Glutathione peroxidase 4 (Gpx4) is uniquely involved in the detoxification of oxidative damage to membrane lipids. Our previous studies showed that Gpx4 is essential for mouse survival and that Gpx4 deficiency makes cells vulnerable to oxidative injury. In the present study, we generated two lines of transgenic mice overexpressing Gpx4 (Tg(GPX4) mice) using a genomic clone containing the human GPX4 gene. Both lines of Tg(GPX4) mice, Tg5 and Tg6, had elevated levels of Gpx4 (mRNA and protein) in all tissues investigated, and overexpression of Gpx4 did not cause alterations in activities of glutathione peroxidase 1, catalase, Cu/Zn superoxide dismutase, and manganese superoxide dismutase. The human GPX4 transgene rescued the lethal phenotype of null mutation of the mouse Gpx4 gene, indicating that the transgene can replace the essential role of mouse Gpx4 in mouse development. Cell death induced by t-butylhydroperoxide and diquat was significantly less in murine embryonic fibroblasts from Tg(GPX4) mice compared with wild type mice. Liver damage and lipid peroxidation induced by diquat were reduced significantly in Tg(GPX4) mice. In addition, diquat-induced apoptosis was decreased in Tg(GPX4) mice, as evidenced by attenuated caspase-3 activation and reduced cytochrome c release from mitochondria. These data demonstrate that Gpx4 plays a role in vivo in the mechanism of apoptosis induced by oxidative stress that most likely occurs through oxidative damage to mitochondrial phospholipids such as cardiolipin.

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, are constantly generated in aerobic organisms during normal respiration. In addition, environmental factors (such as ionizing radiation) and pathological compounds (such as β-amyloid in Alzheimer’s disease) can generate ROS. Although ROS at physiological concentrations may be required for normal cell function, excessive production of ROS can be detrimental to cells, because ROS can cause oxidative damage to lipids, protein, and DNA. Polyunsaturated fatty acids, which are found predominantly in cellular membranes, are especially vulnerable to attack by ROS because of the high concentration of allylic hydrogens in their structure (1). The resulting lipid hydroperoxides can affect membrane fluidity and the function of membrane proteins. In addition, lipid hydroperoxides can undergo iron-mediated, one-electron reduction and oxygenation to form epoxyalgal peroxy radicals, which trigger a chain reaction of free radical-mediated lipid peroxidation (2). The end-products of lipid peroxidation are reactive aldehydes such as 4-hydroxynonenal and malondialdehyde, many of which are highly toxic to cells (3). In addition, reactive aldehydes generated by lipid peroxidation can attack other cellular targets, such as proteins and DNA, thereby propagating the initial damage in cellular membranes to other macromolecules. Because lipid hydroperoxides formed in membranes are an important component of ROS generation in vivo, their detoxification appears to be critical in the survival of an organism to oxidative stress (4, 5).

All cells possess a complex antioxidant system to detoxify reactive oxygen species. Antioxidant enzymes act in concert to remove various ROS produced by free radical reactions. Superoxide dismutases (Cu/Zn-SOD and Mn-SOD) scavenge the superoxide radicals, converting them into hydrogen peroxide and oxygen, whereas catalase and the glutathione peroxidases convert hydrogen peroxide to water. The glutathione peroxidases are a group of selenoproteins that catalyze the reduction of peroxides generated by ROS at the expense of glutathione (6). Four selenoprotein glutathione peroxidases have been identified in mammalian systems: glutathione peroxidase 1 (Gpx1) was the first mammalian selenoprotein to be identified and is the most abundant glutathione peroxidase; glutathione peroxidase 2 (Gpx2) is a glutathione peroxidase expressed in the gastrointestinal tract; glutathione peroxidase 3 (Gpx3) is a plasma form of glutathione peroxidase; and glutathione peroxidase 4 (Gpx4) is a membrane-associated glutathione peroxidase that is also called phospholipid hydroperoxide glutathione peroxidase. Recently, a new glutathione peroxidase, Gpx6, has...
been identified, but its cellular role and significance are not clear (7).

