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Comparison of glutathione peroxidase 1 and peroxiredoxin 6 in protection against oxidative stress in the mouse lung

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Abstract

Peroxiredoxin 6 (Prdx6) and cytosolic GSH peroxidase (GPx1) both GSH-dependent peroxidases, were compared for effects of their “knock-out” on lung injury and lipid peroxidation in: a) mice exposed to 0.85 or 1.0 ata O₂, b) isolated perfused mouse lungs exposed to 5 mM tert-butyl hydroperoxide (t-BOOH) or 1 mM paraquat, and c) primary mouse pulmonary microvascular endothelial cells exposed to 50 μM t-BOOH. Derangements in GPx1 null were similar or slightly greater than wild type for all parameters in the different models of oxidant stress, while Prdx6 null showed markedly increased effects. GSH peroxidase activity with phosphatidylcholine hydroperoxide as substrate in GPx1 null lung homogenate was decreased only slightly vs. wild type while activity in Prdx6 null lungs was decreased by ~ 95%, indicating that Prdx6 is the major enzyme for reduction of oxidized lung phospholipids. Expression levels of oxidant related genes measured with a PCR-based gene array indicated no significant differences between the Prdx6 and the GPx1 null except for the target genes. Thus, Prdx6 null mice are significantly more sensitive to oxidant stress as compared to GPx1 null, suggesting that scavenging of phospholipid hydroperoxides by Prdx6 plays a major role in lung antioxidant defense.

Keywords

lipid peroxidation; protein carbonyls; phospholipid hydroperoxide; tert-butyl hydroperoxide; hyperoxia; paraquat; endothelial cells; perfused lung; oxidative stress superarray

INTRODUCTION

The lung by virtue of its anatomic location and physiologic role is particularly at risk for oxidative stress associated with agents that are either inhaled directly into the lungs or that reach the lung through the blood supply. Defense against oxidant-mediated injury is provided by a broad array of antioxidant enzymes as well as non-enzymatic quenchers. The enzymes include the superoxide dismutases, catalase, and the glutathione peroxidases (GPx).

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The major non-enzymatic scavenger is GSH which has been shown to be an important component of lung antioxidant defense [1]. Because of its relatively slow reaction kinetics with peroxides, GSH is unlikely to function as a direct scavenger of H_2O_2 but rather to serve as a co-factor for peroxidases such as the GPx enzymes. Cytosolic GPx (GPx1) is widely distributed in essentially all cells including the lung epithelium, and is generally considered as the primary scavenger for H_2O_2 and short chain organic hydroperoxides [2]. The role of GPx1 in protection against oxidative injury has been evaluated in mice that are null for GPx1, but these studies have yielded mixed results. In the “knockout” mouse model, GPx1 appeared to be necessary for protection against toxicity due to paraquat at lethal doses (> 30 mg/kg) but not for protection against oxidative injury with hyperoxia [3] or sublethal concentrations (< 12 mg/kg) of paraquat [3-5]. Thus, these results suggest that an alternate GSH-dependent enzyme may play an important role in oxidant scavenging.

Peroxiredoxin 6 (Prdx6) represents an alternate peroxide scavenger that, unlike the other members of the peroxiredoxin family, utilizes GSH as its physiological reductant [6-8]. Both Prdx6 and GPx1 are cytosolic enzymes and can reduce a broad range of hydroperoxide substrates. However, Prdx6 [9], unlike GPx1 but similarly to GPx4 [2], is able to reduce phospholipid hydroperoxides. Within the lung, Prdx6 is expressed at especially high levels in bronchiolar Clara cells and alveolar type II epithelial cells and at lower levels in macrophages, endothelium, and other lung cell types [10]. Recent studies have found that Prdx6 null mice compared to wild type have increased sensitivity to lung injury associated with exposure to oxygen at elevated partial pressure or sub-lethal concentrations of paraquat [11-13] in clear distinction to results with GPx1 deletion [3,4]. However, these studies of GPx1 and Prdx6 “knock out” were carried out in different laboratories under varying experimental conditions so that the published results are not directly comparable. In the present study, GPx1 null and Prdx6 null mice, both on the C57/BL6 background, were bred and exposed to oxidants under identical conditions permitting a direct comparison of their responses to oxidative stress. We propose that increased sensitivity to oxidant stress in the null mouse reflects the anti-oxidant activity of the index enzyme in the wild type mouse.

MATERIALS AND METHODS

Materials

1-Palmitoyl, 2-linoleoyl *sn*-glycero-phosphorylcholine (PLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylcholine hydroperoxide (PLPCOOH) was prepared by controlled oxidation of PLPC using soybean lipoxygenase as described previously [9]. Medical grade O_2 was obtained from Air Products, Allentown, PA. Paraquat (methyl viologen), H_2O_2 , and tert-butyl hydroperoxide (t-BOOH) were from Sigma, St. Louis, MO.

Diphenylpyrenylphosphate (DPPP) was obtained from Dojindo, Gaithersburg, MD; it was solubilized in DMSO and stored at $-20^{\circ}C$. The 8-isoprostane assay kit was from Cayman (Ann Arbor, MI) and the Fe^{2+} -xylenol (FOX) assay kit was from Northwest Life Science (Vancouver, WA). The Live/Dead Cell Viability and Cytotoxicity kit was from Molecular Probes (Eugene, OR). The in situ Cell Death Detection kit was from Roche Diagnostics (Indianapolis, IN). The Oxidative Stress and Antioxidant Defense PCR Array kit (for mouse) was from SABiosciences (Frederick, MD). The polyclonal anti-Prdx6 peptide antibody has been described previously [14]; the polyclonal antibodies to GPx1 and GPx4 were obtained from Frontier Science (Ishikari, Japan).

