Mice Deficient in Cellular Glutathione Peroxidase Develop Normally and Show No Increased Sensitivity to Hyperoxia*

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Glutathione peroxidase, a selenium-containing enzyme, is believed to protect cells from the toxicity of hydroperoxides. The physiological role of this enzyme has previously been implicated mainly using animals fed with a selenium-deficient diet. Although selenium deficiency also affects the activity of several other cellular selenium-containing enzymes, a dramatic decrease of glutathione peroxidase activity has been postulated to play a role in the pathogenesis of a number of diseases, particularly those whose progression is associated with an overproduction of reactive oxygen species, found in selenium-deficient animals. To further clarify the physiological relevance of this enzyme, a model of mice deficient in cellular glutathione peroxidase (GSHPx-1), the major isoform of glutathione peroxidase ubiquitously expressed in all types of cells, was generated by gene-targeting technology. Mice deficient in this enzyme were apparently healthy and fertile and showed no increased sensitivity to hyperoxia. Their tissues exhibited neither a retarded rate in consuming extracellular hydrogen peroxide nor an increased content of protein carbonyl groups and lipid peroxidation compared with those of wild-type mice. However, platelets from GSHPx-1-deficient mice incubated with arachidonic acid generated less 12-hydroxyeicosatetraenoic acid and more polar products relative to control platelets at a higher concentration of arachidonic acid, presumably reflecting a decreased ability to reduce the 12-hydroxyeicosatetraenoic acid intermediate. These results suggest that the contribution of GSHPx-1 to the cellular antioxidant mechanism under normal animal development and physiological conditions and to the pulmonary defense against hyperoxic insult is very limited. Nevertheless, the potential antioxidant role of this enzyme in protecting cells and animals against the pathogenic effect of reactive oxygen species in other disorders remains to be defined. The knockout mouse model described in this report will also provide a new tool for future study to distinguish the physiological role of this enzyme from other selenium-containing proteins in mammals under normal and disease states.

Glutathione peroxidase (GSHPx)1 is believed to play an important role in cellular antioxidant defense by reducing hydroperoxide and various hydroperoxides using glutathione as a reducing agent to form water and corresponding alcohols, respectively (H₂O₂ + 2GSH → 2H₂O + GSSG or ROOH + 2GSH → ROH + GSSG + H₂O) (1). GSHPx is a selenium-containing protein present in both cytosol and mitochondria of eukaryotic cells (2–5). The selenocysteine at the active site of this protein is encoded by an opal nonsense codon in all species of mammals studied (6–13). This modified amino acid is incorporated co-translationally into the protein by a unique species of opal suppressor tRNA (14). There are at least four GSHPx isozymes 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 (15). The plasma GSHPx is detected in milk, plasma, and lung alveolar fluid (16, 17). The phospholipid-hydroperoxide GSHPx, which is capable of reducing hydroperoxides of phospholipids and cholesterol, is found mainly in the testis (18–20). The recently reported GSHPx gastrointestinal tract is expressed predominantly in liver, intestine, and colon (21, 22).

Although GSHPx is capable of reducing cellular hydroperoxides, which can otherwise serve as substrates for metal-mediated Fenton reaction to generate highly reactive hydroxyl radical (for review, see Ref. 23), its function in lung antioxidant mechanism has never been directly evaluated. Exposure of mammals to hyperoxia can cause extensive lung injury. This type of pulmonary damage is due to the overproduction of reactive oxygen species (ROS), which overwhelms the cellular capacity of antioxidant defense in the lung (for review, see Ref. 24). To date, the physiological relevance of GSHPx has been implicated from studies on animals fed with a selenium-deficient diet. Selenium deficiency decreases the activity of rat lung GSHPx and exacerbates pulmonary injury following hyperoxic exposure (95% oxygen) compared with rats fed with a selenium-sufficient diet (25). These observations suggest the critical role of GSHPx in pulmonary antioxidant defense mechanisms. However, selenium deficiency also affects the activity