Among the glutathione peroxidases, Gpx4 is unique in several ways. First, in addition to the common substrates (hydrogen peroxide and alkyl peroxides) reduced by all glutathione peroxidases, Gpx4 reduces hydroperoxide groups on phospholipids, lipoproteins, and cholesterol esters. Second, unlike the other glutathione peroxidases, which are tetrameric enzymes, Gpx4 is a monomeric enzyme and is rich in hydrophobic amino acid residues. Because of its small size and large hydrophobic surface, Gpx4 can interact with complex lipids in membranes and thereby detoxify membrane lipid hydroperoxides (8). The other pathway for removing membrane lipid peroxides from membranes is through the coupled actions of phospholipase A₂ (PLA₂) and Gpx1 (9); PLA₂ first excises the fatty acid hydroperoxide from the phospholipid hydroperoxide in the membrane, and then Gpx1 reduces the fatty acid hydroperoxide to alcohol and water. From kinetic modeling, the Gpx4 pathway is estimated to be far more efficient at removing phospholipid hydroperoxides than the PLA₂-Gpx1 pathway, because the affinity of Gpx4 to membrane lipid peroxides is more than 10⁴-fold greater than PLA₂ (10). Therefore, Gpx4 is considered to be the primary enzymatic defense system against oxidative damage to cellular membranes (6).

Gpx4 is ubiquitously expressed; however, its activity makes up only a fraction of the total cellular glutathione peroxidase activity in most tissues. The exception is the testes, where Gpx4 activity makes up the majority of the glutathione peroxidase activity (11). Despite its relative low cellular abundance, Gpx4 was shown to play a critical role in the antioxidant defense system in our study using mice deficient in Gpx4. The Gpx4 null mutation (Gpx4⁻/⁻) is embryonically lethal; Gpx4⁻/⁻ embryos die at embryonic stage E7.5 to E8.5 (12). In addition, embryonic fibroblasts derived from mice heterozygous for the Gpx4 gene (Gpx4⁺/⁻) have increased lipid peroxidation, more cell death after exposure to oxidizing agents, and experienced growth retardation under high oxygen (13). These data strongly suggest that Gpx4 deficiency makes cells vulnerable to oxidative stress, especially lipid peroxidation.

In the present study, we generated two lines of transgenic mice overexpressing Gpx4 (Tg(GPX4) mice) using a genomic clone containing the human GPX4 gene. Both lines of Tg(GPX4) mice, Tg5 and Tg6, have elevated levels of Gpx4 mRNA and protein in all tissues studied. We further demonstrated that Tg5(GPX4) mice had reduced oxidative injury after oxidative stress. The Tg5(GPX4) mouse therefore is a potentially valuable animal model for studying the role of reduced membrane lipid peroxidation in vivo in the mechanism underlying a variety of pathological conditions.

EXPERIMENTAL PROCEDURES

Generation of Tg(GPX4) Mice—A human P1 library (Genome Systems, Inc., St. Louis, MO) was screened for the human GXP4 gene using a PCR-based method. Positive P1 clones were analyzed for the presence of GXP4 gene and 5'- and 3'-flanking sequences by restriction analysis, Southern blotting, and PCR using sequence information obtained from the human genome data base. The P1 clone (3C15) contained the intact human GXP4 gene (~3 kb) plus ~30 and 20 kb of 5'- and 3'-flanking sequences, respectively. DNA from this clone was isolated and injected to fertilized oocytes derived from B6D2F1 mice to generate transgenic mice by the Transgenic Core of the San Antonio Nathan Shock Aging Center. Tail DNA was isolated from mice derived from the injected oocytes digested with Scellr and analyzed by Southern blotting using a cDNA probe for Gpx4, and β-actin was used to adjust for loading variation (14).

G-protein levels in tissues from Tg(GPX4) and wild type mice were determined by Western blots as described previously (12). To detect Gpx4 protein in tissue subcellular fractions, tissues (testes and liver) were homogenized in buffer 1 (250 mM mannitol, 75 mM sucrose, 500 μM EGTA, 100 μM EDTA, and 10 mM Hepes, pH 7.4) supplemented with protease inhibitor mixture. The homogenates were centrifuged at 600 g for 10 min at 4 °C to pellet nuclei, which were re-suspended and re-centrifuged to eliminate the nuclei. The resulting supernatant was then centrifuged at 14,000 g for 20 min at 4 °C to obtain the mitochondria pellet. The supernatant was further centrifuged at 100,000 g for 60 min at 4 °C to yield the cytosolic fraction and the pellet containing microsomes. Gpx4 protein levels in each fraction were determined by Western blots. Representative protein levels for each fraction were used to adjust for loading (histone–1 for the nuclear fraction, Leb–α for the cytosolic fraction, ATPase for the mitochondrial fraction, and ERP57 for the microsomal fraction).