Animals

The use of animals for these studies was approved by the University of Pennsylvania Animal Care and Use Committee (IACUC). Three groups of mice were studied: a C57BL/6 wild-type (control) group, a GPx1 null group, and a Prdx6 null group. Wild-type mice, free of specific pathogens, were obtained from the Jackson Laboratory (Bar Harbor, ME). GPx1 null and Prdx6 null founders were derived in the laboratory of Dr. Ye-Shih Ho [3,15]. Mice were subsequently bred at the University of Pennsylvania animal facility. GPx1 null mice had been fully back-crossed to the C57 BL/6 background in the laboratory of Dr. Alan Richardson (U. Texas, San Antonio). The Prdx6 null mice also have been fully back-crossed (> 99.9% genetic identity) at the University of Pennsylvania to the same C57BL/6 background as confirmed using microsatellite marker analysis performed by the Jackson Laboratory. Prdx6 and GPx1 null mice grow and reproduce normally with no obvious difference from the wild-type, although Prdx6 null mice do show age-related accumulation of lung phospholipids associated with altered phospholipase A₂ activity [16]. Mice weighed 24-28 g (age 8-11 wk) at the time of study.

Exposure to hyperoxia

Cages containing 3-5 mice were placed in a Plexiglas chamber (Braintree Scientific Inc., Braintree, MA) and exposed to 85% or 100% O₂ at 1 atmosphere absolute in as described previously [11,17]. Oxygen was passed through a bubble humidifier and introduced into the sealed chamber at 6-10 liters/min to provide 5 gas exchanges per hour. The chamber oxygen concentration was measured continuously with an oxygen analyzer (Pacifitech, Temecula, CA) and varied less than 2% from desired concentration. Chamber CO₂ was absorbed with a soda lime filter and was maintained at < 0.2%. Relative humidity in the chamber was 45-50%. Mice were allowed food and water *ad libitum* and maintained on a 12 h dark:light cycle. Cages were opened once daily for 5 min for change of water, food, and bedding.

To study mortality, mice were observed closely during O₂ exposure and those showing signs of extreme lethargy or labored breathing were removed from the chamber and immediately euthanized in a CO₂ chamber. This approach was utilized for the purpose of humane conduct of research. The cages used in the experiments were identified by number rather than phenotype in order to minimize observer bias. The time of death for each mouse was recorded and the time to 50% lethality (LT₅₀) was calculated.

To study injury to the lungs, mice were sacrificed after 60 h of O₂ exposure. After induction of anesthesia (pentobarbital, 50 mg/kg, intraperitoneally), the trachea was cannulated for continuous ventilation, a midline laparotomy/thoracotomy was performed, and mice were exsanguinated by laceration of the abdominal aorta. The hilum of the right upper and middle lobes was ligated, and those lobes were removed for measurement of the wet to dry weight ratio.

The remaining lung lobes were lavaged three times by instillation and aspiration of 0.5 ml PBS containing 0.5 mM EDTA, pH 8.0. This bronchoalveolar lavage fluid (BALF) was placed on ice for cell counting and analysis of protein concentration. Wet:dry weight and BALF analyses were performed immediately after sample collection. To measure the ratio of wet to dry lung weight, specimens were lightly blotted, weighed, and then placed in a drying oven (Scientific Glass Apparatus, Bloomfield, NJ) until repeated weighing demonstrated no change in weight (usually 3d). Total nucleated cells in an aliquot of BALF were counted with a Coulter Counter (Coulter Electronics, Luton, UK). The remaining BALF then was centrifuged for 20 min at 250 rpm and 4°C in order to sediment cells. Protein concentration of the supernatant was measured by binding of Coomassie blue dye (BioRad) using bovine gamma globulin as the standard.

In experiments to measure thiobarbituric acid-reactive substances (TBARS), the pulmonary artery was cannulated via the right ventricle and the pulmonary vasculature of the ventilated lung was flushed with PBS, followed by *en bloc* removal of the heart and lungs. The heart and large airways were dissected away and the cleared lung was rapidly frozen in liquid nitrogen and stored at -80°C. For subsequent assay, an aliquot of frozen lung was homogenized under N₂ in PBS (1:10) containing 0.01% butylated hydroxytoluene; the homogenate was reacted with thiobarbituric acid and the absorbance was measured at 535 nm as described previously [18].

Lung histology was evaluated in mice exposed to 100% O₂ for 60 h (n=5-6 each for wild type, GPx1 null, Prdx6 null). Mice under anesthesia were perfused through the pulmonary artery with 2.5% glutaraldehyde plus 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) followed by inflation of the lungs with 2-3 ml of the same fixative. The trachea was ligated and the lungs were removed from the thorax and stored overnight in the same fixative at 4°C. The fixed tissues were dehydrated in a graded ethanol series followed by xylene and embedded in paraffin. Three longitudinal sections for each lung were cut at a thickness of 6 µm and stained with hematoxylin and eosin. Randomly selected fields (10-15 for each lung section) were examined by a “blinded” observer with experience in the evaluation of lung histology. Lungs were graded on a scale of 1-4 with reference to alveolar and interstitial edema, infiltration with polymorphonuclear neutrophils (PMN), hemorrhage, and cellular necrosis. Thickness of the vessel wall and perivascular space was measured as the ratio of total area to luminal area using the Metamorph Imaging system (Medical Devices, Toronto, Canada) in order to evaluate vascular thickening and perivascular edema.

Isolated perfused mouse lung

Isolated lung perfusion was carried out as described previously [16,18]. Mice were anesthetized and lungs were cleared of blood by perfusion through the pulmonary artery and placed in the perfusion chamber. Lungs were ventilated continuously at 0.3 ml tidal volume with 5% CO₂ in air and perfused at 2 ml/min with perfusate containing 3% fatty acid free BSA and 10 mM glucose in Krebs Ringer bicarbonate (KRB) buffer (pH 7.4) with or without 1 mM paraquat or 5 mM t-BOOH. At the end of 1 h perfusion, the lungs and perfusates were rapidly frozen in liquid N₂ for subsequent assay.