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1 The abbreviations used are: GSHPx, glutathione peroxidase; ROS, reactive oxygen species; HETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; ES, embryonic stem; kb, kilobase pair(s).
of several other selenium-containing proteins in mammals including the type I iodothyronine 5'-deiodinase, the plasma selenoprotein P, and thioredoxin reductase (26–28). Type I iodothyronine 5'-deiodinase catalyzes the 5'-deiodination of thyroxine to produce the more biologically active hormone 3,3',5'-triiodothyronine. Deficiency in selenium is known to affect thyroid hormone metabolism in experimental animals (29). The plasma selenoprotein P has been postulated to also contain antioxidant activity (for review, see Ref. 30). Furthermore, thioredoxin reductase functions to reduce oxidized thioredoxin, one of the major cellular proteins capable of reducing disulfide bonds present in oxidized proteins. Therefore, it is likely that the apparent pathophysiological changes found in the animals following dietary selenium depletion may not completely result from the decreased activity of GSHPx. To further understand the antioxidant role of GSHPx, a new animal model of GSHPx deficiency is needed. Toward this end, we describe the generation and characterization of knockout mice defective in the major cellular GSHPx (GSHPx-1) in this report. These mice are apparently healthy and show no increased oxidative stress or sensitivity to hyperoxia compared with that of wild-type mice. However, the profile of arachidonic acid metabolites is altered in GSHPx-1-deficient platelets relative to that of controls at a higher concentration of substrate. This mouse model will be valuable for understanding the pathogenesis of certain diseases in which the role of GSHPx insufficiency is implicated.

EXPERIMENTAL PROCEDURES

Generation of GSHPx-1-deficient Mice

Eight Gpx1 genomic clones were isolated from a bacteriophage FII genomic library constructed with DNA of 129/SVJ mouse (Stratagene, La Jolla, CA) by hybridization screening using a corresponding rat cDNA clone (12). The genomic insert in clone 21 was released from the bacteriophage vector by SacI digestion and further characterized by restriction mapping and DNA sequencing analysis. A 5.3-kb SacI genomic fragment was found to contain the entire mouse Gpx1 gene with sequence virtually identical to that published by Chambers et al. (6) except a few base substitutions in the region of intron 1 (data not shown). This fragment of DNA was then used in construction of the targeting vector (Fig. 1). The coding sequence was disrupted by insertion of a neomycin resistance gene cassette (neo) derived from plasmid pPNT (Ref. 31, generously provided by Dr. Richard Mulligan of Massachusetts Institute of Technology) into the EcoRI site located in exon 2 (6). The herpes thymidine kinase gene cassette from plasmid pPNT was placed before the coding sequence. The targeting vector linearized with HindIII was transfected into R1 embryonic stem (ES) cells obtained from Dr. András Nagy of Mount Sinai Hospital, Toronto, Canada (32) and selected with 300 µg/ml G418 and 2 µm ganciclovir (a gift from Syntex Inc., Palo Alto, CA). Resistant colonies were isolated and then screened by DNA blot analysis using a probe 3′ external to the targeting sequence as shown in Fig. 1.

Three clones containing the targeted Gpx1 allele were microinjected into C57BL/6 blastocysts according to the method described by Bradley (33), and embryos were re-implanted into the uterine horns of foster mothers. A total of 23 chimeric mice were generated. Four male chimeric mice with more than 95% agouti coat color chimerism were chosen to breed with C57BL/6 female mice. One hundred percent of their F1 offspring showed the agouti coat color, indicating that the microinjected ES cells might reconstitute the entire reproductive organ in the chimeric mice. Germ line transmission of the targeted Gpx1 allele was evident by DNA blot analysis as shown in Fig. 2b.

RNA Blot Analysis—Total RNA was isolated from various tissues using the guanidinium isothiocyanate-CsCl method as described by Chirgwin et al. (34). RNA was denatured with glyoxal and dimethyl sulfoxide and separated on an agarose gel in 10 mM sodium phosphate, pH 7.0 (35). The RNA blot filter was initially hybridized with a rat Gpx1 cDNA probe (12) and then re-hybridized with a rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (36) according to the procedures described by Thomas (37).

Preparation of Tissue Homogenates—Wild-type and knockout mice were anesthetized with pentobarbital sodium. After opening the abdo-

men and thorax, an incision was made on the left atrium, and the entire mouse was then perfused with approximately 10 ml of phosphate-buffered saline through the left ventricle. The tissues were removed and stored at −70 °C. For enzyme activity assay, the tissues were homogenized in 50 mM potassium phosphate, pH 7.0, containing 0.1% Triton X-100 with a Polytron homogenizer, followed by sonication on ice for 30 s with a microprobe at maximum power. The tissue homogenate was clarified by centrifugation at 20,000 × g for 10 min. Protein content in each sample was determined by a bicinchoninic acid protein assay kit (Pierce).

