The Nature of Antioxidant Defense Mechanisms: A Lesson from Transgenic Studies

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Reactive oxygen species (ROS) have been implicated in the pathogenesis of many clinical disorders such as adult respiratory distress syndrome, ischemia–reperfusion injury, atherosclerosis, neurodegenerative diseases, and cancer. Genetically engineered animal models have been used as a tool for understanding the function of various antioxidant enzymes in cellular defense mechanisms against various types of oxidant tissue injury. Transgenic mice overexpressing three isoforms of superoxide dismutase, catalase, and the cellular glutathione peroxidase (GSHPx-1) in various tissues show an increased tolerance to ischemia–reperfusion heart and brain injury, hyperoxia, cold-induced brain edema, adriamycin, and paraquat toxicity. These results have provided for the first time direct evidence demonstrating the importance of each of these antioxidant enzymes in protecting the animals against the injury resulting from these insults, as well as the effect of an enhanced level of antioxidant in ameliorating the oxidant tissue injury. To evaluate further the nature of these enzymes in antioxidant defense, gene knockout mice deficient in copper–zinc superoxide dismutase (CuZnSOD) and GSHPx-1 have also been generated in our laboratory. These mice developed normally and showed no marked pathologic changes under normal physiologic conditions. In addition, a deficiency in these genes had no effects on animal survival under hyperoxia. However, these knockout mice exhibited a pronounced susceptibility to paraquat toxicity and myocardial ischemia–reperfusion injury. Furthermore, female mice lacking CuZnSOD also displayed a marked increase in postimplantation embryonic lethality. These animals should provide a useful model for uncovering the identity of ROS that participate in the pathogenesis of various clinical disorders and for defining the role of each antioxidant enzyme in cellular defense against oxidant-mediated tissue injury. — *Environ Health Perspect* 106(Suppl 5):1219–1228 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1219-1228ho/abstract.html*

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Reactive oxygen species (ROS), which are produced as by-products of normal metabolism, are capable of causing cellular damage, leading to cell death and tissue injury (*1*). Mammalian cells are equipped with both enzymatic and nonenzymatic antioxidant defense mechanisms to minimize the cellular damage resulting from interactions between cellular constituents and ROS [for review see Forman and Fisher (*2*)]. The enzymatic antioxidant mechanism contains various forms of superoxide dismutases (SOD), catalase, and glutathione peroxidase (GSHPx), as well as the enzymes involved in the recycling of oxidized glutathione, such as glutathione reductase and glucose 6-phosphate dehydrogenase, a major enzyme in the pentose phosphate pathway for the generation of NADPH.

Despite the presence of these delicate cellular antioxidant systems, an overproduction of ROS in both intra- and extracellular spaces often occurs upon exposure of cells or individuals to radiation, hyperoxia, and certain chemicals. In mammals, the activated phagocytic cells present in local tissue inflammation, as a result of cell injury from environmental insults or various diseases, can also contribute to an increased production of ROS. An unbalanced production of ROS in localized compartments has been postulated to play a role in the pathogenesis of a number of clinical disorders such as adult respiratory distress syndrome, ischemia–reperfusion injury, atherosclerosis, neurodegenerative diseases, and cancer [for review see Cross (*3*)]. This understanding illustrates the importance of the antioxidant defense system in maintaining normal cellular physiology. However, because of the overlapping activity among some of the antioxidant enzymes, as well as the different intra- and extracellular sites of ROS production and expression of antioxidant enzymes, it is generally difficult to clearly define the physiologic role of each of these enzymes. For example, three distinct isoforms of the superoxide scavenging enzyme, SOD, are present in the mammals. The copper–zinc SOD (CuZnSOD) is predominantly expressed in the cytosol and the manganese SOD (MnSOD) is located in the mitochondria of the cells (*4,5*). In addition, a form of extracellular SOD is present in the intravascular and extracellular fluids such as plasma, lymph, and synovial fluid (*6–8*). Although the protective role of each SOD isozyme has been postulated according to its cellular site of expression, direct evidence demonstrating their role *in vivo* in antioxidant defense mechanism is very limited.

The enzyme GSHPx, which contains selenium at the active site, involves the same complication. GSHPx is believed to play an important role in cellular antioxidant defense by reducing H_2O_2 and various

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Abbreviations used: CuZnSOD, copper–zinc superoxide dismutase; ECSOD, extracellular superoxide dismutase; ES, embryonic stem; GSH, glutathione, reduced form; GSHPx, glutathione peroxidase; GSHPx-1, cellular GSHPx; MnSOD, manganese superoxide dismutase; 'NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; superoxide, superoxide anion radicals.

hydroperoxides using glutathione (GSH) as a reducing agent to form water and corresponding alcohols, respectively ($H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ or $ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$). There are at least four isoforms of GSHPx found in mammals. The major cellular GSHPx (GSHPx-1) is expressed in all tissues and contributes to most of the GSHPx activity present in erythrocytes, kidney, and liver (*9*). The plasma GSHPx is detected in milk, plasma (0.149 U/ml plasma), and lung alveolar fluid (3.6 mU/ml of 25-fold concentrated lavage fluid) of humans (*10,11*). The phospholipid–hydroperoxide GSHPx, which is capable of reducing hydroperoxides of phospholipids and cholesterol, is found mainly in the testis (*12–14*). The recently reported gastrointestinal tract GSHPx is expressed predominantly in liver, intestine, and colon (*15,16*). Although the physiologic relevance of GSHPx has been implicated from the studies performed on animals fed a selenium-deficient diet (*17*), due to the effect of selenium deficiency on other selenium-containing proteins such as thioredoxin reductase (*18*), these studies are hardly conclusive.

Our laboratory and several others have been interested in elucidating the function of antioxidant enzymes using transgenic mouse models in which the expression of these enzymes is altered by transgenic or gene targeting technology. This report summarizes some of the physiologic consequences from the increased activity of various antioxidant enzymes in transgenic animals, as well as our recent work on generation and characterization of knockout mice deficient in CuZnSOD and GSHPx-1.

Altered Responses of Transgenic Mice Overexpressing Various Antioxidant Enzymes to Acute Oxidant Toxicity

Transgenic mice overexpressing various antioxidant enzymes have been generated by several laboratories during the past several years. The altered responses of these mice to a variety of oxidant-mediated tissue injury are summarized in Table 1.

Copper–Zinc Superoxide Dismutase. Lines of CuZnSOD transgenic mice were initially generated by Epstein and colleagues using a piece of 14.5-kb genomic fragment containing the entire human CuZnSOD gene (*19*). Overexpression of CuZnSOD activity to various levels has been found in several major organs including the brain, heart, lung, and liver. The enhanced CuZnSOD activity protects animals against tissue injury induced by a variety of pathogenic conditions that include cold-induced brain edema and infarction (*20*), focal cerebral ischemic injury (*21*), neurotoxicity of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (*22*), hyperoxic lung injury (*23*), alloxan- or streptozotocin-induced diabetes (*24*), and myocardial ischemia–reperfusion injury (*25*). These data clearly indicate the role of superoxide anion radicals, which are generated in or diffused to the cytosolic compartment under these pathogenic conditions, in causing cell and tissue injury, and the effect of the enhanced CuZnSOD activity in cellular protection.

