sup>−/−</sup> mice (row 1) with respect to untreated WT mice. As Supplemental Table S6 shows, when considering the larger fold changes (>2-fold and >2.5-fold), the number of genes altered in the untreated Sod1<sup>−/−</sup> is greater than that in the untreated Gpx1<sup>−/−</sup> mice (43 vs. 14 genes altered >2.5-fold). Thus the gene expression changes in the untreated Sod1<sup>−/−</sup> mice are greater in both number and magnitude compared with the changes in the untreated Gpx1<sup>−/−</sup> mice. This is to be expected, considering that Sod1<sup>−/−</sup> mice show a significant increase in oxidative damage under basal conditions compared with Gpx1<sup>−/−</sup> mice (Figs. 2B and 3B).

Similarity in Gene Expression Between WT Mice Treated With Diquat and Untreated Sod1<sup>−/−</sup> Mice Points to a Similar Response to Exogenous and Endogenous Oxidative Stress

Because diquat and an absence of antioxidant enzymes are both expected to induce overproduction of ROS, we compared the gene expression pattern induced by diquat in WT mice with that induced by the ablation of either Sod1 or Gpx1. We asked whether changes in gene expression in either the Sod1<sup>−/−</sup> or Gpx1<sup>−/−</sup> mice were similar to the changes in gene expression caused by diquat treatment in WT mice at 3–6 h after diquat injection, which was the time interval when oxidative damage was maximal (Figs. 2 and 3). Table 1 shows the number of genes that were significantly altered (in the same direction) in the untreated Sod1<sup>−/−</sup> or Gpx1<sup>−/−</sup> mice and in WT mice treated with diquat at 3 or 6 h. As is shown in Table 1, the
changes in gene expression in WT mice treated with diquat are much more similar to the expression changes in untreated Sod1\(^{-/-}\) mice than Gpx1\(^{-/-}\) mice, which is especially true for highly upregulated genes. For example, of the 321 genes upregulated >1.5-fold in WT mice treated with diquat, 26 were also upregulated >1.5-fold in the Sod1\(^{-/-}\) mice. In contrast, only 2 of 321 genes upregulated >1.5-fold in WT mice by diquat were upregulated in the untreated Gpx1\(^{-/-}\) mice. This comparison (from Table 1) is presented graphically in Fig. 4. The fold change of genes significantly altered by diquat at the either the 3 or 6 h time points (on the x-axis) was plotted against the fold change of the same genes also statistically significantly altered in the untreated Gpx1\(^{-/-}\) or Sod1\(^{-/-}\) mice (on the y-axis). A least-squares regression line was then drawn through the data, with the slopes and \(R^2\) values given in Fig. 4. A slope and \(R^2\) equal to 1 would be obtained if the gene expression changes in the two data sets were identical, while a value of 0 for these parameters indicates no relationship between the data. As can be seen in Fig. 4, B and D, the comparison of WT mice treated with diquat and Gpx1\(^{-/-}\) mice yield a slope of the regression line and the \(R^2\) value very close to 0, as one would expect from randomly distributed data, indicating that the gene expression patterns between Gpx1\(^{-/-}\) mice and WT mice treated with diquat were dissimilar. On the other hand (in Fig. 4, A and C), the comparison of Sod1\(^{-/-}\) and WT mice treated with diquat, yielded a slope of 0.55 with an \(R^2\) of 0.54 (\(P < 0.001\)) and a slope of 0.48 and an \(R^2\) of 0.41 (\(P < 0.001\)) at the 3 h and 6 h time points, respectively. There was little or no similarity in expression response between diquat-treated and untreated Gpx1\(^{-/-}\) mice, but a good degree of similarity between that caused by diquat treatment and the untreated Sod1\(^{-/-}\) mice, which is in agreement with the fact that the Sod1\(^{-/-}\) mice are oxidatively stressed whereas the Gpx1\(^{-/-}\) mice are not (Figs. 2 and 3).