Gpx1, Catalase, Cu/Zn-SOD, and Mn-SOD Activity Determination—Tissues from Tg(GPX4) and wild type mice were homogenized in Dounce homogenizers in ice-cold lysis buffer (2 mM Tris, pH 7.4, 0.05% SDS) and centrifuged for 10 min at 10,000 g at 4 °C. The supernatants were used for antioxidant defense enzymatic activity assay. Gpx1 activity was measured using the cytosolic fraction as described (15). Briefly, cell homogenates were run on 7.5% polyacrylamide gels, and the gels were soaked in 1 mM reduced glutathione. The substrate, cumene hydroperoxide (0.008%), was added to the solution, and the gels were soaked at 37 °C. The gel was illuminated at 560 nm in a light box for 15 min. Under these conditions, the gel stained purple with a chromatic band indicating the presence of SOD activity. The gel images were recorded with a digital-camera imager system (Amersham Biosciences), and the data were analyzed using ImageQuant 5.0 software.

The activities of CuZn-SOD and Mn-SOD were measured using a SOD activity gel as previously described (16). Fifteen micrograms of protein were separated on a 10% polyacrylamide gel. The gel was stained in 1% ferric chloride/1% potassium ferricyanide solution. After staining, the gels became dark green with yellow bands indicating the presence of glutathione peroxidase activity. The gels were photographed using a digital-camera imager system (Amersham Biosciences), and the data were analyzed using ImageQuant 5.0 software.

Mice in this study were reviewed and approved by the IACUC (Institutional Animal Care and Use Committee) of University of Texas Health Science Center at San Antonio and the IACUC of Audie Murphy Veterans Affairs Hospital.

Assays for Gpx4 Expression—Total RNA was isolated from tissues using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. To detect the expression of the human GXP4 transgene, RT-PCR (reverse transcription PCR) was performed with SuperScript™ One-Step RT-PCR Systems (Invitrogen), using a pair of PCR primers (G4F1: TCC CAG TGA GGC ACC GAA, G4R1: TTG TCG ATC AGG AAC TTG GTO) that specifically amplifies the human GXP4 mRNA. Total RNA from human cell line EJ cells was used as positive control for RT-PCR. Total Gpx4 mRNA levels in tissues from Tg(GPX4) and wild type mice were determined as previously described using a cDNA probe for Gpx4, and β-actin was used to adjust for loading variation (14).
further for 10 min. The elution medium was then transferred to a 96-well plate, and absorption of the elution medium was read at 540 nm with a micro-plate reader (Dynex Technologies).

**Assay for Caspase-3 Activity—**The caspase-3 activity in MEFs, with or without t-BuOOH treatment, was determined using the caspase assay system from Promega (Madison, WI). The cells were lysed by repeated freezing and thawing in lysis buffer provided in the kit. Cell lysates corresponding to equal amounts of total protein were used to set up caspase-3 activity assay reactions. After incubation at 37 °C for 20 h, the intensities of color in the reaction wells, which were proportional to caspase-3 activity present in the samples, were measured with a microplate reader at 405 nm. Caspase-3 protein levels in liver tissues were determined by Western blot using an anti-caspase-3 antibody (sc-7148, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The intensities of bands corresponding to p20 of caspase-3 were quantified and used as indicators of caspase-3 activation.

**Plasma Alanine Aminotransferase and F2-isoprostane Levels—**Two groups of Tg(GPX4) and wild type mice (2–4 months of age) were injected intraperitoneally with diquat dissolved in saline at a dose of 50 mg/kg. Six hours after injection, the mice were sacrificed, and plasma was isolated to determine ALT activity and free F2-isoprostanes. Plasma ALT activities were determined using an ALT kit (SGPT, Teco Diagnostics, Anaheim, CA). The levels of F2-isoprostanes were determined using gas chromatography/mass spectrometry as described by Morrow and Roberts (18). Briefly, plasma was added to HPLC (pH 3.0) water, and mixed by vortex. After centrifugation (2,500 × g for 3 min 4 °C), the F2-isoprostanes were extracted from the clear supernatants with C<sub>18</sub> Sep-Pak column and a silica Sep-Pak column. The F<sub>2</sub>-isoprostanes were then converted to pentfluorobenzyl esters and converted to trimethylsilyl ether derivatives, and the F2-isoprostanes levels were quantified by gas chromatography/mass spectrometry. An internal standard, 8-isoPGF2α-d<sub>4</sub> (Cayman Chemical, Ann Arbor, MI), was added to the samples at the beginning of extraction to correct yield of the extraction process. The amount of F2-isoprostanes was expressed as picograms of 8-iso-prostaglandin F<sub>2α</sub> per milliliter of plasma.