Glutathione Peroxidase Assay—The GSHPx activities in tissue homogenates were determined by the indirect, coupled test procedure (38, 39). Briefly, the GSSG produced during GSHPx enzyme reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was monitored as a measurement for the rate of GSSG formation during GSHPx reaction. The enzyme reaction was conducted in a buffer containing 20 mM potassium phosphate, pH 7.0, 0.6 mM EDTA, 0.15 mM NADPH, 4 units of glutathione reductase, 2 mM GSH, 1 mM sodium azide, and 0.1 mM H2O2 at 25 °C, and the rate of decrease in absorption of NADPH at 340 nm was followed. The GSHPx activity is defined as nmol of NADPH consumption per min per mg of tissue protein at 2 mM GSH. Consumption of NADPH was calculated using a m extinction coefficient for NADPH of 6.22.

Catalase Assay—The catalase activity was measured according to the method of Aebi (40). The rate of H2O2 decomposition was followed by monitoring absorption at 240 nm in 50 mM phosphate buffer, pH 7.0, containing 10 mM H2O2 at 25 °C. The activity of catalase is defined as µmol of H2O2 consumed per min per mg of tissue protein. Calculation for H2O2 concentration was made using a m extinction coefficient for H2O2 of 0.0394.

Glutathione Reductase Assay—The activity of glutathione reductase was determined at 25 °C in 54 mM phosphate buffer, pH 6.8, 12 mM NaHCO3, 0.5 mM EDTA, 0.5 mM 2-mercaptoethanol, 1 mM GSSG, and 0.1 mM NADPH by following the decrease of NADPH absorption at 340 nm (41). The glutathione reductase activity is defined as nmol of NADPH consumption/min/mg of tissue protein.

Glucose-6-phosphate Dehydrogenase Assay—The glucose-6-phosphate dehydrogenase activity was measured with a kit purchased from Sigma. Briefly, the rate of production of NADPH from NADP and glucose 6-phosphate was followed by monitoring the absorption of NADPH at 340 nm (42). The activity of 6-phosphogluconate dehydrogenase was inhibited by maleimide. The enzyme activity is defined as nmol of NADPH generation per min per mg of tissue protein.

Assay for Superoxide Dismutases—Activities of copper-zinc and manganese superoxide dismutases were determined by measuring the inhibition of xanthine plus xanthine oxidase-mediated cytochrome c reduction at pH 7.8 (43). The measurement was performed in the presence of 10 µM KCN to eliminate the activity of tissue cytochrome c oxidase. To distinguish the contribution of copper-zinc superoxide dismutase and manganese superoxide dismutase to the total superoxide dismutase activity, the same measurement was also repeated in the presence of 1 mM KCN to inhibit the activity of copper-zinc superoxide dismutase. One unit of superoxide dismutase activity is defined as the enzyme activity needed to inhibit 50% cytochrome c reduction.

Hematological Analysis

Blood samples were collected by cardiac puncture from four normal, heterozygous, and homozygous knockout mice and sent to Consolidated Veterinary Diagnostics, Inc., West Sacramento, CA, for hematological analysis. Smear slides were stained with Wright’s Giemsa or methylene blue for counting leukocyte differentials or reticulocytes, respectively. The total numbers of red cells, reticulocytes, platelets, lymphocytes, neutrophils, monocytes, and eosinophils were determined.

Histological Study

Normal and age-matched homozygous knockout mice were fixed by systemic perfusion with Bouin’s fixative through the left ventricle. The tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Tissue sections were examined under a light microscope.

Determination of Tissue Carboxyl Content (44)

Tissues free of blood were initially sliced with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd., Goose Green, Gomshall, UK) and then gently homogenized in a glass homogenizer equipped with a Teflon probe for 30 s in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 40 µg/ml phenylmeth-
Fig. 1. Targeted disruption of the mouse Gpx1 gene. Genomic structure and partial restriction map of the wild-type mouse Gpx1 locus (top), the targeting vector (middle), and the targeted locus (bottom) are shown. Numbered black boxes represent exons. Striped boxes on top of the restriction map of the Gpx1 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 determining genotypes of mouse progeny. B, BamHI; S, SacI; E, EcoRI; H, HindIII; 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 (31). The sizes of SacI and BamHI restriction fragments of normal and targeted loci hybridized with the probe are shown on the top and bottom of the figure, respectively.

Lipid Peroxidation Assay

The contents of malondialdehyde and 4-hydroxylalkenals in tissue homogenates used for carbonyl assay were determined by a colorimetric assay kit purchased from OXIS International, Inc., Portland, OR., following the manufacturer’s instructions. Standard curve was generated using known quantities of 1,1,3,3-tetramethoxypropane.