**Identification of Oxidative Stress-Responsive Transcripts**

The major goal of this work was to find which genes are altered in response to oxidative stress in vivo. We screen our data for genes whose expression is responsive to oxidative stress by the following criteria: 1) they are altered by diquat in WT animals at the 3- or 6-h time interval (because oxidative damage peaks at these time points) and 2) they are altered in the untreated Sod1\(^{-/-}\) mice, which already exhibit enhanced oxidative damage, though not in the Gpx1\(^{-/-}\) mice, which do not exhibit increased oxidative damage (Figs. 2 and 3). In other words, we asked what genes would be altered by exogenous as well as endogenous oxidative stress. As shown in Table 1, a total of 121 transcripts met these criteria, with 37 transcripts being altered >1.5-fold (26 up, 11 down). The identities of these 37 transcripts are listed in Table 2 (the discrepancy in the numbers between Table 1 and Supplemental Table 6 is because...
duplicate genes were deleted in Supplemental Table 6). Alternatively, genes responsive to oxidative stress were selected in that they are altered by diquat in WT animals at the 3- or 6-h interval and are further altered significantly in the untreated antioxidant knockout mice (5). To verify that they are altered by diquat in WT animals at the 3- or 6-h time points and in untreated antioxidant knockout mice was determined, and the data are presented graphically. The analysis is restricted to genes altered >1.5-fold in both groups. Fold change in expression in the untreated antioxidant knockout mice (Sod1−/−, A and C; Gpx1−/−, B and D) is on the y-axes, and that of WT mice treated with diquat is on the x-axes. Data for the 3 h time point are in A and B, and data for the 6 h time point are in C and D. Each symbol represents 1 specific gene whose x, y coordinates are given by its fold-level expression in antioxidant knockout mice (y) and WT mice treated with diquat (x), in both cases compared with untreated WT control mice. A least-squares regression line was calculated for each data set, with the slope and R² values indicated in each graph. There is a statistically significant correlation (P < 0.001) for the diquat vs. Sod1−/− comparison but not for the diquat vs. Gpx1−/− comparison (especially note the large number of data points at lower right quadrant of B and D, i.e., genes altered in opposite directions in both groups).

Validation of Microarray Results

The changes in gene expression measured by microarrays were confirmed in two ways. First, we compared our data to previous studies in which mRNA transcripts were measured by Northern blots. For example, we observed a dramatic (4- to 8-fold) increase in metallothionein 1 mRNA in the liver after diquat treatment or in Sod1−/− mice (Table 2). Bauman et al. (5) reported a >10-fold increase in metallothionein 1 mRNA levels in the livers of mice after diquat treatment, and Ghoshal et al. (26) and Levy et al. (52) reported a >10-fold increase in metallothionein 1 mRNA levels in the liver of Sod1−/− mice. Because we performed experiments on Sod1 and Gpx1 knockout mice, it follows that the transcripts for Sod1 and Gpx1 should be very low or at the minimum, and that the region of the mRNA that corresponds to the exon that was targeted in the knockout should be either absent or highly downregulated compared with the WT control animals. In the Sod1 knockout mice used in this study, exon 4 is deleted and exon 3 is truncated (41), but a mature mRNA is still expressed. Indeed, Affymetrix ID 1451124_at (which covers exons 3 and 4) was scored as “absent” in the 14 arrays in the comparison (especially note the large number of data points at lower right quadrant of B and D, i.e., genes altered in opposite directions in both groups).
ever, very low levels of a neomycin-gpx1 fusion mRNA are detectable after very long exposure (34). In full agreement with these known data, the probe set (Affymetrix ID 1460671_at) covering the Gpx1 gene was decreased approximately fivefold in the 14 arrays on the Gpx1−/− mice (34), and is in fact the most statistically significant alteration (in other words, with the lowest P value) in the Gpx1−/− mice regardless of the comparison.

