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Characterization of Transgenic Mice That Overexpress Both Copper Zinc Superoxide Dismutase and Catalase

JAMES MELE,[1] HOLLY VAN REMMEN,[1,3,4] JAN VIJG,[2,3,4] and ARLAN RICHARDSON[1,3,4]

ABSTRACT

Transgenic mice overexpressing both Cu/ZnSOD and catalase [*Tg(SOD1/CAT)*$^{+/o}$] were used to evaluate the effects of overexpression of both genes against oxidative stress. Characterization of these transgenic mice revealed that catalase or Cu/ZnSOD activities were two- to fourfold higher in the tissues of transgenic mice compared to wild-type mice, and the activities of the other major antioxidant enzymes were not altered in the tissues of the transgenic mice. The murine embryonic fibroblasts (MEFs) from the *Tg(SOD1/CAT)*$^{+/o}$ and MEFs overexpressing Cu/ZnSOD were more resistant to paraquat cytotoxicity, relative to wild-type MEFs. The MEFs from *Tg(SOD1/CAT)*$^{+/o}$ tended to be more resistant (up to 2.25-fold) to paraquat cytotoxicity than MEFs overexpressing either Cu/ZnSOD or catalase alone. MEFs from *Tg(CAT)*$^{+/o}$ and *Tg(SOD1/CAT)*$^{+/o}$ were equally as resistant to hydrogen peroxide cytotoxicity. However, there were no significant differences in whole animal survival against either paraquat or γ-radiation. *Antioxid. Redox Signal.* 8, 628–638.

INTRODUCTION

Reactive oxygen species (ROS) are produced as by-products of normal cellular metabolism. ROS also appear to play an important role in regulating gene expression through the activation of some transcription factors, which in turn mediate the induction of proteins involved in cellular responses to changes in environmental conditions. Under normal conditions, a steady-state balance exists in the production of ROS and cellular antioxidant systems (2, 8, 52, 62). Despite the presence of these cellular antioxidant systems, overproduction of ROS in intra- or extracellular spaces can occur through exposure to environmental factors (e.g., radiation or toxins) and through the onset of disease states (63). The progressive accumulation of ROS-mediated damage is thought to promote many disease processes (63) and has been proposed to be involved in the aging process (19, 52, 53, 58). Mammalian cells are equipped with both enzymatic and nonenzymatic antioxidant defense systems to minimize the cellular damage resulting from interactions between cellular components and ROS. Typically, this system includes enzymes and small molecular weight species that catalyze the conversion of more reactive to less reactive species.

One major challenge is determining the interplay of the antioxidant enzymes under normal and disease states. The major enzymatic antioxidant defense mechanisms consist of various forms of superoxide dismutases (SODs), catalase, and the glutathione peroxidases (GPXs). The SODs are thought to provide a first line of defense against oxygen radicals, specifically the superoxide anion ($O_2^{\bullet -}$), which is the major ROS produced by mitochondrial respiration and various metabolic reactions (5). Three different forms of SODs have been described: copper/zinc superoxide dismutase (Cu/ZnSOD), manganese SOD (MnSOD), and extracellular copper/zinc SOD (EC-SOD). All of the SODs catalyze the dismutation of two superoxide radicals to hydrogen peroxide (H_2O_2) and molecular oxygen. Cu/ZnSOD constitutes the major proportion (85–90%) of total SOD activity and is

Departments of [1]Cellular and Structural Biology, [2]Physiology, [3]Barshop Institute for Longevity and Aging Studies at the University of Texas Health Science Center at San Antonio, San Antonio, Texas.
[4]Department of Geriatric Research, Education and Clinical Center and Research Service, South Texas Veterans Health Care System, San Antonio, Texas.

thought to be responsible for removing superoxide radicals generated in the cytosol and the nucleus (42, 52, 65). This activity has also been shown to be localized in the mitochondrial intermembrane space (57); however, the physiological role of Cu/ZnSOD in the mitochondrial membrane space is unclear. The expression of Cu/ZnSOD is constitutive and is thought to be a housekeeping gene (65). MnSOD accounts for approximately 10% of the total SOD activity and is found exclusively in the mitochondrial matrix (65). MnSOD expression is inducible under conditions of oxidative stress and by several cytokines (65). MnSOD is believed to play a critical role in dismutating superoxide radicals generated during respiration, which is believed to be a major site of ROS production.

The physiological importance of the SODs was studied by genetic ablation experiments (34, 51). Null mutations in the Cu/ZnSOD (*Sod1*) genes, which code for the major superoxide scavenging activity in the cytoplasm, result in viable mice; however, the lifespan of these mice is reduced to less than 25 months and the mice have a very high incidence of hepatocellular carcinoma and some deficits in motor function (12, 14, 51). At present, it is impossible to say exactly why this knockout is not lethal. Possibly, this phenotype is due to a combination of low cytoplasmic production of superoxide anions and a lack of viability-critical targets in the cytoplasm. Alternatively, the levels of low-molecular weight antioxidants are sufficient to compensate for the lack of Cu/ZnSOD activity in the cytoplasm. Although the null mutation in *Sod1* is not lethal, these mice are more sensitive to oxidative stress and have some defects, including female infertility and increased susceptibility to ischemic injury (22, 27, 30, 40). In contrast, mouse models null for MnSOD present severe phenotypic syndromes characterized in part by embryonic or neonatal lethality, tissue specific loss of the respiratory chain enzymes, NADH dehydrogenase and succinate dehydrogenase, and the TCA-cycle enzyme aconitase (34, 35, 41).