To assess lung injury, samples of the perfusate were assayed for lactate dehydrogenase (LDH) by measuring the rate of reduction of NAD⁺ [18]. Lung and BALF samples were analyzed for lipid peroxides and protein carbonyls. Protein content of BALF, lung homogenate, and tissue extracts were measured by Coomassie blue binding as described. Lung lipid peroxides were determined using several methods. Thiobarbituric acid reactive substances (TBARS) were analyzed as described above in both the homogenized lung and the BALF. For other measurements of lipid peroxidation, lipids were extracted from homogenized lung by the Bligh and Dyer procedure as previously described [18]. The organic fraction was removed by evaporation under N₂ and the lipid residue was dissolved in chloroform-methanol (1:6, vol/vol). 8-Isoprostanes were measured in the lung extracts and BALF while conjugated dienes and lipid hydroperoxides were measured only in the lung extracts. For conjugated dienes, absorbance was measured at 233 nm using a spectrophotometer and conjugated diene concentration was expressed as optical density units per mg protein [18]. For lipid peroxides by the FOX method, absorbance of the Fe³⁺-xylenol orange complex was measured at 550 nm as described by the kit manufacturer. 8-Isoprostanes were measured by immunoassay. Briefly, 50 µl of samples were placed in a 96-well plate that was pre-coated with mouse monoclonal antibody that had been purified using a C-18 solid phase extraction cartridge. The 8-isoprostane tracer (50 µl) and 8-isoprostane antiserum were added into each well and incubated for 18 h at room temperature. After washing with wash buffer, Ellman's reagent (200 µl) containing the acetylcholinesterase

substrate was added. The plates were read at 412 nm and the values of lung 8-isoprostanes levels were calculated from a standard curve.

For analysis of tissue protein carbonyls, an aliquot of frozen lung was homogenized under liquid nitrogen in buffer containing complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN) and centrifuged at 100,000 g for 15 min; the supernatant was reacted with dinitrophenylhydrazine and assayed at 360 nm as described previously [19,20].

Additional lungs were evaluated for lipid peroxidation by the DPPP fluorescence method [21]. DPPP is non-fluorescent but becomes fluorescent when oxidized. DPPP fluorescence was determined with a PTI spectrofluorometer (Photon Technology Int., Bricktown, NJ) equipped with a single photon counting system. Lungs were preperfused with DPPP (50 μ M) for 30 min and then subjected to oxidative stress. At the end of perfusion, lungs were freeze-clamped, extracted, and DPPP fluorescence measured at 380 nm (350 nm excitation) as described previously [21].

Mouse pulmonary microvascular endothelial cells

Mouse pulmonary microvascular endothelial cells (mPMVEC) were isolated from the lungs of wild-type and gene-targeted mice using methods as described elsewhere [22]. Briefly, freshly harvested mouse lungs were treated with collagenase, followed by isolation of cells by adherence to magnetic beads coated with mAb to platelet endothelial cell adhesion molecule (PECAM) (BD Biosciences Pharmingen, Palo Alto, CA). mPMVEC were propagated in DMEM supplemented with 10% FBS, nonessential amino acids, and penicillin/streptomycin. The endothelial phenotype of the preparation was routinely confirmed by evaluating cellular uptake of 1, 1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated low density lipoprotein (Dil-AcLDL) and by immunostaining for platelet-endothelial cell adhesion molecule (PECAM). Cells at passage 4-5 were cultured on 25mm tissue culture plastic dishes and studied at ~90% confluence.

To evaluate oxidative stress in cultured cells, mPMVEC were exposed to 50 μ M t-BOOH for 1-4 h. This exposure regimen was chosen based on a previous study using isolated alveolar type 2 cells [23]. Cell viability was detected by the live/dead cell viability and cytotoxicity kit. mPMVEC were incubated with 0.5mM calcein AM plus 2mM ethidium homodimer-1 (EthD-1) for 20 min at 37°C and examined by fluorescence microscopy. Live cells were determined by green fluorescence (ex/em 495 nm/ 515 nm) obtained by enzymatic conversion of non-fluorescent calcein AM to calcein. Dead cells were indicated by red fluorescence (ex/em 495 nm/635 nm) of EthD-1 which can enter cells through damaged membranes and binds to nucleic acids.

Apoptotic cells were detected and quantified by fluorescence using the In Situ Cell Death Detection Kit for Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) [24]. After oxidant treatment, mPMVEC cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, and incubated with TUNEL reaction mixture (enzyme and labeling solutions) in a humidified atmosphere for 60 min at 37°C in the dark. Double-stranded DNA in nuclei was counterstained with 40,60-diaminido-2-phenylindole dihydrochloride (DAPI, 1 μ l/ml). Cells were evaluated by fluorescence microscopy (ex/em 495 nm/515 nm); for each experiment, the percentage of TUNEL positive cells was determined by counting 500 cells per dish from 5-6 randomly chosen fields. For TUNEL positive control staining, the fixed, permeabilized mPMVEC were incubated with recombinant DNase I (2 U/ml) for 10 min to induce DNA strand breaks. For TUNEL negative control staining, the processed mPMVEC were incubated with the labeling solution of the TUNEL reaction mixture minus the enzyme.

Lipid peroxidation in mPMVEC was detected by the DPPP method [23]. Cells were labeled by incubation at 37°C for 10 min with a micellar suspension of DPPP (400 nmol/ml), washed twice with PBS to remove extracellular DPPP, and then subjected to oxidative stress. The DPPP labeled cells were detached from the culture dish with trypsin-EDTA and resuspended in phosphate-buffered saline by gentle swirling in a standard quartz cuvette. After recording the initial emission spectrum, the cell suspension was excited at 350 nm (1 nm slit) and fluorescence was measured continuously at 380 nm (3 nm slit) over the subsequent 60 s. The total number of cells in the cuvette was counted using a hemocytometer (Hausser Scientific, Horsham, PA). Fluorescence intensity for DPPP was expressed as the average value during 60 s and normalized to the total number of cells.