Second, we used real-time QRT-PCR to validate the expression of five genes: Apoa4, Igfbp1, Pdk4, and the p53 target genes Cdkn1a and Gadd45a. The expression of these genes were measured at 1, 3, 6, and 12 h after diquat treatment and in the untreated and diquat-treated Sod1−/− and Gpx1−/− mice, i.e., we performed 39 comparisons (12 h diquat vs untreated WT comparison for Gadd45a was not available), and these data are shown in Supplemental Table S4. In all samples, the transcripts for Apoa4, Igfbp1, Pdk4, Cdkn1a, and Gadd45a readily amplified by QRT-PCR at the correct melting point. The array ratios of genes are validated when 95% confidence intervals for the QRT-PCR ratios overlap with the corresponding 95% confidence intervals for the array ratios. All values obtained by QRT-PCR overlapped with the microarray data at the 95% confidence interval, validating the microarray data we have obtained for these five genes at all time points and mouse models.2

Confirmation of Selected Transcripts at the Protein Level

We performed Western blot analyses to confirm selected mRNA upregulation at the protein level. In these experiments, we compared the protein levels in liver tissue from Sod1−/− vs. WT mice (Figs. 5 and 6). From the genes in Table 4, we selected two highly upregulated transcripts (Srxn1, Mti1) and three modestly upregulated transcripts (Txn2, Gpx4, Prdx1) for Western blot confirmation. The two highly upregulated transcripts, Mti1 and Srxn1, were also significantly upregulated at the protein level in the Sod1−/− mice (Fig. 5). The Srxn1 transcript was increased 2.3-fold, and sulfiredoxin protein was increased >6-fold. Mti1 transcript was increased 4.5-fold and Mti1 transcript was increased 3.6-fold (Supplemental Table S1), but the Mti2 data did not reach the statistical cutoff (P < 0.0083 vs. P < 0.0005); the protein levels of metallothionein 1 + 2 are increased 4.5-fold (Mti1 and Mti2 have the same molecular

Table 2. Genes altered by both exogenous and endogenous oxidative stress

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>1-h DQ</th>
<th>3-h DQ</th>
<th>6-h DQ</th>
<th>12-h DQ</th>
<th>Gpx1−/−</th>
<th>Sod1−/−</th>
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</tr>
<tr>
<td>Lect1</td>
<td>Leukocyte cell derived chemotaxin 1</td>
<td>-1.30</td>
<td>-1.52</td>
<td>-2.14</td>
<td>-2.64</td>
<td>-2.57</td>
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<tr>
<td>Hsd3b5</td>
<td>Hydroxysteroid dehydrogenase-5, δ &lt;5&gt;3-β</td>
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<td>-9.84</td>
<td>-5.82</td>
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</table>

Genes showing a significant difference of >1.5-fold when we compared untreated WT mice with either WT mice treated with diquat (DQ) or untreated Gpx1−/− and Sod1−/− mice are shown. Duplicates (by gene name or unigene ID) were removed. a Gene is a p53 target based on Refs. 44 and 46; b gene is altered in the same direction by parquat in heart as previously described by Edwards et al. (21); c genes are altered in the same direction in human MCF7 and MRC9 cells in response to 100 μM H2O2 as described by Desaint et al. (19).

2 For an extensive comparison of microarray vs. “true” fold changes see Tables 2 and 3 in Ref. 1.
was increased by 90% in the cytosolic fraction of Hmox1. In addition, we confirmed the increase in the Txnrd1 statistical cutoff from the arrays (Hmox1). The nuclear levels of p53 were significantly elevated (30%) in mice compared with WT mice (Fig. 6). To confirm that the increase in p53 levels in the nucleus of liver from Sod1−/− mice was due to higher activation of p53, we also measured the phosphorylated p53 levels in the nucleus by Western blots. Because the upregulation of several p53 target genes suggested that alterations in p53 might be affected by oxidative stress, we measured the levels of p53 in the nucleus of liver from Sod1−/− mice compared with WT mice (Fig. 6A). To confirm that the increase in p53 levels in the nucleus of Sod1−/− mice was due to higher activation of p53, we also measured the levels of phosphorylated p53 in total homogenate and nuclear fractions obtained from the livers of Sod1−/− and WT mice. The nuclear levels of phosphorylated p53 were significantly higher (40%) compared with WT mice; however, we did not observe this effect when we examined p53 levels in the liver homogenates (Fig. 6B). These data suggest that increased levels in p53 found in the nucleus of liver from Sod1−/− mice were due to an increase in the posttranslational activation of p53 (phosphorylated) rather than an increase in the expression of p53.