The superoxide dismutases remove the potentially dangerous superoxide anions from biological systems by their conversion to H_2O_2. In addition to the SODs, there are a number of enzymatic reactions that generate H_2O_2, for example, mitochondrial monoamine oxidases, microsomal diamine oxidases, peroxisomal glycolate oxidase and acyl-CoA oxidase, and the cytosolic enzyme xanthine oxidase. H_2O_2 is more stable than the superoxide anion and, unlike the superoxide anion, can freely diffuse across membranes. In the presence of transition metals (iron-catalyzed Haber-Weiss reaction), superoxide and H_2O_2 can react to form the hydroxyl radical, which damages all known biological components at diffusion controlled rates (20). The next major components of the enzymatic antioxidant defense system, catalase and the GPXs, serve to control H_2O_2 levels within the cell. Catalase, which is found in peroxisomes, is a major enzyme involved in the detoxification of H_2O_2 (39, 50). This enzyme catalyzes the conversion of two molecules of H_2O_2 to O_2 and two molecules of H_2O. The primary physiological function of catalase is to detoxify the H_2O_2 produced from peroxisomal dehydrogenase reactions. The GPXs are an enzyme family that detoxifies cellular organic peroxides and H_2O_2 while oxidizing two molecules of glutathione (GSH). The most abundant GPX, cGPX, is present in the cytoplasm and mitochondria. Because the K_m of GPX for H_2O_2 is lower than that of catalase, GPX is believed to scavenge low concentrations of H_2O_2 in more cellular compartments than catalase. It has been proposed that under conditions of oxidative stress, catalase becomes important in providing protection against oxidative damage (39, 42, 50, 52, 56). However, other studies have noted that catalase and GPX appear to be equally effective (11, 42) or that catalase is more effective than GPX in eliminating H_2O_2 (39, 56). In addition, catalase overexpression alone has been shown to be beneficial against H_2O_2 toxicity in cells (4, 28) and beneficial or neutral in transgenic models that overexpress catalase in a limited number of tissues or cell types (23).

Despite our knowledge of the activities and properties of the antioxidant enzymes and the fact that they play an important role in the defense against ROS, their relative importance in the cell and their potential cooperation is still a matter of controversy. This is partly due to the heterogeneity of experimental models, cell types, and the oxidative conditions used to generate a type of oxidative stress. Studies in mammalian cell culture (2, 3, 11, 13, 29, 37, 38, 42, 52) strongly suggest that there is a balance in the overexpression of the antioxidant enzymes Cu/ZnSOD and catalase that determines the extent of protection against oxidative insults. The objective of this study was to examine the effect of overexpression of both Cu/ZnSOD and catalase in transgenic mice and determine the sensitivities of cells from transgenic mice to oxidative stress.

MATERIALS AND METHODS

Animals and genotyping

The hemizygous transgenic mice that overexpress Cu/ZnSOD [*Tg(SOD1)*$^{+/o}$], catalase [*Tg(CAT)*$^{+/o}$], and Cu/ZnSOD and catalase [*Tg(SOD1/CAT)*$^{+/o}$] and wild-type control mice were generated by mating *Tg(SOD1)*$^{+/o}$ male and *Tg(CAT)*$^{+/o}$ female mice. The parental *Tg(SOD1)*$^{+/o}$ and *Tg(CAT)*$^{+/o}$ hemizygous mice were generated using human P1 clones, containing large genomic fragments (64 and 80 kb, respectively) as described previously (7) and maintained in the C57Bl/6 genetic background. Due to the large size of the human transgenes in the offspring, the presence of catalase and Cu/ZnSOD transgenes were determined using primer sets designed to amplify three regions of each transgene: human catalase promoter, exon 3, and exon 12/13 and the human Cu/ZnSOD promoter, exon 4, and exon 5, as described previously (7). The mice were maintained in a temperature-controlled environment and fed a mouse chow (LM485) from Harlan Teklad (Madison, WI) *ad libitum* unless otherwise stated. All procedures with the mice were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the Subcommittee for Animal Studies at Audie L. Murphy Memorial Veterans Hospital.

Enzyme activity assays

Catalase, cGPX, MnSOD, and Cu/ZnSOD activities in various tissues of transgenic mice and wild type littermates were determined using standard enzymatic assays. Tissues were homogenized on ice in 10 volumes of homogenation

buffer (10 m*M* KH_2PO_4, pH 7.4, 20 μ*M* EDTA, 30 m*M* KCl) using a polytron homogenizer. The homogenates were centrifuged at 13,600 *g* in a microcentrifuge for 10 min at 4ºC, and the supernatants were collected for measuring protein concentration and enzyme activities. Protein concentrations were determined by Bradford assay using bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA). The activities of MnSOD and Cu/ZnSOD were measured using activity gels as previously described (61). The activities of catalase and cGPX were measured by using the catalase and cGPX activity gels as described by Sun *et al.* (59) with slight modifications. Protein extracts were separated on an 8% native polyacrylamide gel with a 5% stacking gel. For detection of cGPX activity, the gel was incubated in a solution containing 0.008% cumene hydroperoxide and 1.5 m*M* reduced glutathione for 10 min and then stained with a solution containing 1% ferric chloride and 1% potassium ferricyanide until the gel became dark green with yellow activity bands. For detection of catalase activity, the gel was soaked in a solution containing 0.003% H_2O_2 for 10 min and then stained with the same staining solution used for the cGPX activity gel. The gel images were recorded and analyzed by using the image-acquiring system and software as described by Van Remmen *et al.* (61).

Isolation and culturing of murine primary embryonic fibroblasts (MEFs)

Timed matings of *Tg(SOD1)*$^{+/o}$ and *Tg(CAT)*$^{+/o}$ mice were carried out to generate wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ embryos. Embryonic fibroblasts were derived from 13- to 14-day mouse embryos as described previously (23, 24). Briefly, mouse embryos were rinsed in sterile PBS, finely minced with scissors, and passed through an 18 gauge needle attached to a 3 ml syringe. The resulting suspension from each embryo was cultivated individually in 50% DMEM/50% F12 medium supplemented with 15 m*M* HEPES, 10% FBS, 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA), and 1% Glutamax (Invitrogen) in a 75 cm^2 flask and regarded as passage 0. Fetal fibroblasts were split 1:3 and passaged every 3–5 days. All assays were performed in duplicate or triplicate using isolates derived from three animals of each genotype. Assays were carried out using cells from passage 3 to 5.