Enzymatic activity

Peroxidase activity in lung homogenate was assayed using both H₂O₂ and phosphatidylcholine hydroperoxide (PLPCOOH) as substrate by measuring consumption of NADPH in the presence of GSH and GSH reductase [9]. The reaction buffer (3 ml) was 50 mM Tris-HCl (pH 8.0), 2 mM NaN₃, 0.1 mM EDTA, 0.3 mM NADPH, 0.36 mM GSH, and 0.23 units/ml GSH reductase. The homogenate in buffer (50 µg protein/ml) was preincubated for 5 min with continuous stirring. Fluorescence was continuously recorded at 460 nm (340-nm excitation) using the PTI spectrophotometer. After a steady base line was achieved the reaction was started by the addition of substrate (660 µM H₂O₂ or 100 µM PLPCOOH plus 0.1% Triton X-100) and the linear change in fluorescence was recorded for 5-10 min. The change in fluorescence was corrected for the relatively small base-line nonenzymatic oxidation of NADPH. Enzymatic activity was expressed as nmol of NADPH oxidized/min/mg protein.

Gene and protein expression

Real time reverse transcriptase PCR assay was used to determine possible differences in expression of oxidant stress-related genes between the two gene-targeted mouse models (Prdx6 null, GPx1 null). Isolation of mRNA from lung homogenates was accomplished using the Qiagen RNeasy Mini Spin Column-AIAshredder Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Purity and quantity of total RNA were determined by reading optical densities at 260 and 280 nm using a spectrophotometer (model 1000, NanoDrop Products, Wilmington, DE). We used the RT² First Strand Kit, SYBR green RT-PCR Master Mix, and a 384-well format RT² SuperArray containing 84 genes related to oxidant stress and antioxidant defense. Sample preparation and cycle times were performed according to the RT² SuperArray protocol using an Applied Biosystem 7900 instrument.

Western blots for protein expression used the antibodies described above; they were performed using standard techniques under reducing conditions [23] and analyzed by the Odyssey system (Li-Cor, Lincoln, NE) [23]. The secondary antibody was goat anti-rabbit IgG labeled with IrDye 800 obtained from Rockland (Gilbertsville, PA).

Statistical analysis

Data are expressed as mean±SEM. Statistical significance was assessed with SigmaStat software (Jandel Scientific, San Jose, CA). Group differences were evaluated by one-way ANOVA followed by the Bonferroni t test or by Student's t test as appropriate. In order to focus the results to the primary goals of this study, we have indicated the statistical significance between the results only for GPx1 null vs. Prdx6 null and have not indicated differences from wild type. Differences between mean values were considered statistically significant at P < 0.05.

RESULTS

Intact mouse

Consistent with a previous study [11], wild type mice showed 100% mortality within 5 days of beginning exposure to 100% O₂ or 7 days of 85% O₂ and Prdx6 null mice were more sensitive to the effects of hyperoxia (Fig. 1). The previous study [11] utilized Prdx6 null mice of mixed background (C57/Bl6: 129SvJ) while the mice used in the present study were fully backcrossed to the C57/Bl6 background. The novelty of the present study was to compare oxidant stress in the GPx1 and Prdx6 null mouse models. The survival of mice from these two groups was significantly different upon exposure to hyperoxia (Fig. 1). Mortality in Prdx6 null mice exposed to 85% O₂ began at 90 h whereas all GPx1 null mice survived until 110 h (Figure 1A). At 100% O₂, the initial death of Prdx6 null mice occurred at 56 h, but required more than 60 h for GPx1 null mice (Figure 1B). The LT₅₀ for the Prdx6 null mice was slightly greater than 4 days at 85% O₂ and almost 3 days at 100% O₂; at both O₂ concentrations, the LT₅₀ was longer by ~ 25% (P<0.05) in the GPx1 null mice (Figure 1).

Further studies evaluated lungs after 60 h of exposure to 100% O₂ in order to detect pre-lethal hyperoxic injury. Wild type lungs showed increased wet:dry ratio and increased total protein and nucleated cells in the BALF indicating increased alveolar permeability and lung inflammation (Figure 2). The lung content of TBARS was increased with O₂ exposure consistent with lipid peroxidation (Figure 3). These alterations in the lung at 60h of O₂ exposure were significantly greater in the Prdx6 null mouse; values for GPx1 null lungs were intermediate between the wild type and Prdx6 null (Figures 2, 3).

Histological examination showed no differences in control (non-exposed) lungs for the 3 types of mice (n=1 for each) (Figure 4). Lungs fixed after 60h of exposure to 100% O₂ showed patchy areas of alveolar and perivascular edema, hemorrhage, and infiltration with inflammatory cells (Figure 4). Quantitative analysis of vessel walls indicated a greater degree (P < 0.05) of perivascular edema in Prdx6 null lungs compared to GPx1 null (Table 1). There were no other apparent differences in histology for lungs from wild type, GPx1 null, and Prdx6 null animals following O₂ exposure.

Perfused lungs

t-BOOH and paraquat were used to induce oxidant injury in isolated perfused lungs. We measured BALF protein as an index of altered alveolar permeability, BALF LDH content as an index of cell damage, and BALF TBARS and 8-isoprostanes to determine lipid peroxidation. Lipid and protein oxidation were determined in lung extracts. Since control values (unexposed to oxidant) showed no significant differences among wild type and the null mouse models, these are shown only for wild type lungs (Tables 2 and 4). Also, since the same control values apply to both the t-BOOH (Tables 2 and 4) and paraquat studies, the control values are omitted in Tables 3 and 5. Each of the assayed parameters in BALF was increased significantly with t-BOOH exposure in wild type lungs (Table 2). GPx1 null mice showed increased derangement compared to wild type, while Prdx6 null mice showed even greater abnormality (Table 2). With paraquat the degree of change overall in each of the lung models was less severe as compared to t-BOOH (Table 3). As with t-BOOH, injury with paraquat was most marked in the Prdx6 null lungs.