### DISCUSSION

To determine which changes in gene expression are most likely caused by oxidative stress, we compared the expression

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Table 3. Genes hyperinducible or hyperrepressible by diquat in antioxidant knockout mice

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>1-h DQ</th>
<th>3-h DQ</th>
<th>6-h DQ</th>
<th>12-h DQ</th>
<th>Gpx1−/− 1-h DQ</th>
<th>Sod1−/− 1-h DQ</th>
<th>Gpx1−/− WT 1-h DQ vs. Sod1−/− WT 1-h DQ</th>
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<tr>
<td>Atf3a,b,c</td>
<td>Activating transcription factor 3</td>
<td>2.10</td>
<td>3.40</td>
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<td>13.76</td>
<td>1.95</td>
<td>11.70</td>
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<td>Copeb</td>
<td>Kruppel-like factor 6 (Klf6)</td>
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<td>2.04</td>
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<td>2.58</td>
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<td>4.90</td>
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<td>Tiparp</td>
<td>TCDD-inducible poly(ADP-ribose) polymerase</td>
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<td>Apao4</td>
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Genes showing the greatest difference when we compared diquat-treated WT mice with diquat-treated knockout mice (either Sod1−/− or Gpx1−/−) are shown. Duplicates (by gene name or unigene ID) were removed. Fold expression data are expressed with reference to untreated WT control mice, except for columns 10 and 11, where data are expressed with respect to WT mice 1 h after diquat injection. aGene is a p53 target based on Refs. 44 and 46; bgene is altered in the same direction by parataut in heart as described by Edwards et al. (21); cgene is altered in the same direction in human MCF7 and MRC9 cells in response to 100 μM H2O2 as described by Desaint et al. (19).

---

3 Sulfiredoxin, also known as Npo3 or Neoplastic progression 3, is the most strongly inducible Nrf2-dependent gene (see Ref. 48).
Table 4. Genes with antioxidant function upregulated in Sod1−/− mice

<table>
<thead>
<tr>
<th>AffyMetrix Probeset ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Adjusted P Value</th>
</tr>
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<tbody>
<tr>
<td>1422557_s_at</td>
<td>Mt1^M^</td>
<td>Metallothionein 1</td>
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<tr>
<td>1427473_at</td>
<td>Gstm3^N^</td>
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<td>1451260_at</td>
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<tr>
<td>1425351_at</td>
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<td>Sulfiredoxin 1 homolog (S. cerevisiae)</td>
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<td>Selenoprotein W, muscle 1</td>
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Antioxidant or allied function genes significantly upregulated in Sod1−/− mice compared with untreated WT mice or Gpx1−/− mice are shown. *Genes previously shown to be induced in Sod1−/− mice (26, 51); ^known target of Nrf2 (48); "known target of Mtf1 (86).

patterns induced by diquat in WT mice with those of antioxidant knockout mice treated or untreated with diquat. In other words, we asked: Which genes would be altered by both exogenous and endogenous oxidative stress and which genes altered by diquat in WT animals would be enhanced by diquat in antioxidant knockout mice? We identified transcripts based on the following criteria: 1) they were altered by diquat in WT animals at 3 or 6 h after injection because oxidative damage peaks at these time points (Table 2); 2) they were altered in untreated Sod1−/− mice, which have constitutively elevated oxidative damage (Table 2); and 3) they were altered to an even greater extent in the knockout mice, i.e., Sod1−/− or