However, in other studies the overexpressed CuZnSOD caused certain pathologic changes and exacerbated oxidant-mediated tissue injury. Mice with elevated CuZnSOD activity exhibit abnormal neuromuscular junction in their tongues (*26*), impairment of muscle function (*27*), thymus and bone marrow abnormalities (*28*), a decreased serotonin uptake by platelets (*29*), and a retarded rate in secretion of prostaglandin by kidney and cerebellum (*30*). In addition, these mice are also more susceptible to kainic acid-induced excitotoxicity in neurons than control mice (*31*). It is not clear whether the detrimental effect of CuZnSOD overexpression is a result of the associated free radical-generating activity of this enzyme or of its capability in enhancing nitration of tyrosine by peroxynitrite (*32,33*).

Manganese Superoxide Dismutase. We have generated transgenic mice using a human MnSOD expression construct (*34,35*). Transcription of this transgene is controlled by the 5′ flanking sequence and promoter of the human β-actin gene. Overexpression of MnSOD activity was found in the heart, lung, brain, muscle, kidney, spleen, and eye. The overexpressed MnSOD was further localized by immunocytochemical staining to the mitochondria of transgenic cardiomyocytes and various types of lung cells. Because mitochondrion is one of the major subcellular sites capable of generating superoxide anion radicals, it is expected that an enhanced MnSOD activity will be protective in the animals against certain oxidant-mediated tissue injury. Indeed, these transgenic mice are more resistant to cardiotoxicity after treatment with adriamycin, a quinone-containing anthracycline antibiotic, and heart injury resulting from ischemia–reperfusion (*36,37*). However, the overexpressed MnSOD in lung alveolar type I and type II cells, capillary endothelial cells, and fibroblasts does not significantly prolong the survival of transgenic mice compared to nontransgenic littermates upon exposure to greater than 99% oxygen (*35*). This result contradicts that reported by Wispe and colleagues (*38*). In their studies, transgenic mice with an overexpression of MnSOD in alveolar type II cells and nonciliated bronchiolar epithelial cells resulting from the expression of a human surfactant protein C regulatory sequence-driven transgene are markedly more resistant to greater than 99% oxygen than controls. The reason for the discrepancy in the protective effect of MnSOD in these transgenic mice is not understood. One possibility is that the magnitude of increase in MnSOD immunolabeling density in mitochondria of alveolar type II cells in Wispe's transgenic mice (400%) is slightly higher than that found in our homozygous transgenic mice (350%). Alternatively, it may result from the fact that different strains of mice are being used in Wispe's laboratory (FVB/N) and our laboratory (B6C3 hybrid).

Extracellular Superoxide Dismutase. The same human β-actin expression vector has also been employed to overexpress the human extracellular superoxide dismutase (ECSOD) in the brain and heart of transgenic mice (*39*). Surprisingly, ECSOD transgenic mice exhibit an increased susceptibility to oxygen toxicity in the central nervous system induced by hyperbaric oxygen compared to that of nontransgenic littermates. In this study, inhibitor of nitric oxide synthase, an N_ω-nitro-L-arginine methyl ester, protects both nontransgenic and transgenic mice against hyperbaric

Table 1. Altered responses of transgenic mice overexpressing antioxidant enzymes to various pathogenic conditions.

Overexpressed enzyme	Hyperoxia	Cold-induced brain edema	Hyperbaric oxygen	Adriamycin	Hydrogen peroxide	Ischemia–reperfusion	
						Brain	Heart
CuZnSOD	↑ (*23*)	↑ (*20*)	ND	ND	ND	↑ (*21*)	↑ (*25*)
MnSOD	–(*35*), ↑ (*38*)	ND	ND	↑ (*36*)	ND	ND	↑ (*37*)
ECSOD	ND	↑ (*40*)	↓ (*39*)	ND	ND	ND	↑ (*41*)
Catalase	ND	ND	ND	↑ (*42*)	ND	ND	ND
GSHPx-1	–(*43*)	ND	ND	ND	↑ (*45*)	↑ (*49*)	↑ (*44*)

↑, more tolerant; ↓, more susceptible; –, no changes; ND, not determined.

oxygen toxicity. These results suggest that nitric oxide (•NO) is an important pathogenic mediator in tissue injury during exposure of animals to hyperbaric oxygen. It is possible that the enhanced brain activity of ECSOD in transgenic mice causes a decrease in the concentration of superoxide anion radicals. This would prevent the reaction between superoxide and •NO to form peroxynitrite, resulting in an increase in •NO content and •NO-mediated toxicity. On the other hand, ECSOD overexpression does provide protection to the animals against cold-induced brain edema and myocardial ischemia–reperfusion injury (*40,41*).

Catalase. Transgenic mice carrying a rat catalase transgene driven by the 5′ flanking sequence and promoter of the mouse α-myosin heavy chain gene have also been established (*42*). As expected, the regulatory sequence of α-myosin heavy chain gene specifically directs expression of the transgene in the heart. Further studies have shown that the increased catalase activity attenuates adriamycin-induced cardiotoxicity in these animals.

Cellular Glutathione Peroxidase. We have also generated transgenic mice using the entire mouse GSHPx-1 gene contained in a piece of 5.3-kb genomic fragment (*43*). Overexpression of GSHPx-1 was found in the brain, heart, lung, and muscle but not in liver and kidney, where a high level of expression of the endogenous GSHPx-1 enzyme is evident. Although GSHPx-1 transgenic mice show no increased tolerance to hyperoxic exposure (*43*), they are more tolerant to ischemia–reperfusion heart injury (*44*), H_2O_2-induced DNA strand breakage in lens epithelial cells (*45*), and paraquat toxicity (*46*). In addition to our studies, Mirochnitchenko et al. (*47*) have also generated transgenic mice carrying a GSHPx-1 transgene driven by the promoter of the mouse hydroxy-methylglutaryl-coenzyme A reductase gene. Increases in GSHPx activity of 4-fold and 1-fold were found in the brain and skin of transgenic mice, respectively. Interestingly, these transgenic mice exhibit an increased susceptibility to hyperthermia and skin carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene and phorbol ester (*47,48*). However, recent results have shown that these mice are more resistant to focal cerebral ischemia–reperfusion injury (*49*).

Conclusions and Perspectives. Our results and those of others (*19–49*) have shown that the enhanced expression of antioxidant enzymes can protect animals against a variety of tissue injury resulting from different oxidant insults. These data have not only confirmed the physiologic function of antioxidant enzymes in defending cells and tissues against oxidant injury but have also suggested the identity of the injurious ROS in each of the pathogenic conditions. For example, mouse hearts overexpressing either one of the SOD isozymes or GSHPx-1 are more tolerant to ischemia–reperfusion injury than those of controls (*25,37,41,44*). These data clearly indicate that the endogenous level of the corresponding antioxidant enzyme is not adequate to detoxify the increased level of ROS that are generated during the reperfusion period, and that myocardial injury is mediated by superoxide anion radicals, H2O2, and/or fatty acid hydroperoxides.