**Cytochrome c Determination—**Cytosolic and mitochondrial fractions were obtained as described above. Equal amounts of protein were then separated on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to Western blotting with an anti-cytochrome c antibody (Santa Cruz Biotechnology). Data were normalized to loading controls (I<sub>β</sub>B-α for the cytosolic fraction; ATPase for the mitochondrial fraction).

**RESULTS**

**Generation of Tg(GPX4) Mice—**Based on our prior experience, transgenic mice generated with a transgene containing endogenous human gene with large amounts of the 5′- and 3′-flanking sequences give expression patterns that mimic the endogenous mouse gene (19). A human P1 clone, containing the intact human GPX4 gene (about 3 kb) plus about 50 and 20 kb of 5′- and 3′-flanking sequence, respectively, was identified and used to generate transgenic mice (Fig. 1A). Tail DNA isolated from mice derived from the injected oocytes was digested with ScaI and probed with a 32P-labeled human GPX4 probe to detect the presence of human GPX4 gene (Fig. 1B). Two lines of transgenic mice, Tg(GPX4) mice, were generated in this study. They are designated as Tg6 and Tg5. Based on signal intensities analyzed by Southern blot, the Tg6 mice appear to have one copy of the transgene, and the Tg5 mice have three copies of the transgene (Fig. 1B).

**Overexpression of Gpx4 in Tissues from Tg(GPX4) Mice—**To detect the expression of the human GPX4 transgene, total RNA was isolated from tissues of the Tg(GPX4) mouse, and RT-PCR (reverse transcription PCR) was performed using a pair of PCR primers that specifically amplifies the human GPX4 mRNA. As indicated in Fig. 1C, human GPX4 mRNA is detected in heart, cerebral cortex, skeletal muscle, liver, kidney, and testes tissues from Tg6 mice and Tg5 mice (Fig. 1C), indicating that the human GPX4 transgene is indeed expressed in Tg(GPX4) mice. RNA from a human cell line EJ cells was used as a positive control to demonstrate the specificity of the RT-PCR reaction.

We next determined whether Tg(GPX4) mice had increased levels of total Gpx4 mRNA (expression from both the endogenous gene and transgene) in liver, cerebral cortex, and testes tissues using Northern blots. Fig. 2A shows that there were a 50% to 100% increase in Gpx4 mRNA levels in Tg6 mice compared with wild type mice and a 4- to 11-fold increase in Gpx4 mRNA levels in Tg5 mice compared with wild type mice.

To determine if the increased levels of Gpx4 mRNA were translated into increased levels of Gpx4 protein, the total Gpx4 protein levels in tissues from Tg(GPX4) mice were determined with an anti-Gpx4 antibody that recognizes both the murine and human Gpx4 protein. In Tg6 mice, the levels of Gpx4 protein in liver, kidney, heart, testes, and brain regions of...
cerebral cortex, hippocampus, and cerebellum were 50% higher than in the same tissues from wild type mice (Fig. 2B). The total Gpx4 protein levels in tissues from Tg5 mice were 2- to 3-fold higher than their non-Tg littermates (Fig. 2B).

Because Gpx4 is associated with different subcellular organelles, we compared the subcellular localization of Gpx4 in testes and liver of Tg5 and wild type mice. As shown in Fig. 3, there was a 2- to 3-fold increase in Gpx4 protein in nuclei, cytosol, mitochondria, and microsomal fractions from either the testes or liver of Tg5 mice. Thus, the Gpx4 protein level appears to be increased in all subcellular compartments in the Tg(GPX4) mice.

Gpx4 and Gpx1 are two ubiquitously expressed selenoprotein glutathione peroxidases. We next wanted to know whether overexpression of Gpx4 affects the activity of Gpx1 in tissues of Tg(GPX4) mice. Therefore, we measured the Gpx1 activity in liver, kidney, and cerebral cortex from Tg(GPX4) and wild type mice. As indicated in Fig. 4A, there was no significant alteration in Gpx1 activity in tissues from either Tg5 or Tg6 transgenic mice. Catalase is an anti-oxidant defense enzyme that detoxifies hydrogen peroxide. Therefore, we also measured catalase protein levels in tissues from wild type, Tg5, and Tg6 mice and found no difference (Fig. 4B).