Parameters of phospholipid peroxidation (TBARS, conjugated dienes, lipid hydroperoxides, 8-isoprostanes, and DPPP fluorescence) also were measured in homogenates of perfused lungs. These assays measure different indices of lipid peroxidation so that the absolute values are expectedly dissimilar. The percent change with oxidant stress also varied but all were in the same direction. All of these parameters were increased in wild type lungs with t-

BOOH exposure; the increase was larger for GPx1 null lungs and significantly greater still for the Prdx6 null lungs (Table 4). Comparing the tests, the percent change was greatest for the 8-isoprostane assay (more than 3-fold increase compared to control for Prdx6 null lungs) and least for the FOX assay (less than 2-fold increase). With paraquat exposure, lipid peroxidation, like the BALF indices of injury, was essentially similar for wild type and GPx1 null lungs but was greater with Prdx6 null lungs (Table 5).

Protein carbonyls were measured in homogenates of perfused lungs to determine protein oxidation. With t-BOOH, protein carbonyls were increased 50% in wild type lungs, 140% in GPx1 null lungs, and 215% in Prdx6 null lungs (Figure 5). As with lipid peroxidation, the changes in protein carbonyls were less marked with paraquat perfusion; wild type and GPx1 null lungs showed a similar increase of ~40-50% while protein oxidation in Prdx6 null lungs was increased by 100% (Figure 5).

Isolated endothelial cells

mPMVEC in cell culture were analyzed for live/dead ratio based on the uptake of calcein (live) or Eth-D (dead). Upon treatment of cells with t-BOOH (50 μ M), there was a time-dependent decrease in the percentage of live to dead cells (Figure 6). The effect of t-BOOH was most marked in the Prdx6 null cells while the ratio of live to dead GPx1 null cells was intermediate between the results for wild type and Prdx6 null cells. Almost all of the Prdx6 null cells were dead by 2 hrs of exposure to t-BOOH while ~ 30% of the wild type and ~ 22% of the GPx1 null cells remained viable. Cells also were evaluated by the TUNEL assay. The percentage of TUNEL positive cells in the Prdx6 null group was nearly 100% by 2 h of t-BOOH treatment, similar to the result for live/dead assay (Figure 7). The percentage of GPx null cells that were TUNEL positive was intermediate between the results for wild type and Prdx6 null cells (Figure 7). DPPP fluorescence was used to image membrane lipid peroxidation in mPMVEC. DPPP fluorescence was increased to a similar degree in the wild-type and GPx null cells with t-BOOH treatment, but the increase in fluorescence intensity was much more marked in the Prdx6 null cells (Fig. 8).

Enzymatic activity

Western blots showed no significant alteration of Prdx6 expression in GPx1 null lungs or of GPx1 expression in Prdx6 null lungs (Figure 9A, B). Lung homogenates from wild type, GPx1 null, and Prdx6 null mice were analyzed for GSH-dependent peroxidase activity using H_2O_2 and PLPCOOH as substrate. Activity with H_2O_2 substrate was similar for wild type and Prdx6 null lungs but was decreased approximately 90% with GPx1 null lungs (Fig. 10). This result indicates that GPx1 is the major enzyme in lungs for GSH-dependent reduction of H_2O_2 . The level of activity with PLPCOOH in wild type lung homogenate was considerably less compared to H_2O_2 and decreased only slightly in GPx1 null lungs (Figure 10) compatible with the observation that GPx1 does not reduce phospholipid hydroperoxides [2, 9, 25]. On the other hand, activity with PLPCOOH as substrate was essentially absent in Prdx6 null lungs (Figure 10). This result is consistent with the relatively low level of GPx4 in lungs (Figure 9C) and indicates that Prdx6 is the major enzyme for GSH-dependent phospholipid hydroperoxide reduction in mouse lungs.

mRNA and protein expression

Because of the differential degrees of injury in the two null mouse models in response to oxidant stress, we investigated their gene expression profiles (with respect to oxidant-related genes) to determine whether induction of non-target enzymes could play a role in the responses. We compared gene expression using the Oxidant Stress and Antioxidant Defense Superarray (SA Biosciences, Gaithersburg, MD) that contains 84 genes out of which approximately 40 appear to code for enzymes broadly related to anti-oxidant defense. This

includes all of the mammalian GSH peroxidases, peroxiredoxins, SODs, and catalase. mRNA from the lungs of GPx1 and Prdx6 null mice (n=3 independent samples) was analyzed. Statistical analysis was performed for these genes where the mean value for the comparison of GPx1 to Prdx6 was increased or decreased by at least 2-fold. Interleukin 19 showed a statistically significant lower level of expression in GPx1 null compared to Prdx6 null lungs (Table 6). Otherwise, there were no statistically significant differences ($P > 0.05$) indicating a > 2 -fold increase or decrease in lung mRNA expression level between GPx null and Prdx6 null mice for any of the RNAs represented on the array, except for the expected altered expression of the targeted genes (Table 6).

DISCUSSION

GPx1 in concert with its cofactor GSH has long been considered to have an important role in antioxidant defense and to constitute a primary pathway to scavenge intracellular H_2O_2 . Previous studies, supported by the present results, indicate an approximately 90% decrease in GSH-dependent reduction of H_2O_2 by “knockout” of this enzyme in mouse lungs [3] or when the selenoenzyme (GPx1) is depleted by maintaining rats on a selenium deficient diet [26]. It is now recognized that the remaining 10% of GSH-dependent peroxidase activity, at least in the lung, can be accounted for by the presence of Prdx6, a non-seleno GSH-dependent enzyme. The greater importance of GPx1 as compared to Prdx6 for H_2O_2 removal can be accounted for by its rate constant that is approximately an order of magnitude greater ($\sim 10^7 M^{-1}s^{-1}$ for GPx1 vs. $\sim 10^6 M^{-1}s^{-1}$ for Prdx6) [2,8,9,27].