The pathologic changes found in CuZnSOD overexpressing mice reported by Groner and colleagues (*26–31*) are also informative with respect to the functional role of this enzyme. Their studies have shown that overexpression of CuZnSOD results in a chronic oxidative stress (*27,31*). The detrimental effect of CuZnSOD may be derived from its activity in generating hydroxyl radicals using H_2O_2 as a substrate (*32*). This understanding may provide a potential explanation for the pathologic changes observed in patients with Down syndrome, in whom the extra copy of chromosome 21 gives rise to a 50% increase of CuZnSOD activity. Furthermore, these results may exclude the use of CuZnSOD in antioxidant therapy for treatment of oxidant-mediated disorders.

In other cases, overexpression of a single antioxidant enzyme may not be protective in the animals. As mentioned previously, the MnSOD transgenic mice generated in our laboratory show no significant increase in tolerance to hyperoxia (*35*). However, the transgenic mice created by Wispe and colleagues (*38*) with lung-specific overexpression of MnSOD are markedly resistant to hyperoxic exposure. The discrepancy of these results may result from the fact that different strains of mice are used in the transgenic studies as well as from the potential difference in the level of MnSOD overexpression in the lung cells. These speculations also bring out three critical issues in the transgenic studies. First, different genetic determinants in different strains of mice may interact directly or indirectly with the overexpressed proteins to affect the physiologic outcome found in the transgenic mice. Second, the critical role of each antioxidant enzyme in defending cells and tissues against oxidant injury may differ in various tissues and is dependent on the type of oxidant injury. Third, it is impossible to precisely control the specificity of transgene expression in the transgenic mice. Consequently, the level of gene overexpression may vary in different types of cells in a particular tissue. Therefore, a negative result may not be conclusive as to the protective role of an antioxidant enzyme when its expression is enhanced. This also illustrates the limitation associated with the studies using transgenic animals. Toward this end, we and other laboratories have developed lines of knockout mice carrying null mutations in the genes coding for several antioxidant enzymes. Because the defective gene will affect every single type of cell in the animals, the knockout mice may provide an insightful alternative for defining the nature of each antioxidant enzyme in cellular defense mechanisms. If the activity of a particular antioxidant enzyme is critical for protecting the animals against certain oxidants, a diminished or decreased enzyme activity as a result of gene disruption is expected to confer a sensitive phenotype in the animals.

Mice Lacking CuZnSOD Show a Marked Increase in Sensitivity to Acute Paraquat Toxicity and Postimplantation Embryonic Death

Generation and Characterization of CuZnSOD-Deficient Mice. As shown in Figure 1A, exon 5 of the mouse *Sod1* gene and some of the flanking intron sequences were replaced with the neomycin resistance cassette (neo) (*50,51*). This also creates a new PstI restriction site, resulting in a shorter *PstI* genomic fragment from the targeted allele (12.5 kb) than from the wild-type allele (16.5 kb). The targeting vector was linearized with HindIII digestion and transfected into R1 embryonic stem (ES) cells [(*52*); provided by Dr. Andras Nagy, Mount Sinai Hospital, Toronto, Canada]. The homologous recombinant ES cells were identified by Southern blot analysis probed with a 5′ DNA fragment external to the genomic sequence present in the targeting vector. Chimeric mice were then generated by the standard gene-targeting technique (*53*) and used in breeding with C57Bl/6 female mice to obtain heterozygous knockout mice. Mice heterozygous ($Sod1^{+/-}$) for the targeted allele were interbred to generate homozygous knockout ($Sod1^{-/-}$) mice. An example of the DNA blot analysis of mouse DNA is shown in Figure 1B. In addition to the 16.5-kb wild-type and

12.5-kb targeted genomic fragments, the 5′ external probe containing exon 1 sequence also hybridized with a *PstI* fragment of 6.6 kb. This is believed to represent cross-hybridization between the probe and the mouse *Sod1* pseudogene(s) (*54*).

Expression study was then performed to determine the effect of gene disruption on CuZnSOD expression. As expected, an approximate 40 to 60% reduction of CuZnSOD mRNA was found in tissues of $Sod1^{+/-}$ mice compared with that found in tissues of wild-type ($Sod1^{+/+}$) mice (Figure 1C); virtually no CuZnSOD mRNA could be detected in $Sod1^{-/-}$ mouse tissues. Reduction of CuZnSOD activity in tissues of $Sod1^{+/-}$ and $Sod1^{-/-}$ mice was also confirmed by enzyme activity assay (Table 1). The very low level of CuZnSOD activity in the lung samples of $Sod1^{-/-}$ mice presumably represents the activity of ECSOD, as expression of this SOD isoform is relatively high in the lung compared with that in other tissues (*55*). A decrease in CuZnSOD activity apparently had no effect on the expression of other cellular antioxidant enzymes, including MnSOD (Table 2), catalase, and GHSPx (data not shown).

CuZnSOD Plays a Critical Role in Cellular Defense against Paraquat Toxicity. The ratio of three genotypes of mice obtained from the interbreeding of $Sod1^{+/-}$ mice was in agreement with Mendelian inheritance, indicating that there was no lethality in development of $Sod1^{-/-}$ mice. Male and female $Sod1^{-/-}$ mice grew normally and apparently were healthy on observation to 18 months of age. No abnormalities were found by routine histopathologic tudies in tissues of CuZnSOD-deficient mice at 4.5 months of age including the brain, heart, intestine, kidney, liver, lung, testis, uterus, and ovary (data not shown).

We next determined whether a deficiency in pulmonary CuZnSOD activity would render animals more susceptible to hyperoxic exposure, as transgenic mice overexpressing this enzyme survived longer than control mice under hyperoxic exposure (*23*). A virtually identical survival curve with a median survival time of 3.4 ± 0.1 (SE) days was found in both $Sod1^{+/+}$ and $Sod1^{-/-}$ mice, indicating that the function of this enzyme in lung defense against the damage from increased production of superoxide anion radicals during hyperoxia is very limited. However, the $Sod1^{-/-}$ mice showed a pronounced sensitivity to paraquat at a dose of 10 mg/kg bw, with a median survival time of 1.4 ± 0.3 (SE) days