Fig. 5. Antioxidant proteins upregulated in Sod1−/− mice. Protein levels of selected antioxidant genes were measured by Western blots and enzymatic activity (thioredoxin reductase) in Sod1−/− (filled bars) and WT (open bars) mice. Heme oxygenase 1 (HO-1), metallothionein (Mt1) and 2 (the proteins are too similar to be distinguished by size or antigenicity), thioredoxin-1 (Txn1), peroxiredoxin 1 (Prx-1), glutathione peroxidase 4 (Gpx4), sulfiredoxin (Srxn1), and thioredoxin reductase activity (TxnR) were measured in the cytosolic fraction of the liver. Thioredoxin-2 (Txn2) was measured in the mitochondrial fraction of the liver. The results are means ± SE of 4 or 5 animals. *Significantly different from WT mice (P < 0.05). RU, relative units.
**GENE EXPRESSION AND OXIDATIVE STRESS**

We initially expected that a large fraction of the transcripts that respond to increased oxidative stress would have antioxidant functions; however, the list of genes in Tables 2 and 3 are dominated by stress-response genes. With the exception of metallothionein, none of these genes has any obvious antioxidant function. The pattern of gene expression changes we found to be induced consistently by oxidative stress in vivo was the upregulation of p53 target genes, many of which play a role in the genotoxic stress checkpoint response. In Table 2 (genes altered by both endogenous and exogenous oxidative stress), 6 of 24 upregulated genes are p53 targets (44, 46): Cdkn1a/p21 (43, 64), Atf3 (56, 90), Trp53inp1 (65, 81), Ddit4 (23, 75), and Ndrg1 (47, 79). While these genes were upregulated in untreated Sod1⁻/⁻ mice, none of them was upregulated in untreated Gpx1⁻/⁻ mice, which is a control because the Gpx1⁻/⁻ mice do not exhibit elevated oxidative damage. Two additional p53 transcriptional target genes, Btg2 (12, 71) and Plk3 (55, 89), are also in the list of genes in Table 3, while a third, Gadd45a (36, 93), was not significantly induced in WT mice treated with diquat but was significantly induced (>5-fold) in Sod1⁻/⁻ mice treated with diquat (see Supplemental Table S1), suggesting that this gene is responsive to oxidative stress but only at very high levels of stress. Btg2 and Plk3 barely fell under the statistical cutoff in the untreated Sod1⁻/⁻ mice; on the other hand, the induction of these genes was dramatically higher in the Sod1⁻/⁻ and Gpx1⁻/⁻ mice treated with diquat compared with WT mice treated with diquat (Table 3).

P53 is normally present at very low levels in nucleus (or in the cell in general) because it is rapidly tagged for nuclear export and degradation, predominantly by Mdm2 (37). Phosphorylation of p53 blocks the interaction with Mdm2, resulting in increased p53 half-life and increased levels in the nucleus, where it initiates transcriptional activation of its targets. Therefore, we measured the levels of p53 and phospho-p53 in the nuclear fraction of WT and Sod1⁻/⁻ mice to determine whether the increased oxidative stress was correlated to the activation of p53. We found that the levels of p53 and phospho-p53 protein were significantly higher (30–40%) in the nuclear fraction of the livers of Sod1⁻/⁻ mice.

We compared our microarray results with a previous study from Prolla’s laboratory (21) that reported gene expression changes induced in vivo in mouse heart by paraquat. Because paraquat and diquat are both bipyridil compounds that redox cycle and generate superoxide (28, 78), we aligned the arrays (updating the annotations in the process) to determine which genes were commonly altered in these two situations. Of the 228 genes significantly altered in heart by paraquat, 88 genes or 38% were also statistically altered by diquat in the liver (Supplemental Table S1). Tables 2 and 3 are annotated to indicate which gene alterations were also observed in paraquat-treated heart. Almost all the p53 target genes, which we identified in the present study, were also upregulated in heart after paraquat treatment (21). Of the nine p53 target genes just mentioned (Cdkn1a/p21, Gdf15, Atf3, Trp53inp1, Ddit4, Btg2, Gadd45a, Mdm2, and Ndr4), 8 were also significantly altered in heart by diquat compared with WT mice treated with diquat (Table 3).