Consumption of Extracellular H₂O₂ by Lung Slices

The lungs were perfused free of blood through the pulmonary vasculature and then cut into 0.75-mm slices using a McIlwain tissue chopper. This thickness of tissue has been found to exhibit maximum rates of oxygen consumption (45). Hydrogen peroxide is capable of diffusing freely through the cells. Five milligrams of lung slices from wild-type or homozygous knockout mice were incubated at 37 °C in 3 ml of phosphate-buffered saline plus calcium were incubated with 10, 40, and 300 μM hydrogen peroxide at initial concentrations varied less than 2%, and CO₂ concentration was maintained less than 0.5% by providing approximately 12 complete gas changes per h. During the exposure, food and water ad libitum were provided, and the animals were kept in a 12-h on, 12-h off light cycle at all times. The numbers of surviving animals were counted three times a day.

Statistical Analysis

One-way analysis of variance was used to examine differences in each measurement performed on wild-type, heterozygous, and homozygous knockout mice. If a significant difference was observed (p < 0.05), then pairwise comparisons among mice were made using Duncan’s test. Survival of wild-type and GSHPx1-deficient mice exposed to >99% oxygen was analyzed using the Kaplan-Meier method.

RESULTS

Generation of Mice Deficient in GSHPx-1—Fig. 1 shows that the coding region of exon 2 of the mouse Gpx1 gene was disrupted by insertion of a neomycin resistance cassette. The targeting vector also carried a herpes thymidine kinase gene for performing negative selection with ganciclovir. This sequence recombined homologously with the cognate endogenous gene at a very high frequency. Approximately 30% G418 and gancyclovir-resistant ES colonies were found to contain the desired mutated allele for GSHPx-1. We have isolated approximately 100 homologous recombinant clones from about 300 colonies screened. Fig. 2a is an example of DNA blot analysis of DNA from representative ES clones. As shown in Fig. 2a, the 3’ external probe (probe 1 shown in Fig. 1) hybridized with a 5.3-kb SacI and an approximately 11-kb BamHI genomic fragment from the wild-type mouse Gpx1 allele. Insertion of the neomycin selective marker resulted in hybridizing SacI and BamHI fragments with sizes of 7.1 and 4.3 kb, respectively. However, due to unknown reason, this 3’ external probe tended to generate a high hybridization background on the blot filter.
cells or mice containing one wild-type and one targeted allele. The hybridizing 5.3-kb SacI and ~11-kb BamHI fragments are derived from the wild-type Gpx1 allele. The additional 7.1-kb SacI and 4.3-kb BamHI hybridizing fragments are derived from the targeted allele. a, DNA blot analysis of ES clones using probe 1 shown in Fig. 1. b, DNA blot analysis of mouse tail DNA using probe 2 shown in Fig. 1.

Next we assessed whether GSHPx-1 deficiency would increase the cellular burden of oxidative stress by determining the tissue carbonyl content (a measure of oxidatively modified proteins). Additionally, the tissue content of malondialdehyde and 4-hydroxynonenal, a breakdown product of lipid peroxida-

Expression of Antioxidant Enzymes in GSHPx-1-deficient Mice—RNA blot analysis was performed to determine whether the targeted Gpx1 allele is not functional as expected. Fig. 3 shows an approximately 40–60% reduction in amounts of the 0.9-kb Gpx1 mRNA in brain, heart, kidney, liver, and lung of heterozygous knockout mouse compared with those of normal littermates. Furthermore, no Gpx1 mRNA was found in these tissues of homozygous knockout mice. After a longer exposure of the autoradiogram, two additional species of hybridizing RNA of 1.5 and 1.9 kb, as indicated by arrowheads in Fig. 3, became apparent in liver and kidney samples of homozygous knockout mice. They presumably represent the aberrant forms of fusion transcript between the mouse Gpx1 gene and the neomycin resistance gene. To demonstrate that the decreased or diminished expression of Gpx1 mRNA in tissues of knockout mice was not a result of variation in RNA loading, the same blot filter was then re-hybridized with a rat Gapdh probe. With the exception, as shown in Fig. 3, of the hybridization density of the wild-type lung being somewhat less than those of lungs from knockout mice, the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA in other tissues of various types of mice were equivalent.

Activities of various antioxidant enzymes in tissues of wild-type and knockout mice were also measured. Table I shows that the activities of GSHPx in tissues of heterozygous knockout mice were about 40–60% those of corresponding tissues of wild-type mice. Virtually no or very low GSHPx activity could be detected in tissues of homozygous knockout mice. The residual GSHPx activity may result from the expression of other GSHPx isozymes in the tissues. Additionally, no changes in other antioxidant activities were found in these tissues, except the activity of glutathione reductase was found to be slightly higher (18%) in livers of homozygous knockout mice than those of wild-type and heterozygous knockout mice.