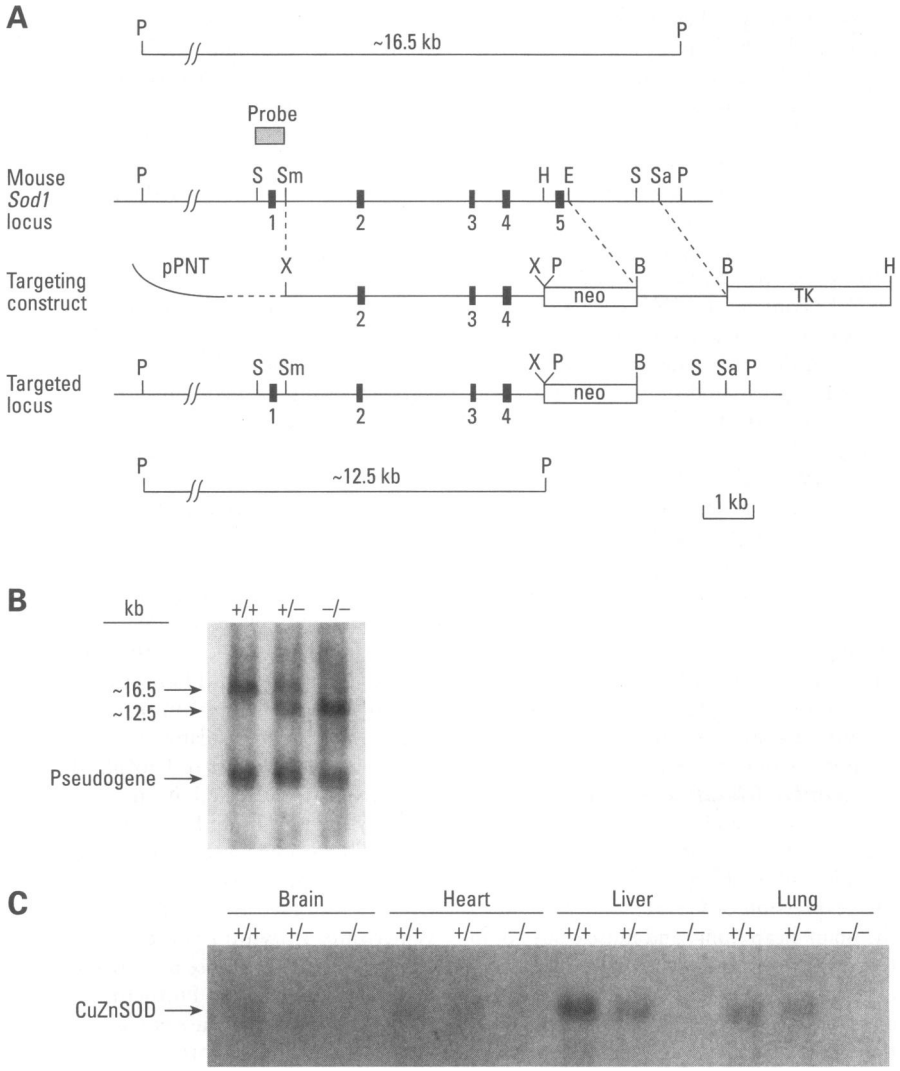

Figure 1. Generation and characterization of CuZnSOD-deficient mice. (*A*) Schematic showing the genomic and partial restriction map of the mouse *Sod1* locus (top), the targeting vector (middle), and the predicted structure of the targeted locus. Numbered black boxes represent exons. Striped box represents the 5′ external sequence used as a hybridization probe. B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SacI; Sa, SalI; Sm, SmaI; neo, neomycin resistance cassette; TK, herpes thymidine kinase gene under the transcriptional control of the 5′ flanking sequence and promoter of the mouse phosphoglycerate kinase-1 gene. The sizes of hybridizing *PstI* restriction fragments of the wild-type and the targeted alleles are shown on the top and bottom of the figure, respectively. (*B*) DNA blot analysis of mouse offspring. Mouse tail DNA was digested with PstI and probed with the 5′ external probe shown in (*A*). +/+, +/−, and −/− represent wild-type, heterozygous, and homozygous knockout mice, respectively. (*C*) RNA blot analysis of total cellular RNA isolated from tissues of $Sod1^{+/+}$, $Sod1^{+/-}$ and $Sod1^{-/-}$ mice. Twenty-five micrograms of total RNA from each tissue were separated on agarose gel for blot analysis. The RNA blot was hybridized with a rat CuZnSOD cDNA probe.

Table 2. Superoxide dismutase activities in tissues of $Sod1^{+/+}$, $Sod1^{+/-}$, and $Sod1^{-/-}$ mice.[a]

Enzyme	*Sod1* genotype	Brain	Liver	Lung
CuZnSOD	+/+	23.0 ± 5.4	61.2 ± 15.9	21.9 ± 1.7
	+/−	10.7 ± 1.2*	28.5 ± 4.5**	11.4 ± 1.3***
	−/−	ND†	ND†	0.6 ± 0.4†
MnSOD	+/+	11.3 ± 3.1	13.0 ± 2.7	9.4 ± 0.9
	+/−	11.1 ± 2.2	12.9 ± 3.1	9.5 ± 1.3
	−/−	11.4 ± 1.4	11.5 ± 1.3	8.3 ± 1.1

ND, CuZnSOD activity was not detected. [a]Values are means ± SD, and $n = 5$ for all tissue samples. *$p < 0.01$ when comparing +/+ to +/− mice. **$p < 0.001$ when comparing +/+ to +/− mice. ***$p < 0.0001$ when comparing +/+ to +/− mice. †$p < 0.0001$ when comparing +/+ to −/− mice, or +/− to −/− mice.

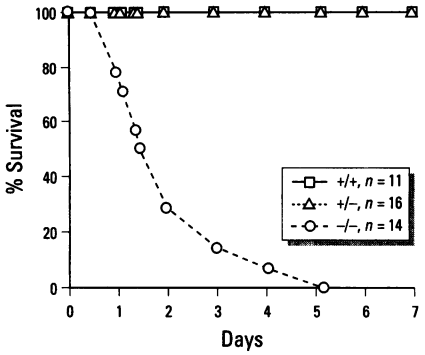

Figure 2. Increased susceptibility of *Sod1*$^{-/-}$ mice to paraquat. The survival times of age-matched, male *Sod1*$^{+/+}$, *Sod1*$^{+/-}$, and *Sod1*$^{-/-}$ mice following intraperitoneal administration of paraquat at a dose of 10 mg/kg were measured.

(Figure 2; $p < 0.0001$ compared with *Sod1*$^{+/+}$ or *Sod1*$^{+/-}$ mice). Remarkably, the *Sod1*$^{-/-}$ mice became listless at about 30 min after peritoneal administration of paraquat, whereas *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ mice were phenotypically normal even at the end of 7 days of observation. In addition, *Sod1*$^{-/-}$ mice showed a marked susceptibility to myocardial ischemia–reperfusion injury (*56*).