Based on the above considerations, GPx1 null mice might be expected to be considerably more sensitive to oxidant stress as compared to mice without Prdx6. However, the present results indicate the inverse. That is, Prdx6 null mice were significantly more sensitive to the lethal effects of hyperoxia and their lungs showed evidence of increased toxic effects as manifested by disruption of the alveolar capillary barrier (protein leak), inflammation (inflammatory cells in BALF), and lipid peroxidation. This conclusion agrees with previous studies indicating that GPx1 null mice do not show increased sensitivity to hyperoxia [3] while Prdx6 null mice are more sensitive compared to wild type [11,17]. These present results were obtained with mice that were bred and exposed under identical conditions. The increased sensitivity of Prdx6 null mice to oxidant stress was not specific for hyperoxia since isolated perfused lungs and isolated endothelial cells from Prdx6 null lungs showed increased injury compared to GPx1 null when treated with t-BOOH (lungs and cells) or paraquat (lungs). Interestingly, t-BOOH and paraquat caused a similar degree of lipid peroxidation in the wild type lungs, while lipid peroxidation in the two null models was greater with t-BOOH. Although both agents result in oxidant stress, they act through differing mechanisms, t-BOOH is a hydroperoxide while paraquat generates $O_2^{\cdot -}$ by auto-oxidation of its reduced form. The differential results are consistent with a role for both GPx1 and Prdx6 in hydroperoxide detoxification. An alternative explanation is that the t-BOOH affects all lung cells while paraquat may be cell specific depending on transmembrane uptake and intracellular metabolism (reduction) of the toxin.

One possibility for the differential responses to oxidant stress in GPx1 null as compared to Prdx6 null mice could be a differential induction of other protective enzymes. However, except for the “knock out” genes and interleukin 19, the mRNA expression assays failed to show any differences between the GPx1 null and Prdx6 null mice in mRNA levels for antioxidant enzymes or other proteins related to oxidant stress. This is consistent with previous studies of protein and mRNA expression levels using a sub-set of oxidant stress-related genes where there were no differences from wild type for either the Prdx6 null [11,13] or the GPx1 null model [28,29]. Further, western blots showed no difference from wild type in GPx1 in Prdx6 null or Prdx6 in GPx1 null (Figure 9). Therefore, possible

compensatory changes in expression levels for anti-oxidant enzymes in GPx1 null mice is unlikely to be the mechanism for the differences in oxidant sensitivity of the two null mouse models.

Another possible explanation for the different sensitivities to oxidant stress that were observed in the two null models is the substrate specificities for the relevant two enzymes (GPx1, Prdx6). Although both enzymes use GSH to reduce H_2O_2 and other short chain hydroperoxides, Prdx6 also is able to reduce phospholipid hydroperoxides. Numerous studies including our own have indicated that GPx1 does not have this activity [2,9,25] and the present results show that knockout of GPx1 has relatively little effect on the ability of the lung homogenate to reduce PLPCOOH. On the other hand, Prdx6 can reduce PLPCOOH with a rate constant similar to H_2O_2 [9] and this activity is greatly decreased in lungs from Prdx6 null mice. While GPx4 also can reduce phospholipid hydroperoxides, there is apparently relatively little expression of this enzyme in the lung [30], as confirmed in the present study by western blot (Fig. 9), and by the nearly total absence of PLPCOOH reduction by the Prdx6 null lung homogenate (Fig. 10). On the other hand it is possible that GPx4 while at low levels in the whole lung is expressed in one or more lung cell types where it could play an important role.

Peroxidation of cell membrane phospholipids is a well recognized manifestation of oxidant stress, possibly mediated by hydroxyl radical ($\cdot OH$) arising from the interaction of superoxide anion ($O_2^{\cdot -}$) with H_2O_2 (in the presence of a transition metal) or with $\cdot NO$. The resultant lipid hydroperoxy radicals can initiate a chain reaction of lipid peroxidation. Prdx6, unlike GPx1, can interrupt the chain reaction and can “repair” peroxidized membrane phospholipids through the direct reduction of phospholipid hydroperoxides. The present studies provide evidence for lipid peroxidation associated with oxidant stress by measurement of TBARS in the intact lung, DPPP fluorescence in isolated cells and lungs and these two tests plus 3 others (8-isoprotanes, FOX assay, conjugated dienes) in the isolated perfused lungs. All of these measurements indicate that the degree of lipid peroxidation was significantly greater in the Prdx6 null compared with GPx1 null. Thus, these results suggest that Prdx6, by reducing phospholipid hydroperoxides can prevent or repair oxidant-induced peroxidation of phospholipids in cell membranes. A schema indicating the essential role of Prdx6 in the reduction of oxidized phospholipids is shown in Figure 11.

The alternative to direct reduction of phospholipids for membrane repair is considered to be excision of the peroxidized fatty acid through the activity of a phospholipase A_2 enzyme followed by reacylation to regenerate the reduced phospholipid [31]. The specific PLA_2 involved in this repair mechanism has not been described, and indeed, the PLA_2 activity of Prdx6 could be responsible [6]. Although Prdx6 has very little PLA_2 activity at pH 7, phosphorylation results in a considerable increase in this activity[32]. However, there are several difficulties with a role for the deacylation/reacylation as a physiologically significant repair mechanism. The first is the relative slowness of the combined two-step repair which has been estimated at 10^4 less efficient as compared with the direct reduction mechanism [33]. A second difficulty is the location of acyltransferases that are required for reacylation of the lysophospholipid. The latter enzymes are contained in the endoplasmic reticulum (ER) and their availability in the intact cell for the *in situ* repair of peroxidized non-ER membranes is questionable. Thus, the peroxidase activity of Prdx6 may represent the major mechanism for repair of peroxidized membranes during oxidative stress and its absence can account for the increased toxicity of oxidants in Prdx6 null cells. This function of membrane repair could be shared with GPx4 in those cells where this latter enzyme is expressed at significant levels. Of note, the reduction of a phospholipid hydroperoxide would generate the phospholipid alcohol which then would require an additional step to regenerate the

reduced phospholipid. A specific enzyme responsible for the final reductive step has not been identified.