Determination of the Growth Rates of the MEFs

The method of Myers (43) was used to determine the growth rates of the MEFs from transgenic and wild-type control mice. Primary MEFs isolated from the various transgenic and wild type mice were seeded at 8×10^3 cells/well in 24-well plates and cultured up to 10 days. At the appropriate time points, cells were fixed with 3.7% paraformaldehyde in PBS, containing 0.1 m*M* $CaCl_2$ and 1.0 m*M* $MgCl_2$. Cells were then permeabilized and stained with SYBR green I (Molecular Probes, Eugene, OR). For permeabilization, fixed cells were treated with 0.1% Trition X-100, in 50 m*M* Tris-HCl pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA, 0.1% gelatin, and 0.05% Nonidet P40 for 30 min at room temperature. Permeabilized cells were stained with SYBR green I (diluted 1:10,000 in water) overnight at 4°C. The plates were then read by scanning on a STORM phosphoimager (Molecular Dynamics, Sunnyvale, CA) using blue fluorescence mode and a PMT tension of 850 V. The fluorescence was then quantitated using ImageQuant software (Molecular Dynamics, Amersham, Piscataway, NJ, USA). Cell number was calculated from a standard curve.

Cytotoxicity assays

MEFs were seeded (2×10^4 cells/well) in 48-well plates in DMEM/F12 supplemented with 10% FBS. For treatment with various agents, growth media was removed, and freshly prepared hydrogen peroxide (0.5–2 m*M*) or *t*-butyl hydrogen peroxide (50–400 μ*M*) in iron-free Minimum Eagle's medium (MEM) was added to MEF cultures. Hydrogen peroxide and *t*-butyl hydrogen peroxide treatments were conducted in iron-free media to prevent the formation of other toxic ROS through Fenton chemical related reactions of peroxides with iron in the culture media. Paraquat and methylmethane sulfonate (MMS) treatments were conducted in DMEM/F12 growth media since these reagents do not directly react with the iron in the culture media. Paraquat (50–400 μ*M*) was added to the cells in DMEM/F12 for 48 h as described (25).

Sensitivity of wild-type, Tg(SOD1)$^{+/o}$*, Tg(CAT)*$^{+/o}$*, and Tg(SOD1/CAT)*$^{+/o}$ *mice to γ-radiation and paraquat*

Animal studies were conducted as described previously (9, 10, 15) to determine the sensitivities of wild-type and transgenic mice to γ-radiation and paraquat. Wild-type and transgenic animals (12 mice per group) were irradiated with a single dose of 10 Gy in the Gamma-Cell 40 irradiator (MDS Nordion, Kanata, OH), and the survival of mice was monitored each day over a 30-day period following the whole body γ-radiation. For the paraquat studies, wild-type (n = 18), *Tg(SOD1)*$^{+/o}$ (n = 15), *Tg(CAT)*$^{+/o}$ (n = 15), and *Tg(SOD1/CAT)*$^{+/o}$ (n = 16) mice were injected with a single intraperitonal dose of 100 mg/kg of paraquat in sterile isotonic saline. Survival of the mice was monitored each day over a 96-h period following the paraquat injection.

RESULTS

Characterization of the antioxidant defense system in Tg(SOD1/CAT)$^{+/o}$ *mice*

Hemizygous transgenic mice [*Tg(CAT)*$^{+/o}$ and *Tg(SOD1)*$^{+/o}$ mice], overexpressing Cu/ZnSOD or catalase alone, were generated using human P1 clones containing large genomic fragments (64 and 80 kb, respectively) as described previously (7). With the selective breeding of these mice, we have generated transgenic mice that overexpress both Cu/ZnSOD and catalase and have characterized their sensitivity to oxidative stress. The data in Figure 1 show that Cu/ZnSOD and catalase activities were significantly higher in all tissues of the *Tg(SOD1/CAT)*$^{+/o}$ mice compared to wild-type littermates. Cu/ZnSOD activities were twofold (skeletal muscle) to fivefold (brain) higher in all tissues of the *Tg(SOD1/CAT)*$^{+/o}$ mice compared to their wild-type litter-

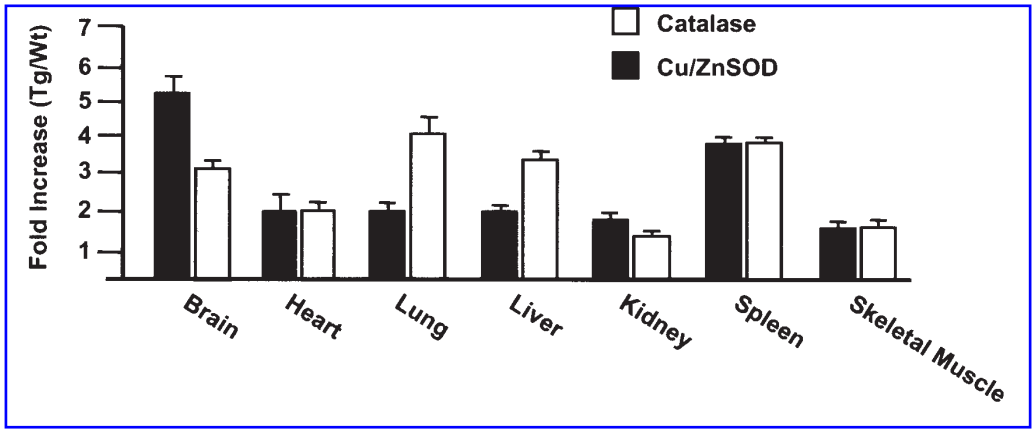

FIG. 1. Cu/ZnSOD and catalase activity in tissues of *Tg(SOD1/CAT)*$^{+/o}$ and wild-type mice. The activities of Cu/ZnSOD and catalase in various tissues of wild type and *Tg(SOD1/CAT)*$^{+/o}$ mice were determined as described in Materials and Methods. The Cu/ZnSOD and catalase activity in various tissues of *Tg(SOD1/CAT)*$^{+/o}$ mice is expressed as the fold increase relative to the wild-type control mice. All values are the mean ± SEM from four mice per genotype. The data were analyzed by Student's *t* test and the levels of Cu/ZnSOD or catalase activity in all tissues of the *Tg(SOD1/CAT)*$^{+/o}$ mice were significantly greater than the wild type mice at the $p \leq 0.05$ level.

mates. Catalase activities were twofold (skeletal muscle) to fourfold (lung) higher in the tissues of the *Tg(SOD1/CAT)*$^{+/o}$ mice compared to the wild-type littermates. The increased Cu/ZnSOD and catalase activities were similar to the increased activities of these antioxidant enzymes that we previously reported in mice overexpressing either Cu/ZnSOD or catalase alone (7). The data in Figure 1 show that *Tg(SOD1/CAT)*$^{+/o}$ mice overexpressed both Cu/ZnSOD and catalase in all tissues.