Female Mice Lacking CuZnSOD Exhibit a Marked Increase in Post-implantation Embryo Death. Surprisingly, the reproductive performance of female *Sod1*$^{-/-}$ mice was inferior to that of female *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ mice. As shown in Table 3, male *Sod1*$^{-/-}$ mice were as fertile as *Sod1*$^{+/+}$ males, and female *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ mice were similarly fertile when bred with either *Sod1*$^{+/+}$ or *Sod1*$^{-/-}$ male mice. However, the fecundity index (number of litters/number of copulations) and size of the litters of *Sod1*$^{-/-}$ females were much less than those of *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ females. The mechanism underlying the poor reproductive performance was further investigated. Examination of vaginal smears indicated that these three types of mice had similar estrus cycles, with an average length of 4 to 5 days. In addition, they also mated at an equivalent frequency (data not shown). These data indicated that the reduced fertility in *Sod1*$^{-/-}$ mice was not a result of altered estrus cycles. The numbers of ova ovulated by these mice at each estrus cycle were then determined and found to be equivalent (*Sod1*$^{+/+}$: 8.0 ± 1.4, $n = 6$; *Sod1*$^{+/-}$: 7.0 ± 1.4, $n = 6$; and *Sod1*$^{-/-}$: 7.6 ± 2.1, $n = 8$; $p = 0.65$). Furthermore, female *Sod1*$^{-/-}$ mice also exhibited a normal ovarian histology, including number, size, or morphology of antral follicles and corpora lutea, compared with those of *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ mice. These results suggested that the reduced fertility in *Sod1*$^{-/-}$ female mice might result from a defect in embryonic implantation in the wall of the uterine horns or premature death of the fetuses.

To further evaluate these two possibilities, we then examined the number and viability of fetuses in *Sod1*$^{-/-}$ female mice bred with *Sod1*$^{-/-}$ male mice between 12.5 to 15.5 days postcoitus. Of 15 mice examined, only 2 were not pregnant. A total of 91 embryos were found to be carried by these female mice. However, 75 embryos in various sizes were dead and in the process of being resorbed. Compared to the normal 12.5-day embryos (Figure 3A), most of the dead and resorbed embryos were loosely attached to the wall of uterine horns without a developed placenta or yolk sac (Figure 3B). In control experiments, 14 of 15 female *Sod1*$^{+/-}$ mice were pregnant from breeding with *Sod1*$^{-/-}$ males. A total of 122 implanted embryos were found, and only 9 of these were dead. Although the mean number of successfully implanted embryos in *Sod1*$^{-/-}$ females (6.3 ± 4.4), including both the live and dead ones, was less than that in *Sod1*$^{+/-}$ females (8.2 ± 3.0), the difference was not statistically significant ($p = 0.2$). These results indicated that the efficiency of embryo implantation in the former mice was equivalent to that in the latter mice. However, the rate of post-implantation embryonic death that occurred in *Sod1*$^{-/-}$ females was significantly higher than that in *Sod1*$^{+/-}$ females ($83 \pm 23\%$ vs $7.0 \pm 7.0\%$, respectively; $p < 0.0001$).

During the course of our study on the CuZnSOD-deficient mice, generation and characterization of a line of mice lacking the entire *Sod1* locus were reported by Reaume and colleagues (*57*). Their results showed that mice lacking the CuZnSOD gene are markedly susceptible to motor neuron loss after axonal injury and transient focal cerebral ischemia (*57,58*). Although we inactivated the *Sod1* gene by a different approach, our results on the apparently normal phenotype of the *Sod1*$^{-/-}$ mice were in agreement with those reported previously (*57*). In addition, we focused on some other antioxidant functions of this enzyme. Our data revealed that although the role of CuZnSOD in defending lungs against lethal exposure of hyperoxia is negligible, it is essential to protect animals against paraquat toxicity. This suggests that the protective role of CuZnSOD is dependent on the cellular site of oxygen radical generation. The mitochondrion is a major subcellular site producing oxygen radicals under normal physiologic conditions (*1*), and the rate of radical production is further enhanced in mitochondria of hyperoxic lungs (*59,60*). Therefore, MnSOD may play a more critical role than CuZnSOD in antioxidant defense mechanism(s) under normal physiologic conditions and in defending against lung injury resulting from hyperoxic insults. This notion is supported by the recent findings that mice lacking MnSOD die at very young ages (*61,62*). However, CuZnSOD is essential for animals to survive a lethal exposure to paraquat, a bipyridyl herbicide capable of generating oxygen radicals through the redox cycling mechanism. This reaction is believed to be catalyzed by the enzyme NADPH-dependent cytochrome P450 reductase, primarily located in the endoplasmic reticulum. Our data suggest that both the cytosolic and microsomal enzymes may be the primary targets of superoxide anion radicals generated during paraquat toxicity. This conclusion is in agreement with the earlier study reported by Phillips and colleagues that *Drosophila* lacking CuZnSOD are hypersensitive to paraquat (*63*).

Table 3. Reproductive performance of *Sod1*$^{+/+}$, *Sod1*$^{+/-}$, and *Sod1*$^{-/-}$ female mice in breeding with *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ male mice.

Sod1 genotype		Number of pups		
Male	Female	from each copulation	Fecundity index[a]	Litter size[b]
+/+	+/+	11, 9, 9, 9, 8, 7, 7, 6, 5, 4	100	7.5 ± 2.1
+/+	+/–	9, 9, 7, 7, 7, 6, 5, 5, 5, 0	90	6.7 ± 1.6
+/+	–/–	5, 3, 3, 1, 0, 0, 0, 0, 0, 0	36*	3.0 ± 1.6**
–/–	+/+	11, 10, 9, 8, 7, 7, 0	88	8.6 ± 1.5
–/–	+/–	14, 11, 11, 9, 9, 8, 8, 7, 5, 2	100	8.4 ± 3.3
–/–	–/–	3, 1, 1, 1, 0, 0, 0, 0, 0, 0	36*	1.5 ± 1.0**

[a]Fecundity index = number of litters/number of copulations × 100. [b]Numbers are means ± SD. *$p < 0.05$ when comparing –/– females to +/+ or +/– females bred with either +/+ or +/– males using one-sided Fisher's exact test. **$p < 0.01$ when comparing –/– females to +/+ or +/– females bred with either +/+ or +/– males.