**Fig. 6.** Increased nuclear levels of p53 and Nrf2 in Sod1⁻/⁻ mice. The nuclear fraction from the livers of Sod1⁻/⁻ (filled bars) and WT (open bars) mice were analyzed by Western blots as described in EXPERIMENTAL PROCEDURES and expressed as a arbitrary units relative to the loading control, histone H1. A: total protein levels of p53 and Nrf2 in nuclear extracts. B: phospho (P)-p53 levels in nuclear and total homogenate extracts. Data are means ± SE of 3 or 4 animals. *Significantly different from WT mice (P < 0.05).
Ndrg1, Btg2, Plk3, Gadd45a), six were upregulated by paraquat in the heart (reanalysis of Prolla’s array indicates that A1849939-unknown, induced >5-fold after paraquat injection, is Ddit4). Trp53inp1 and Plk3 were not present on Prolla’s arrays, while the probe sets for Gdf15 were of poor quality on the MG-U74A array. We also compared our data with the in vitro array data from Toledano’s laboratory (19) in which human cells (MCF7 breast cancerous and MRC9 lung fibroblasts) were oxidatively stressed with a bolus dose of H2O2. Despite the experiments being conducted in different cell types, different species, different gene chips (of different gene composition), and different oxidative stressors, the following gene alterations were conserved: p21, Gadd45a, Aqf3, Btg2, and Ddit4 are upregulated by a large magnitude in vitro in transformed MCF7 and normal MRC9 cells after H2O2 treatment (data from Table I in Ref. 19), in vivo in heart after paraquat treatment (21), and in our present data set [regarding these comparisons, several of these genes appear under different synonyms in different papers, for example, Btg2 is also known as TIS21 or PC3 (71) and Gdf15 is also known as PLAB]. Thus the data indicate that the upregulation of p53 targets is a conserved response to oxidative stress across diverse organs and species.

The question arises as to whether the upregulation of p53 target genes is due to oxidative stress or arises as a consequence of massive cell damage triggering apoptosis. The microarray data do not support that the upregulation is involved in apoptosis because the classic p53 targets that are related to apoptosis, e.g., BAD, PUMA, NOXA (reviewed in Ref. 39), were not significantly increased by diquat treatment in WT mice, in the untreated Sod1−/− mice, or even in the diquat-treated Sod1−/− mice (Supplemental Table S1). The p53 target genes that we observed to be oxidative stress responsive (e.g., p21, GADD45a, GDF15, Btg2) are predominantly involved in cell cycle arrest rather than apoptosis (reviewed in Ref. 92).