To determine if overexpression of catalase and Cu/ZnSOD altered the activities of other components of the antioxidant defense system, we measured the activities of MnSOD and cGPX in various tissues of *Tg(SOD1/CAT)*$^{+/o}$ mice. The data in Figure 2 show that the activity levels of the other two major antioxidant enzymes were not altered in the transgenic mice as compared to the wild-type mice.

Sensitivity of murine embryonic fibroblasts (MEFs) to oxidative stress

To determine how the overexpression of catalase and Cu/ZnSOD in *Tg(SOD1/CAT)*$^{+/o}$ mice affects resistance to oxidative stress, we tested the sensitivity of MEFs to various agents known to induce oxidative stress and cytotoxicity. In these experiments, cell viability was measured by the neutral red assay. We hypothesized that cells with elevated antioxidant defenses would exhibit a greater protection against agents that cause acute oxidative stress and result in cell

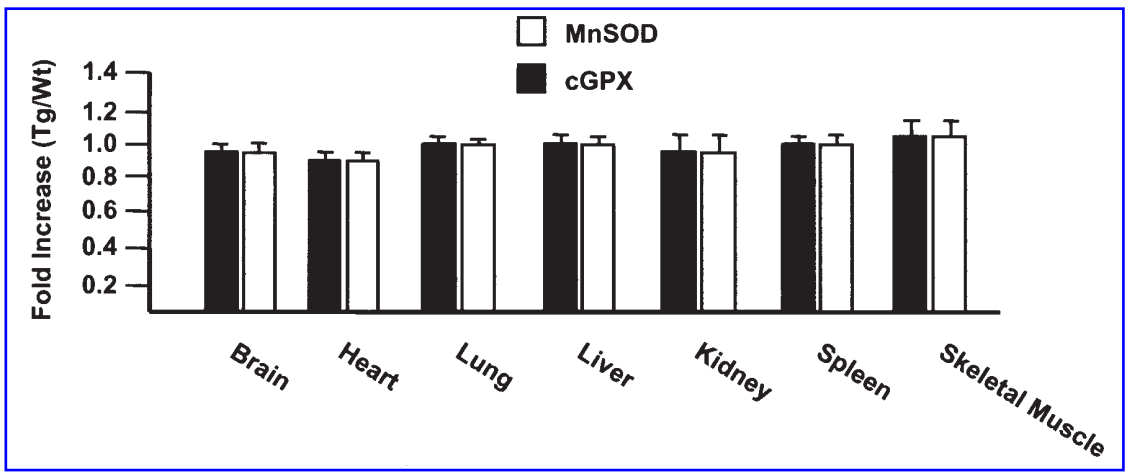

FIG. 2. MnSOD and cGPX activity in tissues of *Tg(SOD1/CAT)*$^{+/o}$ and wild-type mice. The activities of MnSOD and cGPX in various tissues of wild type and *Tg(SOD1/CAT)*$^{+/o}$ mice were determined as described in Materials and Methods. The MnSOD and cGPX activity in the various tissues of *Tg(SOD1/CAT)*$^{+/o}$ mice is expressed as the fold increase relative to the wild-type control mice. The data are expressed as the mean ± SEM from four mice. No significant difference ($p \leq 0.05$) was found for MnSOD or cGPX activities between wild-type and transgenic mice.

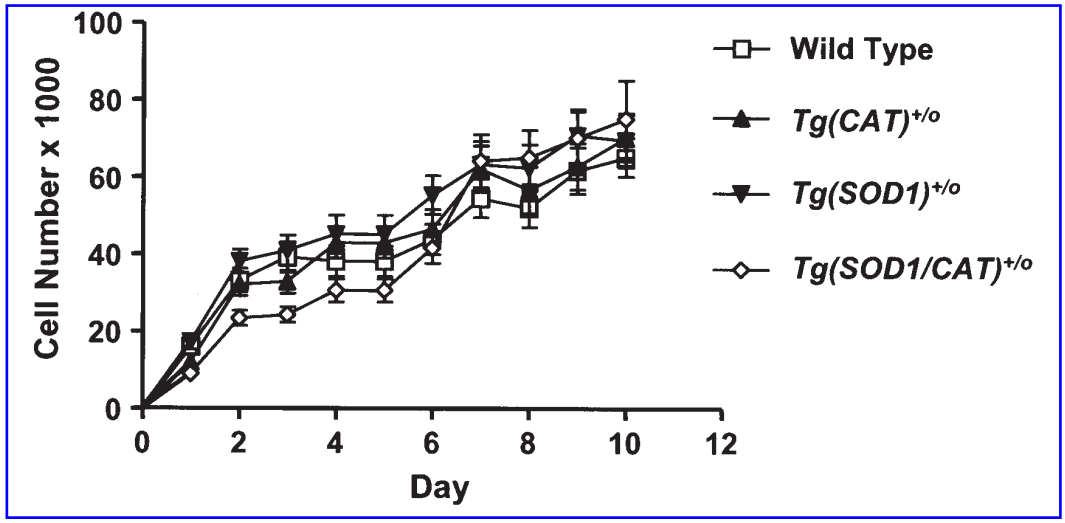

FIG. 3. Growth curves for MEFs isolated from wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ mice. Primary cultures of MEFs were seeded on 24-well plates (8×10^3 cells/well) in DMEM/F12 supplemented with 10% fetal bovine serum and were grown at 37°C with 5% CO_2 for the indicated times. The cell number was determined by measuring DNA content using SYBR Green I as described in Materials and Methods. All values represent the mean ± SEM from experiments repeated with fibroblasts derived from three animals. The data were analyzed by a two-way ANOVA. No significant difference ($p \leq 0.05$) was found in the growth rates of the fibroblasts isolated from wild-type and transgenic mice.

death. We first measured the growth rates of MEFs isolated from the four experimental groups. The data in Figure 3 show that the overexpression of catalase and/or Cu/ZnSOD did not alter the growth rates of the MEFs from any of the transgenic mice relative to their wild-type controls.