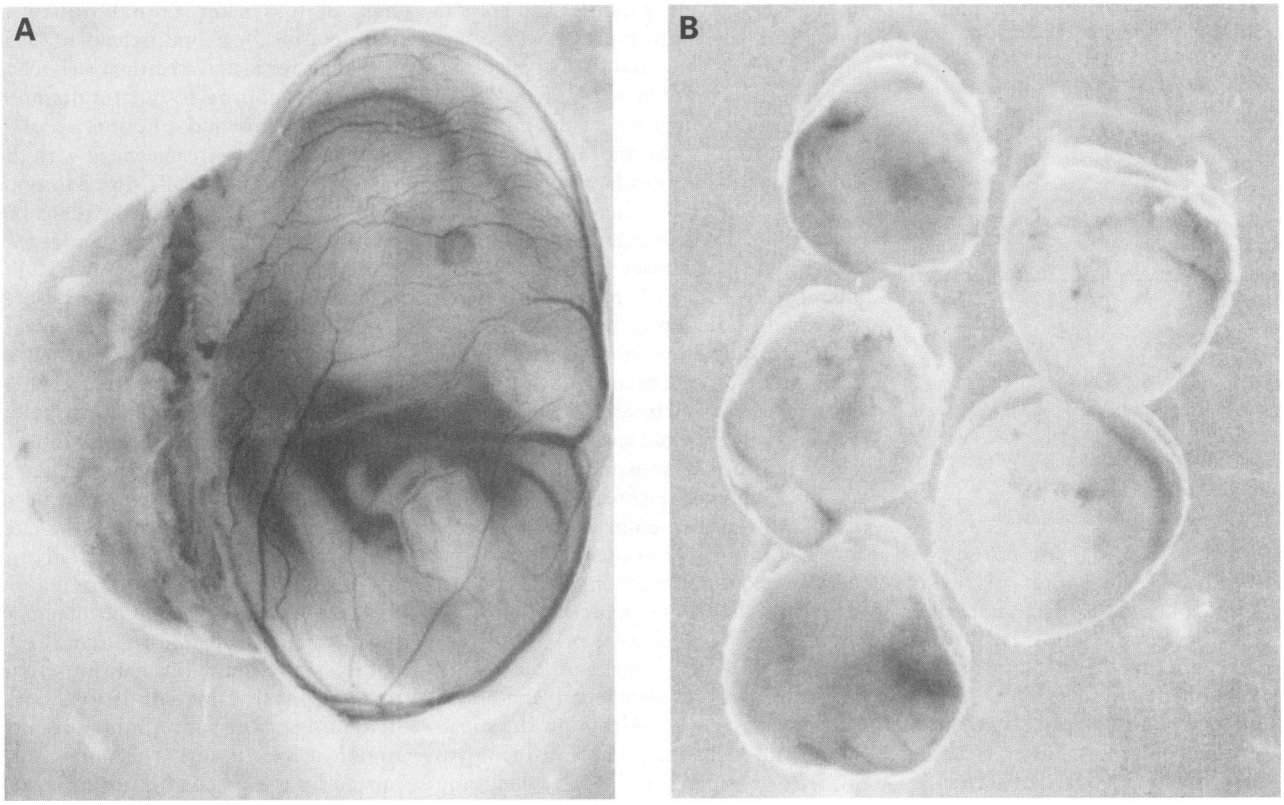

Figure 3. Morphology of normal and resorbed embryos. (*A*) Morphology of a typical and normal 12.5-day fetus carried by the *Sod1*$^{+/-}$ female mice. Note the well-developed placenta, yolk sac, and umbilical cord shown in the middle of the fetus. (*B*) An example of dead and resorbed embryos found in the uteri of pregnant *Sod1*$^{-/-}$ female mice.

The most intriguing observation in this study is the reduced fertility of female mice lacking this enzyme. Apparently, this defect is associated with CuZnSOD deficiency in the female mice and is unrelated to the genotypes of the fetuses. Interestingly, male CuZnSOD-deficient *Drosophila* are sterile and females show markedly reduced fertility (*63*). These results and ours suggest that CuZnSOD plays a critical role in female reproduction. The mechanism underlying the observed reduced fertility in female CuZnSOD-deficient mice, as well as its implication in human reproductive dysfunction, remain to be defined.

Mice Deficient in Cellular GSHPx Are More Susceptible to Acute Paraquat Toxicity and Myocardial Ischemia–Reperfusion Injury

Generation and Characterization of Mice Deficient in Cellular GSHPx. As shown in Figure 4A, the coding region of exon 2 of the mouse *Gpx1* gene was disrupted by insertion of a neomycin resistance cassette (*64*). The HindIII-linearized targeting vector was then transfected into R1 ES by electroporation. The homologous recombinant ES clones, which were identified by Southern blot analysis using a 3′ external probe (Figure 4A, probe 1), were used to generate chimeric mice. The heterozygous mice that descended from the chimeric mice were used in breeding to generate homozygous GSHPx-1 knockout (*Gpx1*$^{-/-}$) mice. An example of Southern blot analysis is shown in Figure 4B. However, for unknown reasons, the 3′ external probe tended to generate a high hybridization background on the blot filter carrying mouse tail DNA, contributing to difficulties in genotyping of mouse progeny. The DNA fragment containing exon 2 and the adjacent 3′ sequence (Figure 4A, probe 2) was then used for identifying the targeted allele in knockout mice. The 5.3-kb *SacI* and an approximately 11-kb *BamHI* hybridizing genomic fragments were derived from the wild-type *Gpx1* allele. Insertion of the neomycin resistance cassette resulted in hybridizing *SacI* and *BamHI* fragments from the targeted allele with sizes of 7.1 kb and 4.3 kb, respectively. Approximately 25% of the offspring from heterozygous breeding were homozygous for the mutated *Gpx1* allele, indicating there was no reduction in viability of GSHPx-1-deficient mice.

The effect of gene disruption on GSHPx-1 expression was initially examined by RNA blot analysis. A 50% reduction of GSHPx-1 mRNA level was found in brain, heart, kidney, liver, and lung of heterozygous knockout (*Gpx1*$^{+/-}$) mice compared with those of wild-type (*Gpx1*$^{+/+}$) littermates. Furthermore, no GSHPx-1 mRNA was found in these tissues of homozygous knockout mice. Interestingly, two additional species of hybridizing RNA of 1.5 kb and 1.9 kb became visible in liver and kidney samples of homozygous knockout mice after a longer exposure of the autoradiograph (Figure 4C, arrowheads). They presumably represent the aberrant forms of fusion transcript between the mouse *Gpx1* gene and the neomycin resistance gene.

The tissue activity of GSHPx in these mice also reflected the *Gpx1* genotypes (Table 4). Virtually no or very low GSHPx activity could be detected in tissues of *Gpx1*$^{-/-}$ mice. The residual GSHPx activity may result from the expression of other