Phenotypic Analysis of GSHPx-1-deficient Mice—Male and female homozygous knockout mice grew normally and were apparently healthy upon observation up to 20 months of age. These mice were also fertile. Histological survey performed at 4 and 15 months of age using light microscopy revealed no evidence for abnormalities in tissues of homozygous knockout mice including brain, heart, intestine, kidney, liver, lung, and spleen (data not shown). In wild-type animals, tissues with the highest levels of GSHPx-1 expression were liver and kidney. In addition, GSHPx-1 was also highly expressed in erythrocytes and is believed to play a protective role against the ROS-mediated damage in these cells. To understand whether a deficiency in GSHPx-1 will affect the homeostasis of erythrocytes, a hematological profile of the wild-type, heterozygous, and homozygous knockout mice was obtained. Total blood cell counts were normal. The numbers of red cells, reticulocytes, and differential leukocyte counts including lymphocytes, monocytes, neutrophils, eosinophila, and platelets were equivalent in all three types of mice (data not shown).
Gpx1 Knockout Mice

**Activity of antioxidant enzymes in tissues of wild-type (Gpx1+/+), Gpx1+/−, and Gpx1−/− mice**

The enzyme activities are defined as following: GSHPx (glutathione peroxidase), nmol of NADPH/min/mg protein assayed at 2 mM GSH; GR (glutathione reductase), nmol of NADPH/min/mg protein; catalase, μmol of H₂O₂/min/mg protein assayed at 10 mM H₂O₂; G6PDH (glucose-6-phosphate dehydrogenase), nmol of NADPH/min/mg protein. Numbers are mean ± S.D. n = 5 for all tissues, except n = 11 for GR activity measurement in liver samples.

| Enzyme     | Gpx1 genotype | Brain | Heart | Kidney | Liver | Lung |
|------------|---------------|-------|-------|--------|-------|------|
| GSHPx      | ++/−           | 24.1 ± 2.6 | 32.0 ± 3.4 | 619.4 ± 129.7 | 1292.0 ± 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 |
| GR         | ++/−           | 25.6 ± 8.7 | 13.5 ± 2.4 | 80.2 ± 11.1 | 39.5 ± 5.8 | 46.8 ± 7.9 |
|            | ++/−           | 25.1 ± 3.4 | 13.6 ± 2.9 | 84.9 ± 10.9 | 38.5 ± 4.8 | 48.6 ± 12.1 |
|            | −/−            | 24.8 ± 6.0 | 14.1 ± 4.7 | 78.0 ± 20.0 | 45.5 ± 5.3 | 47.7 ± 9.5 |
| Catalase   | ++/−           | 2.5 ± 0.7 | 19.6 ± 3.1 | 242.1 ± 52.1 | 510.8 ± 25.4 | 75.9 ± 13.1 |
|            | ++/−           | 2.6 ± 1.2 | 20.2 ± 3.4 | 238.8 ± 60.4 | 471.2 ± 68.2 | 92.6 ± 12.9 |
|            | −/−            | 2.8 ± 0.3 | 19.8 ± 2.9 | 211.5 ± 43.7 | 474.5 ± 43.7 | 78.9 ± 16.4 |
| G6PDH      | ++/−           | 29.6 ± 5.5 | 12.9 ± 1.9 | 31.3 ± 3.5 | 12.5 ± 0.7 | 71.8 ± 11.3 |
|            | ++/−           | 29.9 ± 2.3 | 13.9 ± 3.2 | 32.2 ± 3.9 | 13.5 ± 2.7 | 70.7 ± 16.0 |
|            | −/−            | 31.0 ± 1.6 | 12.2 ± 2.4 | 34.0 ± 3.3 | 13.7 ± 2.0 | 75.3 ± 6.1 |
| CuZnSOD    | ++/−           | 59.2 ± 7.7 | 132.7 ± 18.3 | 75.7 ± 15.4 | 154.6 ± 29.4 | 27.2 ± 3.9 |
|            | ++/−           | 54.3 ± 12.5 | 125.2 ± 18.9 | 74.4 ± 10.2 | 146.8 ± 19.6 | 22.0 ± 5.3 |
|            | −/−            | 55.6 ± 8.1 | 127.7 ± 40.9 | 68.6 ± 12.5 | 152.1 ± 35.7 | 27.5 ± 4.6 |
| MnSOD      | ++/−           | 5.7 ± 1.3 | 24.1 ± 2.8 | 15.6 ± 1.6 | 11.7 ± 0.8 | 2.0 ± 0.5 |
|            | ++/−           | 6.3 ± 0.3 | 25.9 ± 2.9 | 16.1 ± 1.0 | 11.4 ± 0.9 | 2.5 ± 0.7 |
|            | −/−            | 5.9 ± 0.7 | 25.1 ± 4.9 | 16.3 ± 1.3 | 12.6 ± 1.5 | 1.7 ± 0.4 |