Figure 4 shows the sensitivity of MEFs to the oxidative stressors paraquat, hydrogen peroxide, and *t*-butyl hydrogen peroxide. The data in the left graph in Figure 4 show that the cell viability of MEFs from *Tg(SOD1)*$^{+/o}$ and *Tg(SOD1/CAT)*$^{+/o}$ mice were significantly higher than MEFs from *Tg(CAT)*$^{+/o}$ and wild-type mice when exposed to paraquat at concentrations of 100–400 μ*M* for 48 h. There was no significant difference in the sensitivity of MEFs from wild-type and *Tg(CAT)*$^{+/o}$ mice at any of the doses of paraquat tested. MEFs from *Tg(SOD1)*$^{+/o}$ mice showed a 40–400% increase in resistance to paraquat cytotoxicity at doses from 100 to 400 μ*M* paraquat. MEFs from *Tg(SOD1/CAT)*$^{+/o}$ mice showed a 60–900% increase in resistance to paraquat cytotoxicity at doses from 100 to 400 μ*M* paraquat. Relative to MEFs from *Tg(SOD1)*$^{+/o}$ mice, the

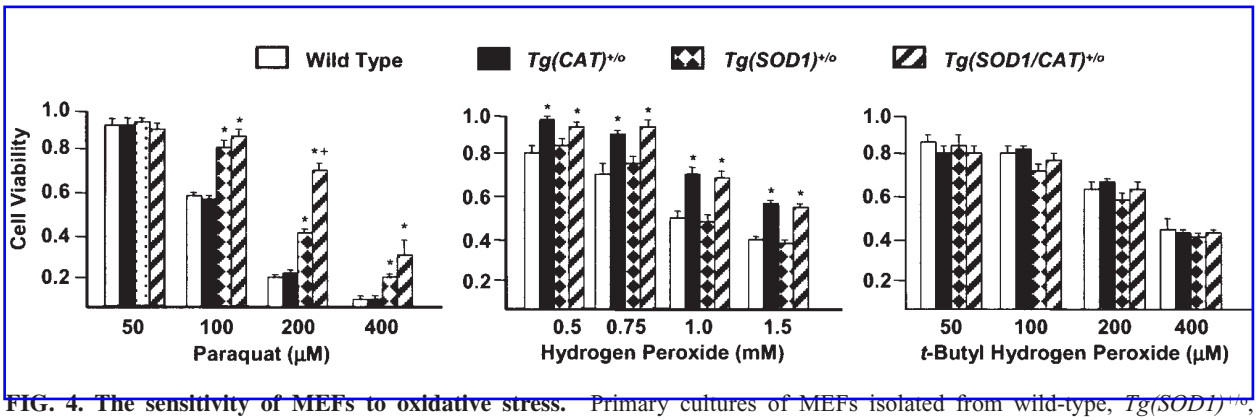

FIG. 4. The sensitivity of MEFs to oxidative stress. Primary cultures of MEFs isolated from wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ mice were treated with various doses of paraquat for 48 h (*left graph*), hydrogen peroxide for 2 h (*middle graph*), and *t*-butyl hydrogen peroxide for 2 h (*right graph*). Cell viability was measured by the neutral red assay as discussed in the Materials and Methods. All values represent the mean ± SEM from experiments repeated with fibroblasts derived from three animals. The data from wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ fibroblasts were compared at various concentrations of the agents by a two-way ANOVA with a followup Tukey's multiple range test. The means that show a significant difference ($p \leq 0.05$) from wild type are shown by (*); those means that are significantly different ($p \leq 0.05$) from the *Tg(SOD1)*$^{+/o}$ are shown by (+).

MEFs from *Tg(SOD1/CAT)*$^{+/o}$ mice tended to be more resistant to paraquat; however, this difference was significant only at a dose of 200 μ*M* paraquat.

The data in Figure 4 (middle graph) also show the effect of hydrogen peroxide on the viability of MEFs isolated from *Tg(CAT)*$^{+/o}$ and *Tg(SOD1/CAT)*$^{+/o}$ mice. MEFs from *Tg(SOD1/CAT)*$^{+/o}$ and *Tg(CAT)*$^{+/o}$ mice showed a significant (24–47%) increase in resistance to hydrogen peroxide cytotoxicity at all concentrations tested. *Tg(SOD1/CAT)*$^{+/o}$ mice were not significantly more protected than *Tg(CAT)*$^{+/o}$ mice at any doses of hydrogen peroxide tested. MEFs from *Tg(SOD1)*$^{+/o}$ and wild-type cells mice were equally susceptible to hydrogen peroxide cytotoxicity. The data in the right graph in Figure 4 show that overexpression of Cu/ZnSOD and/or catalase in MEFs isolated from the transgenic mice did not alter the sensitivity of the MEFs to *t*-butyl hydrogen peroxide. There was no significant difference in viability in any of the transgenic groups treated with doses of 50–400 μ*M* *t*-butyl hydrogen peroxide.

Sensitivity of mice to paraquat and γ-radiation

To determine if the *Tg(SOD1/CAT)*$^{+/o}$ mice are more resistant to oxidative stress, we measured the sensitivity of whole animals to paraquat and γ-radiation. Figure 5, Panel A shows the survival curves for the wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ mice treated with paraquat. The survival of transgenic mice overexpressing Cu/ZnSOD or catalase was similar to wild-type mice (survival at 96 h ranged from 7% to 17%). However, the survival of mice overexpressing both Cu/ZnSOD and catalase was consistently higher than wild-type mice. For example, after 96 h, the survival of the *Tg(SOD1/CAT)*$^{+/o}$ mice was 31% compared to 17% for wild-type mice. However, statistical analysis of the data in Figure 5A indicates no significant difference in the survival of any of the transgenic mice compared to the wild type mice. Figure 5, Panel B shows the survival curves of the wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ mice to whole body γ-radiation. None of the transgenic mice showed increased survival to γ-radiation compared to the wild-type mice. In fact, the *Tg(CAT)*$^{+/o}$ and *Tg(SOD1/CAT)*$^{+/o}$ mice tended to be more sensitive to γ-radiation, which is consistent with our previous study showing that transgenic mice homozygous for the catalase transgene were more sensitive to γ-radiation (6). However, this difference was not statistically significant.