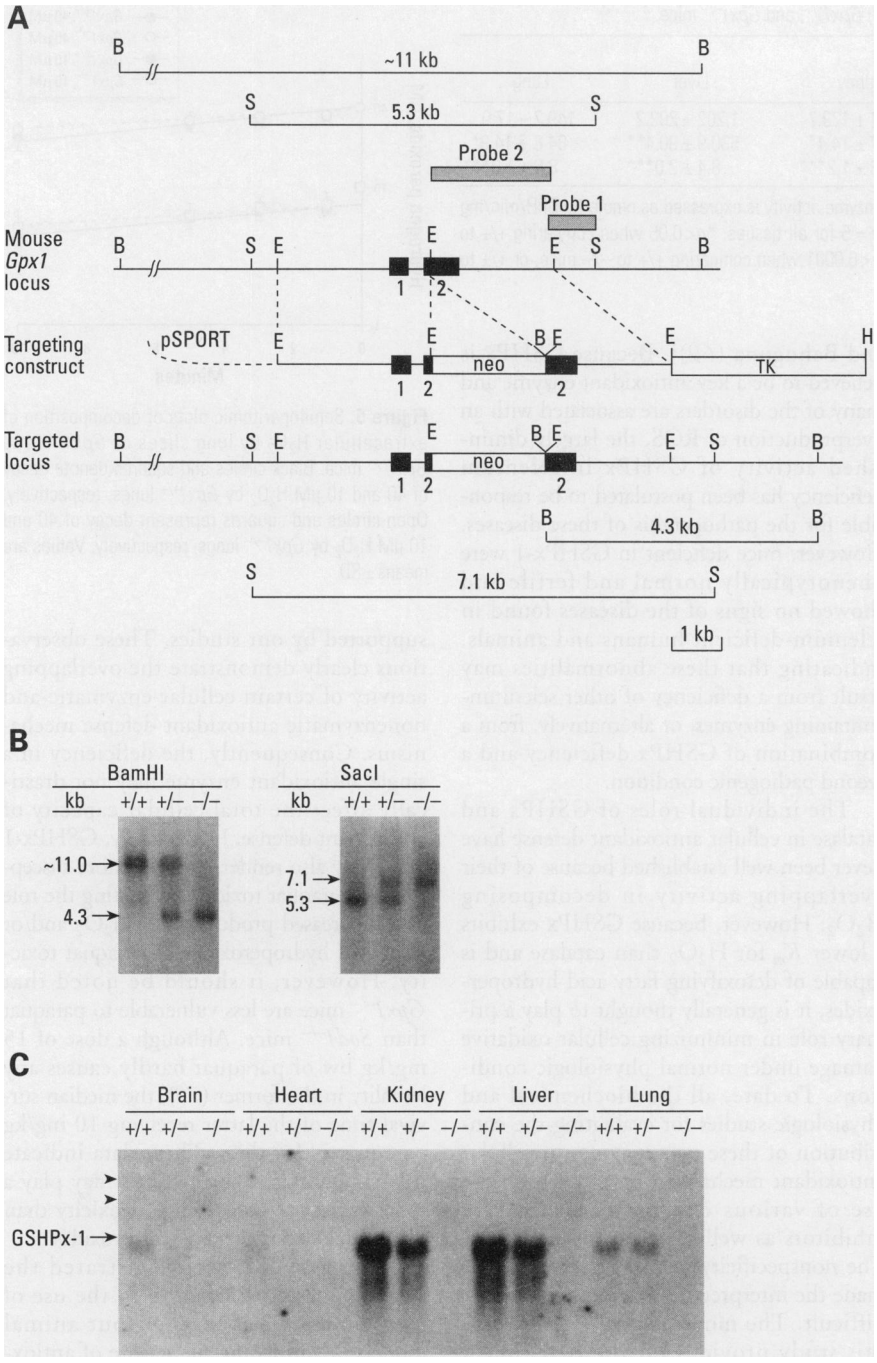

Figure 4. Generation and characterization of GSHPx-1-deficient mice. (*A*) Targeted disruption of the mouse GSHPx-1 gene. Genomic structure and partial restriction map of the wild-type mouse GSHPx-1 locus (top), the targeting vector (middle), and the targeted locus (bottom) are shown. Numbered black boxes represent exons. Shaded boxes on top of the restriction map of the GSHPx-1 locus represent the sequences used for probing the DNA blot filters. Probe 1, which is 3′ external to the genomic sequence used in the targeting vector, was used to screen the ES clones. Probe 2 containing exon 2 and the adjacent 3′ sequence was used for genotyping of mouse progeny. B, *BamHI; E, EcoRI; S, SacI.* The sizes of *SacI and BamHI* restriction fragments of the wild-type and the targeted alleles hybridized with the probe are shown on the top and bottom of the figure, respectively. (*B*) DNA blot analysis of mouse tail DNA. The Southern blot filter was hybridized with probe 2 as shown in *A*. $^{+/+}$, $^{+/-}$, and $^{-/-}$ represent wild-type, heterozygous, and homozygous knockout mice, respectively. (*C*) RNA blot analysis of total cellular RNA isolated from tissues of $Gpx1^{+/+}$, $Gpx1^{+/-}$, and $Gpx1^{-/-}$ mice. Fifty micrograms of total RNA from each tissue were separated on an agarose gel for blot analysis. The RNA blot filter was initially hybridized with a rat GSHPx-1 cDNA probe. The types of tissues are shown on the top of the autoradiography. The two presumably GSHPx-1-neo fusion mRNA are shown by the arrowheads.

GSHPx isozymes in the tissues. These data also indicate that GSHPx-1 is the major isozyme of GSHPx expressed in the tissues examined. In addition, the activities of other antioxidant enzymes including CuZnSOD, MnSOD, and catalase were unchanged in these five tissues of $Gpx1^{+/-}$ and $Gpx1^{-/-}$ mice compared to those of $Gpx1^{+/+}$ mice (data not shown).

Gpx1$^{-/-}$*Mice Show No Changes in Susceptibility to Hyperoxia and Consumption of Extracellular H_2O_2.* Male and female $Gpx1^{-/-}$ mice were phenotypically normal and healthy on observation up to 20 months of age. These mice were also fertile. Furthermore, no apparent pathologic changes could be found in tissues of $Gpx1^{-/-}$ mice, including brain, heart, intestine, kidney, liver, lung, and spleen at 4 and 15 months of age (data not shown).

GSHPx-1 is highly expressed in erythrocytes and is believed to play a protective role against the ROS-mediated damage in these cells. To determine if a deficiency in GSHPx-1 would affect the homeostasis of erythrocytes, a hematologic profile of the $Gpx1^{+/+}$, $Gpx1^{+/-}$, and $Gpx1^{-/-}$ mice was obtained. Total blood cell counts were normal. The numbers of red cells, reticulocytes, and differential leukocyte counts including lymphocytes, monocytes, neutrophils, eosinophils, and platelets were equivalent in all three types of mice (data not shown).

The tissue content of malondialdehyde and 4-hydroxyalkenal, a breakdown product of lipid peroxidation, and protein carbonyl (a measure of oxidatively modified proteins) was found unchanged in tissues of brain, kidney, liver, and lung of $Gpx1^{-/-}$ mice compared to those of $Gpx1^{+/+}$ mice. These results suggest that the cellular burden of oxidative stress may not be increased in $Gpx1^{-/-}$ mice under normal physiologic conditions.

We next determined whether a near-95% decrease in pulmonary GSHPx activity would render the animals more susceptible to hyperoxia. Surprisingly, $Gpx1^{+/+}$ and $Gpx1^{-/-}$ mice exhibited identical median survival times of 4.7 (± 0.3 SE) and 4.7 (± 0.4 SE) days when exposed to greater than 99% oxygen, respectively.