*a p < 0.01 when comparing ++/− an ++/−− mice.
*b p < 0.05 when comparing ++/− and ++/−− mice.
*c p < 0.0001 when comparing ++/− and −/−− mice or ++/− and −/−− mice.
*d p < 0.05 when comparing −/−− mice with ++/− or ++/−− mice.

Deficiency in GSHPx-1 Does Not Alter the Mean Survival Time of Mice Exposed to Hyperoxia and Rate of Extracellular H₂O₂ Consumption—We next determined whether a near 95% decrease in pulmonary GSHPx activity would render the animals more susceptible to hyperoxia. Fig. 4 shows that the Gpx1+/− and the Gpx1−/− mice have a median survival time of 4.7 (±0.3 S.E.) and 4.7 (±0.4 S.E.) days when exposed to >99% oxygen, respectively. Although the median survival times were not statistically different, the knockout mice did show a broader range in time to death than the normal mice.

We have also measured the rate of clearance of extracellular H₂O₂ at 40 and 10 μM by lung tissues for assessing the effect of a decreased antioxidant capacity in the GSHPx-1-deficient mice. These two concentrations of hydrogen were chosen according to findings that GSHPx has a lower Kₘ for H₂O₂ relative to that of catalase and may play a major role in decomposing hydrogen peroxide at concentrations below 10 μM (48). Fig. 5 shows that although the average rates of H₂O₂ degradation by knockout lung slices are slower than those by wild-type lung slices at both concentrations, these differences are not statistically significant. Additionally, no differences were found in either tissues in the decomposition rate of a higher concentration (300 μM) of H₂O₂ (data not shown). In control experiments, no decay of H₂O₂ was found in the same buffer without lung tissue for the same incubation period (data not shown). Furthermore, the rates of extracellular H₂O₂ consumption by tissue slices of heart, liver, and kidney of homozygous knockout mice were unchanged compared with those of wild-type mice (data not shown).

**Analysis of Arachidonic Acid Metabolism in GSHPx-1-deficient Platelets and Peritoneal Macrophages**—The products of lipoxygenase reaction, hydroperoxyeicosatetraenoic acids (HPETEs), are highly reactive and are presumably reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) by GSHPx at the expense of reduced glutathione (HPETE + GSH → HETE + GSSG + H₂O) (49). To directly test this hypothesis, intact platelets and peritoneal macrophages from wild-type and GSHPx-1-deficient mice were examined for their ability to metabolize arachidonic acid to various polar oxygenated metabolites. The 12-lipoxygenase product 12-HETE was not detected in any incubations by reverse phase-high performance liquid chromatography using a chromatographic system that clearly separates 12-HETE and 12-HFETE (data not shown). Instead, 12-HETE, the reduced product was detected in all incubations. There was no difference in the profile of labeled metabolites in macrophages from wild-type or homozygous knockout mice. In platelets, at low arachidonic acid concentrations (25 μM) there did not appear to be any significant difference in the profile of metabolites between the two groups of samples. However, at a higher concentrations (75 μM) there

![FIG. 4. Survival analysis of wild-type and homozygous Gpx1 knockout mice under hyperoxia. The survival times of age-matched wild-type and GSHPx-1-deficient mice from several litters were measured under >99% oxygen.](Image)
were more polar products and less 12-HETE produced in the GSHPx-1-deficient platelets than in the wild-type control samples (Fig. 6). Although the polar products were not identified, they migrated in the expected region of decomposition products (epoxy alcohol and trihydroxy derivatives) of the 12-HETE precursor and were not cyclooxygenase-derived products (thromboxane B₂ or 12-hydroxy-heptadecenoic acid).