DISCUSSION

The reactions catalyzed by the various antioxidant enzymes have been well characterized; however, the interaction among the various antioxidant enzymes to remove different types of ROS is less understood. For example, the superoxide dismutases scavenge the superoxide radicals, converting them into hydrogen peroxide and oxygen. The hydrogen peroxide produced by this and other reactions can be harmful to the cell if not converted to water and molecular oxygen or GSSG by catalase or cGPX, respectively. Overexpression of Cu/ZnSOD in cellular models has been shown to be either beneficial or detrimental, depending on the cell type and level of overexpression (2, 13). Numerous studies have reported the deleterious effects of an imbalance between Cu/ZnSOD and catalase or cGPX in multiple cell types, suggesting that the ratio between the enzymes may be more important than the absolute levels of activity of the individual enzymes (1, 2). An increase in Cu/ZnSOD was noted to be

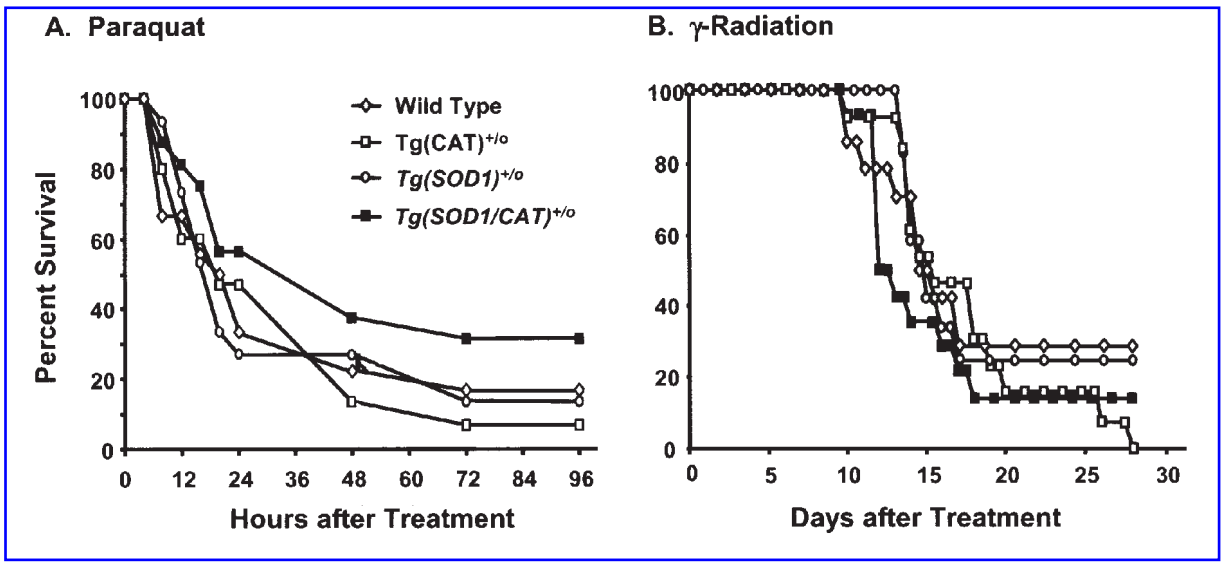

FIG. 5. The sensitivity of mice to paraquat and γ-radiation. Age-matched wild-type, *Tg(SOD1)*$^{+/o}$, *Tg(CAT)*$^{+/o}$, and *Tg(SOD1/CAT)*$^{+/o}$ mice were subjected to a 100 mg/kg i.p. dose of paraquat (**A**), or to 10 Gy whole body γ-radiation (**B**). Survival was measured over a 96-h period following the paraquat treatment or over a 30-day period following γ-radiation. The survival curves were analyzed by the Wilcoxon and Log-Rank test. There was a significant decline in survival among the groups of mice after exposure to paraquat or whole body γ-radiation ($p \leq 0.05$). However, there was no significant difference ($p \geq 0.05$) among the different groups of mice in terms of survival after exposure to either paraquat or whole body γ-radiation.

protective against oxidative stress induced by the superoxide anion generator paraquat (24, 25, 33). Other groups have noted that a compensatory increase in cGPX with the introduction of a Cu/ZnSOD expression vector in murine L cells, neuroblastoma cells, and NIH3T3 cells is required for protection against paraquat toxicity. In addition, murine epidermal JB6 cells overexpressing Cu/ZnSOD displayed increased DNA breakage and growth inhibition when exposed to the xanthine/xanthine oxidase superoxide anion generating system (1, 2, 13). These observations are thought to result from an increased production of H_2O_2 in cells overexpressing Cu/ZnSOD. The increase in H_2O_2 resulting from increased Cu/ZnSOD activity has been correlated to the level of Cu/ZnSOD overexpression and the cell type in which the enzyme is overexpressed. Subsequent transfection of catalase or cGPX into these Cu/ZnSOD overexpressing cells compensated for the sensitizing effect of Cu/ZnSOD (1). Similarly, co-overexpression of Cu/ZnSOD and catalase in insulin producing RINm5F cells conferred greater protection against oxidative stressors compared to cells that overexpressed Cu/ZnSOD or catalase alone (37). Thus, data from cell studies indicate that an increase in a peroxide removing enzyme is required to compensate for the increased production of H_2O_2 resulting from the increase in Cu/ZnSOD activity. Investigators have also proposed that coordinate overexpression of SOD and a peroxidase is more protective than overexpression of the individual enzymes since the enzymes would be able to be maintained in an active form rather than being inactivated by ROS (31, 55).