The effect of a decreased GSHPx-1 activity on the rate of clearance of extracellular H_2O_2 at 40 and 10 μM by lung tissues was also determined. These two concentrations of hydrogen were chosen according to findings that GSHPx-1 has a lower K_m for H_2O_2 relative to that of

Table 4. Glutathione peroxidase activity in tissues of *Gpx1*$^{+/+}$, *Gpx1*$^{+/-}$, and *Gpx1*$^{-/-}$ mice. [a]

Gpx1 genotype	Brain	Heart	Kidney	Liver	Lung
+/+	24.1 ± 2.6	32.0 ± 3.4	619.4 ± 129.7	1,202 ± 202.2	149.2 ± 17.0
+/–	12.3 ± 1.2**	19.6 ± 2.0**	284.7 ± 14.4*	530.9 ± 90.4**	84.8 ± 14.9*
–/–	ND***	3.9 ± 0.5***	9.8 ± 1.2***	8.4 ± 2.0***	8.6 ± 1.9***

[a] ND, activity was not detected in four of five samples. [b] The enzyme activity is expressed as nmole NADPH/min/mg protein assayed at 2 mM GSH. Numbers are means ± SD; $n = 5$ for all tissues. *$p<0.05$ when comparing +/+ to +/– mice. **$p<0.01$ when comparing +/+ to +/± mice. ***$p<0.0001$ when comparing +/+ to –/– mice, or +/± to –/– mice.

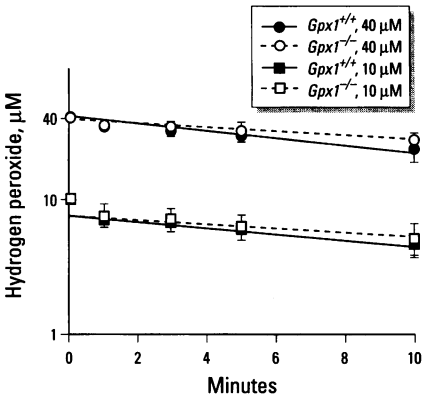

Figure 5. Semilogarithmic plots of decomposition of extracellular H_2O_2 by lung slices of *Gpx1*$^{+/+}$ and *Gpx1*$^{-/-}$ mice. Black circles and squares denote decay of 40 and 10 µM H_2O_2 by *Gpx1*$^{+/+}$ lungs, respectively. Open circles and squares represent decay of 40 and 10 µM H_2O_2 by *Gpx1*$^{-/-}$ lungs, respectively. Values are means ± SD.

catalase and may play a major role in decomposing H_2O_2 at concentrations below 10 µM (*65*). Figure 5 shows that although the average rates of H_2O_2 degradation by knockout lung slices are slower than those by wild-type lung slices at both concentrations, these differences are not statistically significant. Furthermore, the rates of extracellular H_2O_2 consumption by tissue slices of heart, liver, and kidney of *Gpx1*$^{-/-}$ mice were unchanged compared with those of *Gpx1*$^{+/+}$ mice (data not shown). However, when the lens of *Gpx1*$^{-/-}$ and *Gpx1*$^{+/+}$ mice were subjected to oxidant insult generated by photochemical reaction using riboflavin, the former showed a slower rate in removing extracellular H_2O_2 than the latter (*66,67*).

Gpx*$^{-/-}$ *Mice Are More Susceptible to Acute Paraquat Toxicity and Myocardial Ischemia–Reperfusion Injury. Although mice deficient in GSHPx-1 showed no increased susceptibility to hyperoxia, they did display a marked sensitivity to paraquat-induced lethality. The mean survival time of *Gpx1*$^{-/-}$ mice was 5.0 hr in comparison to 69 hr *Gpx1*$^{+/+}$ mice at a dose of 50 mg/kg bw of paraquat administrated intraperitoneally (*46*). The hearts of *Gpx1*$^{-/-}$ mice also displayed a poorer recovery in contractile function following 60 min ischemia–reperfusion compared to *Gpx1*$^{+/+}$ hearts (*68*). In addition, the lens epithelial cells of *Gpx1*$^{-/-}$ mice were also more susceptible to H_2O_2-induced DNA stand breakage (*45*).

The physiologic relevance of GSHPx has previously been implicated from studies on animals fed with a selenium-deficient diet. Selenium deficiency results in a variety of pathologic changes that include cardiomyopathy, nutritional muscular dystrophy, liver necrosis, certain types of cancers, and female infertility [for reviews see Burk and Hill (*17*) and Bedwal and Bahuguna (*69*)]. Because GSHPx is believed to be a key antioxidant enzyme and many of the disorders are associated with an overproduction of ROS, the largely diminished activity of GSHPx in selenium deficiency has been postulated to be responsible for the pathogenesis of these diseases. However, mice deficient in GSHPx-1 were phenotypically normal and fertile and showed no signs of the diseases found in selenium-deficient humans and animals, indicating that these abnormalities may result from a deficiency of other selenium-containing enzymes, or alternatively, from a combination of GSHPx deficiency and a second pathogenic condition.

The individual roles of GSHPx and catalase in cellular antioxidant defense have never been well established because of their overlapping activity in decomposing H_2O_2. However, because GSHPx exhibits a lower K_m for H_2O_2 than catalase and is capable of detoxifying fatty acid hydroperoxides, it is generally thought to play a primary role in minimizing cellular oxidative damage under normal physiologic conditions. To date, all the biochemical and physiologic studies for evaluating the contribution of these two enzymes to cellular antioxidant mechanism have relied on the use of various enzyme and substrate inhibitors as well as selenium depletion. The nonspecificity of these treatments has made the interpretation of the results more difficult. The mouse model generated in this study provides for the first time a unique experimental system to dissect the function of these two enzymes. Our results have shown that the role of GSHPx-1 in animals under normal developmental and physiologic conditions and in pulmonary defense against hyperoxia is unexpectedly limited. In addition, many of the functions of GSHPx previously proposed, such as protection of erythrocytes from premature hemolysis or splenic clearance, are not supported by our studies. These observations clearly demonstrate the overlapping activity of certain cellular enzymatic and nonenzymatic antioxidant defense mechanisms. Consequently, the deficiency in a single antioxidant enzyme may not drastically affect the total cellular capacity of antioxidant defense. Interestingly, GSHPx-1 deficiency also renders animals more susceptible to paraquat toxicity, indicating the role of an increased production of H_2O_2 and/or fatty acid hydroperoxides in paraquat toxicity. However, it should be noted that *Gpx1*$^{-/-}$ mice are less vulnerable to paraquat than *Sod1*$^{-/-}$ mice. Although a dose of 15 mg/kg bw of paraquat hardly causes any lethality in the former (*70*), the median survival time of the latter receiving 10 mg/kg paraquat is 1.4 days. These data indicate that superoxide anion radicals may play a more critical role in paraquat toxicity than H_2O_2 and/or fatty acid hydroperoxides.

These studies have illustrated the application and limitation in the use of transgenic and gene knockout animal models in addressing the nature of antioxidant enzymes. It is believed that these genetically engineered animals will provide a powerful tool for uncovering the identity of ROS that participate in the pathogenesis of many clinical disorders as well as for defining the role of each antioxidant enzyme in defending cells and tissues against tissue injury and diseases whose progression is associated with an unbalanced production of ROS.

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