**DISCUSSION**

Reactive oxygen species (ROS) are generated as by-products of normal cellular metabolism (for review, see Ref. 24). Mammalian cells are equipped with both enzymatic and nonenzymatic antioxidant activities to minimize the cellular damage caused by interaction between cellular constituents with ROS (for review, see Refs. 24 and 50). The enzymatic antioxidant mechanism contains various forms of superoxide dismutases, catalase, and 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 generating NADPH. However, an unbalanced production of ROS occurs frequently in cells particularly following exposure to various chemicals, radiation, hyperoxia, and hypoxia-reperfusion or during tissue inflammation. Among those pathogenic conditions, pulmonary oxygen toxicity has been observed in patients receiving oxygen therapy for treating respiratory insufficiency. Similarly, adult rats die within 3 days of exposure to >99% oxygen. However, rats can survive under >99% oxygen after pre-exposing to a sublethal concentration of oxygen (85%) for 5–7 days (51). Associated with the development of tolerance to hyperoxia is an increase in the lung activities of all the antioxidant enzymes described above. Although these results suggest a role of antioxidant enzymes in protecting lung against the toxicity of hyperoxia, the importance of each of these enzymes in lung antioxidant defense has yet to be defined. This study aims to understand the functional role of GSHPx under normal physiological conditions and in pulmonary antioxidant defense against hyperoxia using mice deficient in this enzyme.

The mouse Gpx1 gene was inactivated by insertion of a neomycin resistance cassette into the region of exon 2. RNA blot analysis of homozygous knockout mice showed that most of the Gpx1-neo fusion mRNA was degraded, except very little amounts of the aberrant mRNA were found in kidney and liver where GSHPx-1 is highly expressed (Fig. 2). These results indicate that cells are capable of effectively recognizing and degrading abnormal mRNA. Since the mammals express at least four GSHPx isoforms, each of which is expressed at different levels in different tissues, the relative activity of each GSHPx isoform in a particular tissue is very difficult to determine. The knockout mice generated in this study have allowed us to determine the contribution of GSHPx-1 to the total cellular GSHPx activity. The total GSHPx activity in brain, heart, kidney, liver, and lung of homozygous Gpx1 knockout mice was greatly diminished (Table 1), indicating that this isoform contributes to most of the measured GSHPx activity in these tissues.

The physiological relevance of GSHPx has previously been implicated from studies on animals fed with a selenium-deficient diet. Depletion of selenium results in a variety of pathologic changes including cardiomyopathy, nutritional muscular dystrophy, liver necrosis, certain types of cancers, and female infertility (for review, see Refs. 52 and 53). Since GSHPx is believed to be a key antioxidant enzyme and many of the pathogenic conditions 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 mice deficient in GSHPx-1 also provide a new model for testing the role of this enzyme in cellular antioxidant defense. GSHPx-1 is believed to protect erythrocyte hemoglobin from oxidative damage and maintain membrane fluidity by removing lipid hydroperoxides, thereby preventing premature clearance of erythrocytes in the spleen (54, 55). Indeed, erythrocytes isolated from selenium-deficient rats are more sensitive to tert-butyl hydroperoxide-induced hemolysis than selenium-sufficient erythrocytes in the presence of GSH (56). In contrast to this understanding, Gpx1 knockout mice showed a normal profile of red cells and reticulocytes. Their red cells were also as resistant to H₂O₂ or tert-butyl hydroperoxide-induced hemolysis as those from wild-type mice (data not shown). In addition, we did not observe an increase in cellular burden of oxidative stress in knockout mice relative to that of wild-type mice as determined by the content of protein carbonyl groups and lipid peroxidation. The knockout mice also showed no increased sensitivity to hyperoxia. These data suggest a very limited antioxidant role of this enzyme in mice under normal physiological conditions and in defense against hyperoxia. This notion...
is in agreement with the conclusion made by Burk and colleagues (57) in an earlier study. Their study has shown that supplementation of selenium by intraperitoneal injection of sodium selenite provides a rapid and drastic protection to selenium-deficient rats against the lipid peroxidation and mortality induced by treatment with diquat, a dipyril herbicide capable of generating superoxide anion radicals through the redox cycling mechanism. This observed protection is not associated with a recovery of tissue GSHPx activity, suggesting the role of this enzyme in defending animals against diquat toxicity is negligible.

Nonetheless, our studies do not rule out the protective effects of GSHPx-1 when overexpressed against certain pathogenic conditions. Overexpression of GSHPx-1 in human breast cancer cells, following transfection with an expression construct, confers an increased resistance to the toxicity of H2O2, cumene hydroperoxide, and menadione compared with that of parental cells (58). Transgenic mice overexpressing GSHPx-1, relative to those of their nontransgenic littermates, exhibit a decreased content of peroxides in the brain as well as an increased tolerance to heart injury induced by ischemia/reperfusion (59, 60). These studies have suggested the role of an unbalanced production of H2O2 and/or other organic hydroperoxides in these pathogenic conditions. Further studies will be needed to determine whether a deficiency in GSHPx-1 will render animals more susceptible to some other diseases whose pathogenesis is associated with an overproduction of ROS.