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Abbreviations

GPx1	Glutathione peroxidase 1
Prdx6	peroxiredoxin-6
t-BOOH	tert-butyl hydroperoxide
PLPC	1-palmitoyl, 2-linoleoyl, <i>sn</i> -3-glycero-phosphorylcholine
PLPCOOH	PLPC hydroperoxide
DPPP	diphenylpyrenylphosphate
BALF	bronchoalveolar lavage fluid
TBARS	thiobarbituric acid-reactive substances
LDH	lactate dehydrogenase
mPMVEC	mouse pulmonary microvascular endothelial cells

Figure 1.

Survival of wild type, GPx1 null, and Prdx6 null mice in hyperoxia. The survival times of age-matched (10 wk) mice were measured during exposure to 85% (panel A) or 100% (panel B) oxygen. The time to 50% lethality (LT_{50}) is shown in the inset. * $P < 0.05$ for Prdx6 null versus GPx1 null type mice.

Figure 2.

Lung wet to dry weight ratio and bronchoalveolar lavage fluid (BALF) analysis. Lungs were analyzed after exposure to 85% or 100% O_2 for 60 hours. A) Ratio of wet to dry lung weight. B) Protein content in BALF. C) Nucleated cells in BALF. Results are mean $\pm$ SE (n =5) for wild-type, GPx1 null and Prdx6 null mice at 60 hours of hyperoxia. *P <0.05 for Prdx6 null versus GPx1 null.

Figure 3.

Thiobarbituric acid-reactive substances (TBARS) were measured in wild type, GPx1 null and Prdx6 null mouse lung homogenates before (control) and after 60 hours of exposure of mice to either 85% or 100% O₂. Values are mean $\pm$ SE ($n=4$). * $P < 0.05$ for Prdx6 null versus GPx1 null.

Figure 4.

Lung morphology with hyperoxia (100% O₂ for 60 h). After exposure, lungs were fixed, sectioned, and stained with hematoxylin and eosin. Lungs of GPx1 null and Prdx6 null and age-matched wild-type mice showed similar histologic appearance before oxygen exposure. With 60 h exposure to hyperoxia, lungs show perivascular edema, hemorrhage cellular necrosis, and cellular infiltration. The Prdx6 null lungs showed thickening of the vascular wall and perivascular edema that was greater than that observed for wild type and GPx1 null lungs. There were no other observed differences among the lungs from the 3 groups. Photomicrographs are representative of n=5-6 lungs (3 sections each, 10-15 fields per section) for each oxygen exposed group. The *scale bar* is 100 μ m. A, airway; V, vessel.

Figure 5. Measurements of protein carbonyls in isolated mouse lungs before (control) and after perfusion with 5mM t-BOOH or 1 mM paraquat. Results are mean $\pm$ SE ($n = 5$). * $P < 0.05$ for Prdx6 null versus GPx1 null.

Figure 6.

Effect of t-BOOH on mouse pulmonary microvascular endothelial cells (mPMVEC). Cells were treated with 50 μ M t-BOOH for 0, 1, 2 or 4h. A) Confocal fluorescence images with calcein to detect “live” cells (green) and ethidium homodimer to detect “dead” cells (red). The calcein stain is diffuse in the cytoplasm while the ethidium stain is concentrated in the nucleus. Original magnification, 20X. B) Percentage of “live” cells from experiments shown in A determined from approximately 200 total cells in 5 microscopic fields. Values are mean $\pm$ SE for n=3 separate experiments. *P < 0.05 for Prdx6 null versus GPx1 null.

Figure 7.

TUNEL staining of mPMVEC treated with t-BOOH. A) mPMVEC from wild type, GPx1 null and Prdx6 null mice were treated with 50 μ M t-BOOH for 0, 1h or 2 hr and then evaluated by TUNEL. B) Percentage of mPMVEC TUNEL positive cells. Values are mean $\pm$ SE for n=3 separate experiments. *P < 0.05 for Prdx6 null versus GPx1 null.

Figure 8.

Lipid peroxidation of mPMVEC cellular membranes with t-BOOH treatment as indicated by DPPP fluorescence. mPMVEC from wild-type, GPx1 null, and Prdx6 null mice were loaded with DPPP and then exposed to 50 μ M t-BOOH for 2h. DPPP fluorescence was measured by spectrofluorometry. Values are means $\pm$ SE for n=3 experiments. *P<0.05 for Prdx6 null compared to GPx1 null.

Figure 9.

Western blot of lung homogenates. All lanes were loaded with 50 μ g protein; β -actin was used as a loading control. A) Wild type and Prdx6 null probed with anti-GPx1. B) Wild type and GPx1 null probed with anti-Prdx6. C) Wild type lungs and testes probed with anti-GPx4.

Figure 10.

GSH-dependent peroxidase activity in mouse lung homogenate with either H_2O_2 or 1-palmitoyl, 2-linoleoyl, *sn*-phosphatidylcholine hydroperoxide (PLPCOOH) as substrate. The initial rate of reaction was calculated from the rate of NADPH oxidation in the presence of a GSH-regenerating system. Values are mean $\pm$ SE for $n=3$. * $P < 0.05$ for Prdx6 null compared to GPx1 null.

Figure 11.

Scheme for intracellular generation of oxidants. H_2O_2 can be reduced by multiple enzymes but oxidized phospholipids (i.e. peroxidized cell membranes) are reduced in lungs only by peroxiredoxin 6 (Prdx6). SOD, superoxide dismutase; Prdx, peroxiredoxins; PL, phospholipid; PLOOH, phospholipid hydroperoxide; PLOH, hydroxyphospholipid. Reproduced with modifications from [8].

Table 1Perivascular edema in lungs of mice exposed to 100% O₂ for 60 h

	Total vessel area / luminal area
Air control	1.5 ± 0.1 (3)
Wild type	4.2 ± 0.8 (3)
GPx1 null	4.8 ± 0.9 (4)
Prdx6 null	7.0 ± 1.3* (4)

Arterioles in the peripheral lung parenchyma of fixed lung sections were examined by light microscopy and the luminal and total (vessel plus perivascular space) areas were measured. The air control group consisted of 1 lung from each type of mouse. Each mouse generated 3 lung sections and 10-15 fields were examined for each lung section. Values are mean ± SE for number of experiments in parentheses; each experiment represents the mean of 2-4 mice.