We also measured the Cu/Zn-SOD and Mn-SOD activities in tissues from Tg(GPX4) and wild type mice, and we did not detect any significant differences between either Tg5 (or Tg6) and wild type mice (Fig. 4C and D). Thus, the overexpression of Gpx4 in either line of Tg(GPX4) mice had no significant effect on the expression of the other major antioxidant enzymes.

The above studies indicated that the GPX4 transgene is overexpressed in all cells. To determine if its expression is comparable to the endogenous murine Gpx4 gene, we asked if the transgene could rescue the null phenotype observed in the Gpx4 homozygous null embryos (Gpx4−/−) die at E7.5 to E8.5 (12). In this study, the Tg6 mice, which express Gpx4 at the lower level, were crossed to mice heterozygous for the murine Gpx4 gene (Gpx4+/-) to generate Gpx4−/− mice that have the human GPX4 transgene, i.e., Gpx4−/−/Tg(GPX4) mice. Male and female Gpx4−/−/Tg(GPX4) mice were then bred, and the offspring were genotyped for the presence of the GPX4 transgene, as well as for the status of the endogenous mouse Gpx4 gene. Fig. 5A

**FIG. 2.** Elevated Gpx4 levels in tissues from Tg(GPX4) mice. A, total Gpx4 mRNA levels in tissues from Tg(GPX4) mice and wild type mice as determined by Northern blotting. The data represent the mean ± S.E. of data obtained from the tissue of three mice, and the differences in levels of Gpx4 mRNA (wt versus Tg6, wt versus Tg5) were statistically significant (p < 0.05, Student’s t test). B, total Gpx4 protein levels in tissues from Tg(GPX4) mice and wild type mice as determined by Western blot. The data represent the mean ± S.E. of data obtained from the tissue of three mice, and the differences in levels of Gpx4 protein (wt versus Tg6, wt versus Tg5) were statistically significant (p < 0.05, Student’s t test).

**FIG. 3.** Elevated Gpx4 levels in subcellular fractions from Tg(GPX4) mice. Total Gpx4 protein levels in subcellular fractions from testes (A) and liver (B) of Tg5 (solid bar) and wild type (open bar) mice. The data represent the mean ± S.E. of data obtained from the tissue of three mice, and the differences in levels of Gpx4 protein between Tg5 and wild type mice were statistically significant (p < 0.05, Student’s t test).
shows an example of the genotyping results from two litters of new born mice. Gpx4−/− genotype was detected in mice that contained the human GPX4 transgene. Fig. 5B is the summary of the genotye information from 75 pups generated from this breeding scheme. Eighteen Gpx4−/− Tg(GPX4) mice were found in the 75 mice. The number of Gpx4−/− Tg(GPX4) genotypes was similar to the expected number, indicating that the human GPX4 transgene can completely replace the endogenous Gpx4 gene in mice without functional endogenous Gpx4 gene. These data also demonstrated that the human GPX4 transgene was properly expressed during development. The Gpx4−/− Tg(GPX4) mice develop into adulthood (6 months of age), and there is no obvious abnormality in these mice.

**Sensitivity of Embryonic Fibroblasts from Tg(GPX4) Mice to Cell Death Induced by Oxidative Stress**—MEFs were derived from wild type and Tg(GPX4) embryos, and MEFs from Tg6 and Tg5 lines of Tg(GPX4) mice showed 0.5- and 2-fold increases, respectively, in Gpx4 protein levels over MEFs from wild type mice (data not shown). Because t-BuOOH is an oxidizing agent that is known to generate lipid peroxidation (20), MEFs were exposed to various concentrations of t-BuOOH. Data in Fig. 6A show that MEFs from Tg(GPX4) mice had significantly higher survival rates than MEFs from wild type mice after exposure to t-BuOOH. The data in Fig. 6A also indicate that MEFs from the Tg5 line of Tg(GPX4) mice had higher survival rates than MEFs from Tg6 line, presumably due to their higher levels of Gpx4. We also compared the sensitivity of MEFs from the Tg(GPX4) mice to superoxide anions by incubating MEFs with the bipyridyl herbicide diquat, which generates superoxide (21). As shown in Fig. 6B, MEFs from Tg(GPX4) mice had significantly higher survival rates than MEFs from wild type mice after diquat treatment. Again, MEFs from the Tg5 line of Tg(GPX4) mice had higher survival rates than MEFs from Tg6 line. To determine if the differences in cell survival was due to the MEFs from the Tg(GPX4) mice having reduced apoptosis in response to t-BuOOH treatment, we compared the activity of caspase-3 in MEFs from Tg(GPX4) mice (Tg5) and wild type mice. MEFs from Tg(GPX4) mice had a lower basal level of caspase-3 activity compared with cells from wild type mice, and treatment with t-BuOOH increased caspase-3 activity in MEFs from both Tg(GPX4) and wild type mice. However, caspase-3 activity was significantly lower in the MEFs from the Tg(GPX4) mice than MEFs from the wild type mice at 3 h after treatment with t-BuOOH (Fig. 6C). Thus, the reduced cell death of MEFs from Tg(GPX4) mice appears to be associated with reduced apoptosis.