While short-term oxidative stress induced by diquat treatment of WT, Sod1−/−, and Gpx1−/− mice did not significantly induce the expression of the classic antioxidant enzymes (e.g., the SODs, peroxiredoxins, glutaredoxins, catalases, glutathione reductases, or other GPXs) in the livers of the mice, even though diquat treatment resulted in a dramatic increase in oxidative damage, long-term oxidative stress in the Sod1−/− mice did result in the significant upregulation of less well-known antioxidant genes in untreated Sod1−/− mice compared with WT and Gpx1−/− mice (Table 4). As shown in Table 4, the most upregulated antioxidant gene was the small cysteine-rich protein metallothionein 1 (~4.5-fold increase). Metallothionein 2 was also upregulated (~3.5-fold increase) but fell just below the statistical cutoff (P < 0.0083 vs. P < 0.005). Using Western blots, we observed an approximately fivefold increase in the levels of metallothionein 1 and 2. We also observed an approximately twofold increase in glutamate-cysteine ligase (Gclc) mRNA in the livers of Sod1−/− mice. Glutamate-cysteine ligase plays a critical role in the glutathione antioxidant system as the rate-limiting enzyme in glutathione biosynthesis (17, 74). The upregulation of Gclc is consistent with the observation by Marklund’s group (73) that levels of reduced glutathione (GSH) were increased in the livers of Sod1−/− mice. We also found several glutathione-dependent enzymes to be upregulated (between 1.2-fold and 3-fold) in Sod1−/− mice, e.g., Gpx4 and several glutathione S-transferases, which exhibit GPX activity against fatty acid, lipid, and organic hydroperoxides but not H2O2 (91). Lei et al. (51) recently reported an increase in overall glutathione S-transferase enzymatic activity in the livers of Sod1−/− mice, which is in agreement with our array data. This same study also reported an ~50% increase in thioredoxin 1 reductase activity (51). We found that the Trmxrl transcript was significantly upregulated in our arrays, and we also found that the activity of thioredoxin reductase was also significantly increased (~90%) in the livers of the Sod1−/− mice. The transcript of selenoprotein W was also found to be significantly increased 45% in the liver of Sod1−/− mice. Selenoprotein W is a small glutathione interacting protein (7) whose exact function is unknown; however, it appears to have antioxidant properties and is also upregulated in the liver during cadmium oxidative stress (86). When ectopically expressed in cell culture, selenoprotein W provides protection against hydrogen peroxide (42). Perhaps most interesting of all changes in gene expression in the livers of Sod1−/− mice was the more than twofold upregulation of the sulfiredoxin (Srxn1) transcript and the sixfold increase in sulfiredoxin protein. Sulfiredoxin was identified in yeast as an enzyme that is capable of reversing what was previously thought to be irreversibly oxidative modification of cysteine (i.e., sulfonic acid; Ref. 10). Our study is the first to demonstrate a direct association between oxidative stress and elevation in sulfiredoxin expression in mammals.

The common thread in these observations is a concerted upregulation of the thiol antioxidant system (metallothionein, glutathione, thioredoxin, sulfiredoxin) and its associated enzymes in the Sod1−/− liver. The upregulation of so many components of the thiol antioxidant system in the Sod1−/− liver could be physiologically protective because free thiols react with superoxide at ~10^5-10^6 M−1 s−1 (9, 87), thereby scavenging the excess superoxide in the absence of CuZn-SOD. Thiol antioxidants, e.g., glutathione, N-acetylcysteine, cysteine, and metallothionein, can rescue Sod1−/− yeast (80, 94), and elevated glutathione biosynthesis rescues neuroblastoma cells in which CuZn-SOD was knocked down by RNA interference (2). Of the antioxidant genes upregulated in the Sod1−/− mice listed in Table 4, none was statistically significantly altered in the Gpx1−/− mice, which exhibit no significant elevation in oxidative damage in the absence of exogenous oxidative stressors.

Many of the antioxidant genes altered in the livers of Sod1−/− mice (listed in Table 4) are known to be under the control of the transcription factor Nrf2 [e.g., glutathione S-transferases, sulfiredoxin, glutamate-cysteine ligase, and thioredoxin reductase 1 (48)]. Nrf2 is normally sequestered in the cytoplasm by its binding partner, Keap1. When Keap1 is oxidized, Nrf2 is activated and transitions to the nucleus to induce the transcription of its target genes (48, 86). We observed no significant change in the Nrf2 transcript in our array data. However, the level of Nrf2 protein in the nuclear fraction of the liver of Sod1−/− mice was increased ~4.5-fold compared with WT mice, which is consistent with oxidative stress inducing the transcription of glutathione S-transferases, sulfiredoxin, glutamate-cysteine ligase, and thioredoxin reductase 1 through the activation of Nrf2.

In conclusion, the expression response to elevated oxidative stress in vivo does not constitute an upregulation of classic antioxidant genes, although long-term oxidative stress in the
mice leads to a significant upregulation of thiol antioxidants. Rather, we found that an upregulation of p53 target genes was a common and robust feature of oxidative stress in vivo. Our retrospective review of the literature shows that an upregulation in p53 target genes is a conserved expression response to oxidative stress across different organs and species, and holds true in vitro and in vivo. Thus our study points to p53 playing an important role in the induction of gene expression in response to oxidative stress.

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REFERENCES


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