* P < 0.05 vs. GPx1 null.

Table 2

Lung Injury in Isolated Mouse Lungs Perfused with 5mM t-BOOH for 1 h

	Control*	Wild Type	GPx1 -/-	Prdx6 -/-
Lavage Protein (μ /g lung dry wt)	8.6 $\pm$ 0.4	9.1 $\pm$ 0.6 6%	9.9 $\pm$ 0.8 15%	11.2 $\pm$ 0.5 [†] 30%
LDH in perfusate (nmol/g lung dry wt)	0.55 $\pm$ 0.04	1.41 $\pm$ 0.02 156%	2.76 $\pm$ 0.07 402%	4.15 $\pm$ 0.2 [†] 655%
TBARS in perfusate (pmol/g lung dry wt)	1.1 $\pm$ 0.2	2.7 $\pm$ 0.5 145%	4.5 $\pm$ 0.6 309%	5.6 $\pm$ 0.3 [†] 409%
8-Isoprostanes in perfusate (pg/g lung dry wt)	3.9 $\pm$ 0.2	13.8 $\pm$ 1.0 254%	27.1 $\pm$ 2.1 595%	32.5 $\pm$ 2.5 [†] 733%

The upper row shows the mean value $\pm$ SE for n=3 lungs; the lower row shows the % increase in mean values from control.

* Control is the value prior to addition of the oxidant.

[†] P<0.05 for Prdx6 null vs. GPx1 null.

Table 3

Lung Injury in Isolated Mouse Lungs Perfused with 1mM Paraquat for 1 h

	Wild Type	GPx1 -/-	Prdx6 -
Lavage Protein (mg/g dry wt)	8.9 ± 0.8 3%	8.8 ± 0.6 2%	10.1 ± 0.6* 17%
LDH in perfusate (nmol/g dry wt)	1.35 ± 0.40 145%	1.68 ± 0.10 205%	1.89 ± 0.02* 244%
TBARS in perfusate (pmol/g dry wt)	2.5 ± 0.04 125%	2.1 ± 0.05 91%	3.9 ± 0.08* 254%
8-Isoprostanes in perfusate (pg/g dry wt)	13.4 ± 0.5 243%	14.3 ± 0.4 267%	20.7 ± 0.5* 431%

See Table 2 for control values. The upper row is the mean value ± SE for n=3 lungs; the lower row shows the % increase in mean values from the control shown in Table 2.

* P<0.05 for Prdx6 null vs. GPx1 null.

Table 4

Lipid Peroxidation in Isolated Mouse Lungs Perfused with 5mM t-BOOH for 1 h

	Control*	Wild Type	GPx1 -/-	Prdx6 -/-
TBARS (pmol/mg prot)	55.3 ± 2.0	79.2 ± 3.6 43%	157 ± 5 184%	186 ± 2 [†] 236%
Conjugated Dienes (pmol/mg prot)	189 ± 5	318 ± 10 68%	594 ± 28 214%	787 ± 57 [†] 316%
Lipid Hydroperoxide/ FOX (pmol/mg prot)	14.6 ± 0.5	17.7 ± 0.4 21%	33.4 ± 0.4 129%	41.7 ± 0.5 [†] 186%
8-Isoprostanes (pmol/mg prot)	0.20 ± 0.01	0.38 ± 0.01 90%	0.76 ± 0.01 280%	0.87 ± 0.02 [†] 335%
DPPP Fluorescence 10 ⁶ counts/sec/mg prot	3.4 ± 0.1	4.9 ± 0.4 44%	10.4 ± 0.5 206%	15.0 ± 0.5 [†] 341%

The upper row is the mean value ± SE for n=3 lungs; the lower row shows the % increase in mean values from control.

* Control is the value prior to addition of the oxidant.

[†] P<0.05 for Prdx6 null vs GPx1 null.

Table 5

Lipid Peroxidation in Isolated Lungs Perfused with 1 mM Paraquat for 1 h

	WT	GPx1 -/-	Prdx6 -/-
TBARS (pmol/mg prot)	90.5 ± 1.4 64%	85.1 ± 2.8 54%	113 ± 2* 104%
Conjugated Dienes (pmol/mg prot)	322 ± 10 70%	302 ± 17 60%	431 ± 17* 128%
Lipid Hydroperoxide/ FOX (pmol/mg prot)	21.5 ± 0.4 47%	21.6 ± 0.3 48%	32.0 ± 0.8* 119%
8-Isoprostanes (pmol/mg prot)	0.42 ± 0.01 110%	0.43 ± 0.02 115%	0.68 ± 0.02* 240%
DPPP Fluorescence 10 ⁶ counts/sec/mg prot	6.1 ± 0.4 79%	5.2 ± 0.2 53%	9.2 ± 0.5* 171%

See Table 4 for control values. The upper row is the mean value ± SE for n=3; the lower row shows the % increase in mean values from control.

* P<0.05 for Prdx6 null vs. GPx1 null.

Table 6

Gene expression in GPx1 null vs. Prdx6 null lungs

Gene Name	Symbol	Fold Change*	p-value
Copper chaperone for SOD	Ccs	3.0	0.53
Peroxiredoxin6	Prdx6	11	0.00088
Xin actin-binding repeat containing1	Xirp1	-2.6	0.14
Eosinophil peroxidase	Epx	-3.8	0.19
Glutathione peroxidase 1	GPx1	-9.5	0.000001
Glutathione peroxidase 6	GPx6	-2.2	0.35
Interleukin 19	Il19	-5.0	0.027
Interleukin 22	Il22	-4.9	0.32
NADPH oxidase organizer1	NoxO1	-2.5	0.13
Recombination activating gene2	Rag2	-2.7	0.36
Thyroid peroxidase	Tpo	-3.7	0.34

The Mouse Oxidative Stress and Antioxidant Defense RT² Profiler™ PCR Array profiles the expression of 84 genes related to oxidative stress. Only genes with 2-fold or greater change are shown.

* Mean value for n=3.