**Sensitivity of Tg(GPX4) Mice to Diquat**—The bipyridyl herbicide diquat is a potent pro-oxidant that generates superoxide anions through redox cycling (21). When injected into mice, diquat causes liver damage associated with lipid peroxidation (22), and diquat has been used to study the protective role of antioxidant enzyme against oxidative stress in mice (23). Therefore, we compared the responses of Tg(GPX4) and wild type mice to diquat treatment. Plasma levels of ALT activity was studied as a marker of liver damage. In untreated mice, no difference in plasma ALT levels was observed between Tg(GPX4) mice and wild type mice (Fig. 7A). Diquat injection resulted in a 6.1-fold increase in plasma ALT activity in wild type mice compared with a 2.2-fold increase in the Tg(GPX4) mice. Thus, the Tg(GPX4) mice showed less liver damage (i.e. decrease in plasma ALT activity) in response to diquat treatment than wild type mice. Diquat also induced lipid peroxidation as measured by plasma F2-isoprostane levels (24). Isoprostanes are a group of prostaglandin-like compounds that arise from free radical attack on membrane phospholipids, and the level of isoprostanes is an excellent marker of lipid peroxidation (25). The data in Fig. 7B show that the plasma F2-isoprostane levels were similar in untreated Tg(GPX4) mice and wild type mice; however, after diquat treatment, Tg(GPX4) mice had significantly lower levels of F2-isoprostanes (29% lower) than wild type mice, indicating that diquat-induced lipid peroxidation was reduced in the Tg(GPX4) mice.
Because our studies with MEFs overexpressing Gpx4 showed that reduced apoptosis was correlated to reduced induction of cell death and because other laboratories showed cells transfected with Gpx4 had a decline in apoptosis induced by a variety of oxidative stressors (26–28), we next examined whether the Tg(GPX4) mice showed reduced apoptosis in response to diquat treatment. We first measured the levels of caspase-3 in the livers of the diquat-treated mice, because it is a very important downstream caspase that is activated by cleavage of upstream caspases (29) and because its activation is a good marker of apoptosis. The levels of activated caspase-3 in liver tissue from wild type and Tg(GPX4) mice were determined by Western blot. As indicated in Fig. 8, diquat treatment resulted in cleavage of pro-caspase-3 (p32) to activated caspase-3 (p20). There was no difference in activated caspase-3 (p20) in livers from untreated Tg(GPX4) and wild type mice. However, diquat treatment induced a 7.3-fold increase in activated caspase-3 in liver tissue from wild type mice (Fig. 8, A and C), compared with a 1.8-fold increase in Tg(GPX4) mice (Fig. 8, B and C). Thus, it appears that the Tg(GPX4) mice show reduced apoptosis in liver after diquat treatment.

We also measured cytochrome c release from mitochondria in the liver after diquat treatment, because cytochrome c release from mitochondria is a very important event in the initiation of apoptosis through the endogenous apoptosis pathway. As shown in Fig. 9 (A and B), diquat treatment resulted in an increase in cytochrome c in cytosol fraction of the liver of both wild type and Tg(GPX4) mice. In wild type mice, there was a 3.6-fold increase in cytosolic cytochrome c levels after diquat treatment. However, there was only a 1.4-fold increase in cy-
tochrome c levels in cytosolic fraction of the liver of Tg(GPX4) mice (Fig. 9, C). We also compared the cytochrome c levels in liver mitochondria before and after diquat treatment (Fig. 9, D–F). As expected, there was 51% loss of cytochrome c levels in liver mitochondria of wild type mice after diquat treatment (Fig. 9, D and F). However, no significant loss of cytochrome c in mitochondria fraction of liver tissue from diquat-treated Tg(GPX4) mice was detected (Fig. 9, E and F). Thus, cytochrome c release from mitochondria into cytosol after diquat treatment was suppressed in liver tissues from Tg(GPX4) mice.