Although the data with cells suggest that the simultaneous overexpression of Cu/ZnSOD and catalase in cells is beneficial over the overexpression of just Cu/ZnSOD alone, there have been only a limited number of studies with whole animals. The most persuasive evidence on the benefits of overexpressing both Cu/ZnSOD and catalase came from a study by Orr and Sohal (47). They reported that overexpression of both Cu/ZnSOD and catalase resulted in a significant increase (14–34%) in the lifespan of *Drosophila* and resulted in lower levels of protein and DNA oxidation. Their previous studies showed that overexpression of Cu/ZnSOD or catalase alone had only a very small positive effect or no effect on lifespan, respectively (45, 46). However, transgenic studies in *Drosophila* using P-element mediated transformation are problematic because the control and experimental lines have different genetic backgrounds, a factor that has been shown to alter lifespan independently of any transgenic manipulation (26). To overcome these parental genotypic effects, Tower's laboratory used an inducible yeast FLP/FRT recombination system to induce the expression of various antioxidant enzymes (58). Overexpression of Cu/ZnSOD resulted in an increase in lifespan of up to 48%, and a more typical increase was around 20%. However, these investigators found no added benefit of overexpressing both catalase and Cu/ZnSOD. More recently, Orr *et al.* (44) reported that the introduction of Cu/ZnSOD and catalase transgenes failed to extend the lifespan of flies from a long-lived genetic background. These studies highlight that both starting lifespan and epistatic interactions affect the lifespan extension resulting from transgene overexpression. Furthermore, these findings illustrate the importance of the subcellular site and activity levels of the overexpressed enzymes. In mice, neutral or deleterious effects were shown when catalase and/or the superoxide dismutases were overexpressed in their natural or mitochondrial-targeted compartment (54). In contrast, overexpression of catalase to the mitochondrial compartment in transgenic mice has been shown to have a positive effect on life span (54). Therefore, ectopic expression of Cu/ZnSOD and/or catalase may or may not have adverse effects on life span in all species. Therefore, it is important to validate these findings in other transgenic models.

The enhanced expression of Cu/ZnSOD in transgenic mouse models has protective effects against many types of ROS-mediated tissue injury, including ischemia and reperfusion injury, hypoxic lung injury, brain trauma, and neurotoxic drugs (8). However, overexpression of Cu/ZnSOD has been reported to have several deleterious effects, for example, neuromuscular abnormalities and premature thymic involution (48, 49). The negative effects of Cu/ZnSOD overexpression are thought to result from increased H_2O_2 production resulting from increased dismutation of superoxide anions. To date, no transgenic model has been generated that allows one to test the effect of coordinated overexpression of Cu/ZnSOD and catalase in mice. The transgenic mice we have generated that globally overexpress either Cu/ZnSOD or catalase, will allow us to determine for the first time the effect of the overexpression of both Cu/ZnSOD and catalase compared to the overexpression of each gene alone.

Because the antioxidant defense system is a complex, integrated system, an alteration in the activity of one component of the system may result in a compensatory response by other components of the system. Several previous studies showed that overexpression of one antioxidant enzyme resulted in alterations in other antioxidant enzymes. Kelner (29) reported that transfected fibroblasts that overexpress Cu/ZnSOD have increased activity of cGPX as well as decreased MnSOD activity. In the present study, we demonstrated that overexpression of Cu/ZnSOD and catalase did not alter the levels of the other major antioxidant enzymes, specifically MnSOD and cGPX. Previously, Chen *et al.* (6) showed that a three- to four-fold overexpression of catalase in tissues of transgenic mice had no effect on the other major antioxidant enzymes. This observation is not limited to transgenic models of Cu/ZnSOD or catalase overexpression because the partial ablation of MnSOD or complete ablation of cGPX does not cause alterations in the activities of the other major antioxidant enzymes (9, 15, 61). Thus, major changes in one or more of the antioxidant enzymes in tissues of transgenic or knockout mice have very little effect on the expression of the other antioxidant enzymes. Thus, the overall antioxidant defense would appear to be enhanced by Cu/ZnSOD and catalase overexpression in the transgenic mice.

To determine if the hypothesis that overexpression of both Cu/ZnSOD and catalase would be more protective than the overexpression of each antioxidant enzyme alone, we measured the resistance of MEFs from each transgenic group to the oxidative stressors paraquat, hydrogen peroxide, and *t*-butyl hydrogen peroxide. Huang *et al.* reported that fibroblasts isolated from transgenic mice that overexpress Cu/ZnSOD are protected against the superoxide generator paraquat (25). Likewise, overexpression of catalase in transgenic mice has been shown to protect against hydrogen per-

oxide (6, 21, 32). Consistent with these studies, we showed that cells from transgenic mice that overexpress either Cu/ZnSOD or catalase were resistant to paraquat and hydrogen peroxide, respectively. Given these results, we determined how the dual overexpression of Cu/ZnSOD and catalase affected cell viability against paraquat and hydrogen peroxide. At low concentrations of paraquat, there was no significant difference in viability for the fibroblasts from wild type and transgenic mice. Catalase overexpression alone provided no significant protection against paraquat toxicity. Fibroblasts from *Tg(SOD1)*$^{+/o}$ were more resistant to paraquat cytotoxicity at most of the doses tested. This finding is consistent with past studies showing that the EC_{50} for paraquat toxicity is significantly elevated in cells that overexpress Cu/ZnSOD (25). Overexpression of Cu/ZnSOD appears to be necessary and sufficient to provide protection against oxidative stress resulting from a 100 μ*M* dose of paraquat. Although overexpression of Cu/ZnSOD alone does provide protection against paraquat, there is a trend for greater protection by co-overexpression of both antioxidant enzymes. Paraquat generates superoxide anions, which are dismuted by Cu/ZnSOD to hydrogen peroxide. As noted above, several studies have suggested that overexpression of Cu/ZnSOD can lead to a toxic buildup up hydrogen peroxide, which can be eliminated by the additional increase in catalase activity. Alternatively, superoxide anions have been shown to inhibit catalase (31, 55). Therefore, we would expect to observe the cooperation between the activities of Cu/ZnSOD and catalase in cells exposed to an increased superoxide anion flux generated by paraquat. Thus, the increased expression Cu/ZnSOD and catalase could be more protective against paraquat by either the elimination of H_2O_2 or by keeping catalase in an active form by the increased elimination of superoxide anions. In contrast, it has been suggested that the species responsible for the cytotoxicity of hydrogen peroxide is the hydroxyl radical (20). Because the hydroxyl radical reacts with all major biological molecules at diffusion controlled rates, additional Cu/ZnSOD activity should not provide any additional protection against hydrogen peroxide toxicity.