The contribution of GSHPx and catalase to removal of extracellular cellular H2O2 has been studied quite extensively. Since GSHPx has a relatively lower Km for H2O2 than does catalase, it is generally believed that GSHPx plays a major role in removing H2O2 at relatively low H2O2 concentrations in cells, and this function is taken over by catalase at high concentrations of H2O2. This hypothesis is supported by a number of studies on erythrocytes, hepatocytes, and fibroblasts (48, 61–64). Makino et al. (48) have shown that in a cultured human fibroblast cell line, up to 80–90% extracellularly added H2O2 is degraded by GSHPx at concentrations of H2O2 lower than 10 μM. In their studies, diethyl maleate was used to react and deplete GSH. Interestingly, tissue slices from wild-type and GSHPx-1-deficient mice showed no differences in decomposing extracellular H2O2 at concentrations of 10, 40, and 300 μM. One possible explanation is that depletion of GSH will not only block the activity of GSHPx but also inhibit the function of selenium-independent GSHPx, namely certain isoforms of glutathione S-transferase. Thus, a retardation in removal of extracellular H2O2 may not completely result from the inhibition of GSHPx activity. Our results suggest that tissues can effectively decompose H2O2 at both low and high concentrations of H2O2, presumably by antioxidant enzymes such as catalase and selenium-independent GSHPx and/or by a nonenzymatic mechanism involving interaction between H2O2 and cellular constituents such as lipids, without a functional aid from GSHPx-1.

There is also evidence suggesting the function of GSHPx in metabolism of arachidonic acid by either cyclooxygenase or lipoxygenase pathways. Purified GSHPx, presumably GSHPx-1, has been shown to be capable of reducing prostaglandin G2 to form mainly prostaglandin F2α, and reducing 15-HPETE (65). Subsequent studies have also demonstrated an altered metabolic activity for arachidonic acid in lymphocytes or neutrophils isolated from selenium-deficient cows or rats, respectively, in comparison with those isolated from corresponding selenium-sufficient animals (66–68). However, as discussed above, these studies are complicated by the effect of selenium deficiency on many other selenium-containing enzymes in addition to GSHPx. The model of Gpx1 knockout mice should provide a new tool to circumvent this complication. Our studies reveal an identical profile of arachidonic acid metabolites in peritoneal macrophages isolated from either GSHPx-1-deficient or wild-type mice. However, at a high concentration of substrate (75 μM), decomposition productions of 12-HETE were apparently accumulated in the platelets from GSHPx-1-deficient mice but not in those from wild-type mice, suggesting the role of GSHPx-1 in metabolism of arachidonic acid at a high substrate concentration. It should be noted that no accumulation of 12-HETE was detected in platelets or peritoneal macrophages from GSHPx-1-deficient mice. This observation indicates that both types of cells can rapidly metabolize 12-HETE without functional GSHPx-1. On the other hand, Sandstrom et al. (69) have shown that human immunodeficient virus-infected T cells, which exhibit a partial deficiency in the activity of GSHPx, are less efficient in converting 15-HETE to 15-HETE. Also, Bryant et al. (70) showed increased levels of 12-HETE in platelets from rats with selenium deficiency and an accumulation of epoxyhydroxy and trihydroxy degradation products. Those results and our results implicate that the contribution of GSHPx to arachidonic acid metabolism may be cell type-, species-, and substrate concentration-dependent.

In summary, the individual role of GSHPx and catalase in cellular antioxidant defense has never been well established due to their overlapping activity in decomposing H2O2. However, since GSHPx exhibits a lower Km for H2O2 than does 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 physiological conditions. To date, all the biochemical and physiological 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, for the first time, provides 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 physiological 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. However, it should be noted that although our results are largely negative in regard to the protective role of GSHPx-1 in a few models of tissue injury induced by oxidants, these studies do not rule out the antioxidant function of this enzyme in defending cells and animals against the increased oxidative stress in the pathogenesis of some other diseases. Future studies using this mouse model should help define the role of GSHPx-1 in certain other disorders, whose progression is associated with accumulation of oxidative damage, such as ischemia/reperfusion injury, atherosclerosis, neurodegenerative diseases, cancer, and aging.

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