**DISCUSSION**

In 2003, we described the first mouse model with a targeted mutation in the Gpx4 gene (12). The homozygous null mutation (Gpx4<sup>−/−</sup>) is lethal: Gpx4<sup>−/−</sup> embryos die at embryonic stage E7.5 to E8.5. Among the glutathione peroxidase genes, knockout mice have been generated for the Gpx1, Gpx2, and Gpx4 genes. The phenotype of Gpx4 null mutation is much more severe than the phenotypes reported for null mutations in the other glutathione peroxidases. For example, mice null for the Gpx1 (30) and Gpx2 (31) genes appear normal under normal housing conditions, although they tend to be more sensitive to oxidative stress. More recently, knockout mice for catalase were generated, and these mice null for catalase appear normal as well (32). Thus, based on observations of the phenotypes of knockout mice null for Gpx1, Gpx2, and catalase, which detoxify hydrogen peroxide and alkyl peroxides (for the glutathione peroxidases) but not the complex lipid hydroperoxides found in membranes, the embryonic lethal phenotype of Gpx4 knockout mice was the first mouse model to demonstrate that Gpx4 null mutation is much more severe than the phenotypes of null mutations in the other glutathione peroxidases.

![Reduction in liver damage and lipid peroxidation in Tg(GPX4) mice](image1.png)

**FIG. 7.** Reduced liver damage and lipid peroxidation in Tg(GPX4) mice. Tg(GPX4) mice (line Tg5) and wild type mice were treated with diquat (50 mg/kg) for 6 h, and plasma ALT activity (A) and isoprostane levels (B) were determined as described under “Experimental Procedures.” The values are presented as the mean ± S.E. of data collected from four mice. The increases in plasma levels of ALT activity and isoprostane levels after diquat treatment in both the Tg(GPX4) mice and wild type mice, and the differences in levels of ALT activity and isoprostane levels between Tg5 mice and wild type mice after diquat treatment are statistically significant (p < 0.05, Student’s t test).

![Caspase-3 activation in liver of diquat-treated Tg(GPX4) mice](image2.png)

**FIG. 8.** Caspase-3 activation in liver of diquat-treated Tg(GPX4) mice. Tg(GPX4) mice (line Tg5) and wild type mice (four per group) were treated with diquat (50 mg/kg) for 6 h, and caspase-3 activation was measured as described under “Experimental Procedures.” A, Western blot showing p32 (pro-caspase-3) and p20 (a subunit of activated caspase-3) in wild type mice with or without diquat treatment. B, Western blot showing p32 (pro-caspase-3) and p20 (a subunit of activated caspase-3) in Tg5 mice with or without diquat treatment. C, the levels of p20 in wild type and Tg5 mice after diquat treatment as determined from the Western blots. The p20 level in control (untreated) mice is assigned as 1 arbitrarily, and relative level in treated mice is calculated and expressed as mean ± S.E. The increases in p20 after diquat were statistically significant in both the Tg5 mice and wild type mice, and the differences in levels of p20 between Tg5 mice and wild type mice after diquat treatment were statistically significant also (p < 0.05, Student’s t test).
mouse points to the critical importance of Gpx4 in repairing membrane lipid hydroperoxides.

Over the past 8 years, several investigators have studied the effect of overexpressing Gpx4 in various cell lines. For example, the overexpression of Gpx4 in RBL2H3 and 104C1 cells has been shown to reduce ROS-induced toxicity (33, 34). Gpx4, especially mitochondrial Gpx4, also has been suggested to play a role in the protection against apoptosis. Potassium cyanide-induced lipid peroxidation and apoptosis are effectively prevented in RBL2H3 cells overexpressing mitochondrial Gpx4 (26). Furthermore, RBL2H3 cells overexpressing mitochondrial Gpx4 are resistant to apoptosis induced by 2-deoxyglucose, etoposide, staurosporine, UV irradiation, cycloheximide, and actinomycin D, but not to apoptosis induced by Fas-specific antibodies (35). Mitochondrial Gpx4 also suppresses apoptosis induced by oxidized low density lipoprotein in rabbit aortic SMC (27), by photosensitizers in human breast cancer cells (28), and by cholesterol hydroperoxide in breast tumor cell lines (36).