We also determined the sensitivity of the transgenic mice to an acute dose of paraquat to evaluate the resistance of the whole animal to this oxidative stressor. Regardless of its route of administration, paraquat is rapidly distributed in most tissues, with the highest concentrations found in the lungs and kidneys (60). The compound accumulates slowly via an energy-dependent process in the Clara and types I and II alveolar epithelium cells in the lungs (60). Paraquat undergoes a one-electron reduction by the flavoenzyme NADPH-cytochrome P450 reductase, which results in the generation of the paraquat radical (18). In the presence of oxygen, the paraquat radical rapidly auto-oxidizes to produce a superoxide radical and regenerates the paraquat di-cation. Thus, in the presence of a sufficient supply of reducing equivalents, repeated cycles of paraquat di-cation reduction and reoxidation can occur, producing large amounts of reactive oxygen species, oxidative stress, and lipid peroxidation (16, 17, 64). Past studies have shown that mice treated with antioxidants (e.g., liposomal Cu/ZnSOD and catalase) are more resistant to paraquat toxicity (36, 60). However, these studies are not conclusive because the antioxidant enzymes are not targeted to their proper intracellular locations, and the enzymes are present in much higher concentrations than observed *in vivo* or obtainable with transgenic models. Therefore, we measured for the first time the effect of the global overexpression of Cu/ZnSOD and/or catalase on paraquat toxicity in whole animals. Overexpression of catalase provided no protection against paraquat, which was predicted from our data with cells from the *Tg(CAT)*$^{+/o}$ mice. Interestingly, we also observed no enhanced protection against paraquat in the Cu/ZnSOD transgenic mice. This was surprising because cells from the *Tg(SOD1)*$^{+/o}$ mice were resistant to paraquat toxicity *in vitro* and because previous studies with other types of oxidative stress showed that transgenic mice overexpressing Cu/ZnSOD are resistant to various oxidative stresses, for example, ischemia and reperfusion injury, hypoxic lung injury, brain trauma, and neurotoxic drugs (8). However, these studies failed to test the effects of paraquat toxicity. Whole animal treatment of the transgenic animals in our study did not display resistance to paraquat toxicity. This would be consistent with our *in vitro* data for the *Tg(CAT)*$^{+/o}$ mice, but not for the *Tg(SOD1)*$^{+/o}$ and *Tg(SOD1/CAT)*$^{+/o}$ mice. The differences in the data sets may be explained by relative protective effects of the antioxidant enzymes in different tissues versus those effects observed in the *in vitro* assays. To protect the whole organism against a global oxidative stressor, it would be necessary for every cell type impacted by the stressor to have a sufficient elevation in antioxidant enzyme level(s) in order to observe any benefit. We may also have mosaicism in expression of the antioxidant enzymes in the transgenic models used in our studies. If these scenarios are true, then the rate limiting cell-type (i.e., least protected against the oxidative stressor) within a tissue could lead to organ failure and lethality. In contrast, the *in vitro* situation represents very limited subset of how certain cell types would respond against a particular oxidative stress.

In addition to paraquat, we have studied the effects of the simultaneous overexpression of Cu/ZnSOD and catalase on the sensitivity of cells and animals to other types of oxidative stress. Overexpression of both Cu/ZnSOD and catalase provided no significant additional protection when MEFs were exposed to either hydrogen peroxide or *t*-butyl-hydroperoxide. This result demonstrates that the protective effects of Cu/ZnSOD and/or catalase overexpression is specific to certain types of ROS (superoxide anions and hydrogen peroxide) and does not provide protection against other ROS which trigger oxidative stress via lipid peroxidation (*t*-butyl hydrogen peroxide). Protection against small hydrophobic hydroperoxides would require the upregulation of the GPX system, given that the GPX system catalyzes the reduction of peroxides that are not suitable substrates for catalase. In addition, we found no protection from the overexpression of either Cu/ZnSOD or catalase alone or in combination on the survival of mice exposed to γ-radiation. The lack of protection against γ-radiation may indicate a deficiency in the appropriate levels of activity of Cu/ZnSOD or catalase in a particular cellular compartment or tissue. Alternatively, γ-radiation is known to generate hydroxyl radicals and cause double-strand DNA breaks which would not be prevented by increasing Cu/ZnSOD or catalase activities.

In summary, we have generated transgenic mice that globally overexpress both Cu/ZnSOD and catalase and have shown that there is a benefit from the co-overexpression of these antioxidant enzymes *in vitro* against superoxide anions generated from paraquat. These data are consistent with studies with transfected cell lines showing that the co-overexpression of Cu/ZnSOD and catalase is more protective against a higher concentration of a superoxide anion generator than overexpression of single enzymes alone. However, the co-overexpression of Cu/ZnSOD and catalase does not appear to provide any additional protection to other types of oxidative stress.

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ABBREVIATIONS

γ-radiation, gamma-radiation; cGPX, cytoplasmic glutathione peroxidase; Cu/ZnSOD, Cu/Zn superoxide dismutase; EC-SOD, extracellular copper/zinc superoxide dismutase; GPXs, glutathione peroxidases; GSH, glutathione; H_2O_2, hydrogen peroxide; MEFs, murine embryonic fibroblasts; MEM, minimum Eagle's medium; MMS, methylmethane sulfonate; MnSOD, manganese superoxide dismutase; $O_2^{\bullet -}$, superoxide anion; ROS, reactive oxygen species; *Sod1*, gene for Cu/Zn superoxide dismutase; SODs, superoxide dismutases; *Tg(CAT)*$^{+/o}$, transgenic mice that overexpress catalase; *Tg(SOD1)*$^{+/o}$, transgenic mice that overexpress Cu/Zn superoxide dismutase; *Tg(SODCAT)*$^{+/o}$, transgenic mice that overexpress Cu/Zn superoxide dismutase and catalase.

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Address reprint requests to:
Arlan Richardson, Ph.D.
Department Cellular and Structural Biology
University of Texas Health Science Center at San Antonio
7703 Floyd Curl Drive, MSC 7762
San Antonio, Texas 78229–3900

E-mail: richardsona@uthscsa.edu

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