Although studies using mammalian cell lines overexpressing Gpx4 indicate that Gpx4 can protect cells from oxidative stress and oxidative stress induced apoptosis, there is no information on the effect of overexpressing Gpx4 in tissues of whole animals. Therefore, we generated transgenic mice overexpressing Gpx4. Because Gpx4 is an evolutionarily conserved gene (the human and mouse protein are 95% identical), transgenic mice...
were produced using the human GPX4 gene with large amounts of the 5'- and 3'-flanking sequences. Our data show that the human GPX4 transgene is expressed in a tissue-specific, subcellular pattern that is similar to the endogenous mouse GPx4 gene and that the human GPX4 transgene rescues the lethal phenotype of the Gpx4 null mutation. Therefore, the expression of the human GPX4 transgene in the transgenic mice that we have generated appears to closely mimic the expression of the endogenous murine Gpx4 gene.

Using MEFs from Tg(GPX4) mice, we found that overexpression of Gpx4 increased survival after oxidative stress insult compared with MEFs from wild type mice and that the increased survival was correlated to reduced apoptosis. This observation is consistent with findings from our previous study using MEFs from Gpx4<sup>−/−</sup> mice in which we showed that MEFs from Gpx4<sup>−/−</sup> mice have reduced cell survival and increased apoptosis compared with MEFs from wild type mice upon oxidative stress (13). This result also is consistent with previous studies by other laboratories showing that the overexpression of Gpx4 is protective against oxidative injuries in several cell types (26, 27, 37). To extend our studies with MEFs from the Tg(GPX4) mice to the whole animal, we compared the extent of oxidative induced injury in the liver of Tg(GPX4) and wild type mice using a diquat model. Diquat, a potent pro-oxidant, which generates superoxide anions through redox cycling (21), induces lipid peroxidation and liver damage in mice (22, 23). We found that the plasma F<sub>2</sub>-isoprostane level, a marker of lipid peroxidation, was significantly reduced in diquat-treated Tg(GPX4) mice compared with diquat-treated wild type mice. Gpx4 overexpression also reduced diquat-induced hepatotoxicity, as indicated by the reduced plasma activity of ALT. To compare the level of apoptosis between the livers of Tg(GPX4) and wild type mice after diquat treatment, we measured the activation of caspase-3, because caspase-3 is a downstream effector caspase of apoptosis, and its activation indicates the commitment of cells to apoptosis (29). We found very little activated caspase-3 (p20) in liver tissues of untreated Tg(GPX4) and wild type mice. Diquat treatment induced caspase-3 activation in both Tg(GPX4) and wild type mice. However, the activation of caspase-3 was significantly less in Tg(GPX4) mice, e.g., a 1.8-fold increase of activated caspase-3 in Tg(GPX4) mice versus a 7.3-fold increase in wild type mice. Therefore, the Tg(GPX4) mice showed reduced apoptosis in liver after diquat treatment. This is the first evidence showing that the overexpression of Gpx4 prevents oxidative stress induced apoptosis in vivo.

Because Gpx4 appears to protect cells/tissues from oxidative stress-induced apoptosis, we studied the effect of overexpressing Gpx4 on the mitochondrial pathway of apoptosis. The release of cytochrome c (cyt. c) from mitochondria is a key step in the initiation apoptosis by the activation of caspase-9. cyt. c is strongly associated with cardiolipin (CL) (38), a phospholipid found exclusively in the inner mitochondrial membrane that makes up the bulk of the mitochondrial phospholipids. Because of its high content of polyunsaturated fatty acids, especially linoleic acid (39), CL is prone to oxidation into cardiolipin hydroperoxide (CLOOH). The oxidation of CL to CLOOH has been shown to be a 1.8-fold fold increase of activated caspase-3 in Tg(GPX4) mice prevented oxidative stress induced apoptosis. The mice showed reduced apoptosis in liver after diquat treatment.

In summary, we have generated transgenic mice that overexpress Gpx4 in all tissues in a manner that mimics the expression of the endogenous Gpx4 gene. Because the Tg(GPX4) mice show no alterations in the activities of the other major antioxidant enzymes, especially Gpx1 and catalase, the primary phenotype of the Tg(GPX4) mice appears to be their ability to remove complex lipid hydroperoxides found in membranes. Thus, the Tg(GPX4) mouse provides investigators with a valuable animal model for studying the role of membrane lipid peroxidation in the mechanisms of a variety of pathological and physiological conditions. For example, our data demonstrate that overexpression of Gpx4 protects the liver in vivo against apoptosis induced by oxidative stress through a mechanism that prevents cyt. c release from mitochondria through the detoxification of CLOOH in mitochondrial membrane.

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